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Analysis Of Secreted Phosphoprotein (2ar; Osteopontin) Gene Expression And Association With Carcinogenesis

Ann Marie Craig

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ANALYSIS OF SECRETED PHOSPHOPROTEIN (2AR; OSTEOPONTIN) GENE EXPRESSION AND ASSOCIATION WITH CARCINOGENESIS

by

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ABSTRACT

The murine cDNA encoding secreted phosphoprotein 1 (SPP) was cloned in this lab on the basis of induction of the mRNA (originally designated 2ar) by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate in JB6 epidermal cells. The polypeptide predicted from the cDNA sequence is 84% identical to rat osteopontin (44 kDa bone phosphoprotein; sialoprotein I). Many primary structural features are conserved, including an ArgGlyAsp (RGD) cell adhesion site and an unusual series of 9-10 consecutive aspartate residues. SPP mRNA is most abundant *in vivo* in bone and kidney, and was localized to the medulla region of adult mouse kidney by *in situ* hybridization. Portions of the predicted SPP polypeptide were expressed as cro-ß-galactosidase fusion proteins, and polyclonal antisera were obtained. The SPP antisera recognized the major [^{32}P]orthophosphate-labeled protein secreted by all mouse and rat cell lines studied. This observation led to the discovery that SPP is closely related or identical to a transformation-related phosphoprotein, also known as pp69. SPP mRNA was transiently induced by treatment of mouse epidermis *in vivo* with 12-O-tetradecanoylphorbol-13-acetate, and was constitutively expressed in 2 of 3 epidermal papillomas and 7 of 7 squamous cell carcinomas. SPP expression was elevated in T24 H-*ras*-transformed mouse fibroblasts, and the level of expression correlated with the metastatic ability of these cells. It is proposed that SPP may act as an autocrine adhesion factor for tumor cells. SPP is encoded by a single copy mouse gene which spans approximately 9 kilobasepairs (kb). A genomic clone containing the first 6 exons and 16 kb of 5' flanking DNA was isolated. The SPP promoter region contains a TATA-like box (TTTAAA), a CCAAT box (reverse

complement), and a number of potential regulatory elements. A small fragment (-253 to +79) was able to direct high level tumor promoter-inducible expression of a fused marker gene.

To my parents

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my supervisor, Dr. David T. Denhardt, for his guidance, support, and unfailing example of dedication and enthusiasm. I am grateful to my advisory committee members, Drs. G.A. Mackie and B.D. Sanwal, and to Dr. W. Flintoff for helpful discussions. The collaboration of Drs. G.T. Bowden, A.F. Chambers, A.H. Greenberg, B.B. Mukherjee, M. Nemir, and J.H. Smith is gratefully acknowledged. Many members of the (former) Cancer Research Laboratory have made my stay here more interesting and enjoyable. I would particularly like to thank Marilyn McLeod for excellent technical assistance, especially with the *in situ* hybridizations; Helen Gilman for help with the affinity purification; Beth Orphan for artistic contributions; and Alison, Michele and Carol for their advice and encouragement.

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LIST OF ABBREVIATIONS

A	adenine
aa	amino acid(s)
Arg	arginine
Asp	aspartate
ATP	adenosine 5'-triphosphate
bp	basepair(s)
bFGF	basic fibroblast growth factor
BSA	bovine serum albumen
BP	benzo(a)pyrene
C	cytosine
cAMP	adenosine-3',5'-monophosphate
cpm	counts per minute
CsCl	cesium chloride
DG	diacylglycerol
DMBA	7,12-dimethylbenz(a)anthracene
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside 5'-triphosphate
DTT	dithiothreitol
E. coli	Escherichia coli
ECDGF	embryonal carcinoma-derived growth factor
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
g	gravity
G	guanine
Gln	glutamine
Glu	glutamate
Gly	glycine
GTP	guanosine 5'-triphosphate
IP_3	inositol 1,4,5-trisphosphate
IPTG	isopropyl ß-D-thio-galactopyranoside

kb	kilobasepair(s)
kDa	kilodalton(s)
Klenow	Klenow fragment of E. coli DNA polymerase I
LB	Luria broth
m.o.i.	multiplicity of infection
M_r	relative molecular mass
mRNA	messenger ribonucleic acid
NEN	New England Nuclear
nt	nucleotide(s)
ODC	ornithine decarboxylase
P^-	non-promotable
P^+	promotable
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PEG	polyethylene glycol
p.f.u.	plaque forming units
PIPES	piperazine-N,N'-bis(2-ethane-sulfonic acid)
PKC	protein kinase C
RGD	arginine-glycine-aspartate
RNA	ribonucleic acid
rpm	rotations per minute
SDS	sodium dodecyl sulfate
Ser	serine
SSC	standard saline citrate
SPP	secreted phosphoprotein
T	thymine
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
TGFß	transforming growth factor-ß
TPA	12-O-tetradecanoylphorbol-13-acetate
tRNA	transfer ribonucleic acid
U	unit(s)
Xgal	5-brom-4-chlor-3-indolyl-ß-D-galactopyranoside
YT	yeast-tryptone broth

CHAPTER 1

GENERAL INTRODUCTION

1.1 Carcinogenesis

A cancerous growth, or neoplasm, can be defined as: "an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change" (Willis, 1952). The word "cancer" originates from the Latin word meaning crab, which attests to its persistent and claw-like tendency to spread throughout the body (Skinner, 1961). The disease has been described as early as 1770 B.C., in the Ebers papyrus (from Mainwaring, 1986), but has only recently become amenable to study at the cellular and molecular levels.

Cancers are malignant tumors, most commonly arising from epithelium (carcinomas), mesenchyme (sarcomas), or blood cells (leukaemias) (Franks, 1986). The hallmark of malignancy is the ability to invade surrounding tissues. It is this invasive nature and the ability to metastasize (form a tumor at a secondary site) that most often leads to the death of cancer patients. In contrast, benign tumors are local growths which do not spread to distant sites. However, benign tumors can progress to become malignant. Diagnosis is usually based on histology, which can reveal the invasive, disorganized, and often anaplastic (dedifferentiated) growth characteristic of malignancy (Franks, 1986).

The bulk of evidence supports the idea that multiple changes must occur in a normal cell to bring about the uncontrolled growth characteristic of neoplasia. The concept of multistage carcinogenesis was first experimentally applied to mouse skin tumor formation (Friedwald and Rous, 1944; Boutwell, 1964). In this system, two stages have been operationally

defined: initiation, the production of potentially tumorigenic cells by exposure to a sub-threshold dose of a carcinogen, is followed by promotion, the completion of neoplastic transformation as the result of subsequent chronic treatment with appropriate agents that are not intrinsically carcinogenic (Friedwald and Rous, 1944). A typical initiation-promotion regimen is a single application of 10 nmol 7,12-dimethylbenz(a)-anthracene (DMBA) followed by twice weekly applications of 2 μg 12-O-tetradecanoylphorbol-13-acetate (TPA) each. This results in the formation of a large number of benign papillomas after 6-20 weeks and a lesser number of squamous cell carcinomas after 20-60 weeks (Slaga, 1984).

The relevance of these studies to human skin cancer is not entirely clear since, obviously, direct experiments cannot be done. However, urethane initiation and TPA promotion of human skin grafted onto nude mice did lead to the formation of some papillomas (Yuspa et al., 1979). TPA was also able to stimulate the growth of epithelial cells from adenomatous polyps while inhibiting the growth of normal human colonic epithelial cells in culture (Friedman, 1981). Thus, at least some aspects of two-stage carcinogenesis can occur in human tissues.

Two-stage carcinogenesis involving initiation and promotion by TPA, hormones, or other agents has been described in other mammalian organ systems, including liver, lung, colon, bladder, pancreas, and mammary gland (Slaga, 1983; Yuspa and Poirier, 1988). However, in most of the studies, the strict definitions of initiation-promotion defined in mouse skin were relaxed slightly. For example, multiple carcinogen treatments were given, or the initiation-promotion regimen was accompanied by partial excision of the organ under study to induce regenerative hyperplasia. There is an intriguing report that transplacental administration of

urethane or DMBA to a mouse fetus followed by application of TPA to the skin of the newborn mouse led to tumors of several internal organs as well as skin tumors (Goerttler and Löhrke, 1977). Many promoting agents are tissue-specific, and can influence the site of tumor formation (Yuspa and Poirier, 1988).

Controversy exists over the mechanism of action of complete carcinogens (high or repeated doses of some initiating agents). The most common interpretation is that complete carcinogens have both initiating and promoting ability (Slaga, 1984; Weinstein, 1988), while another interpretation is that promotion is not necessary for complete carcinogenesis (Scribner and Scribner, 1982).

Biochemical, cytogenetic, molecular genetic and immunological evidence indicates that tumors are clonal in origin, i.e. arise from a single altered cell (Nowell, 1986; Nicolson, 1987). However, cancers are not static, but rather are composed of heterogeneous sub-populations of cells undergoing a dynamic evolution, most often resulting in invasion and metastasis, but occasionally resulting in regression (Nowell, 1986; Nicolson, 1987). The term progression was initially used to refer to the total development of a neoplasm, beginning from the first initiated cell (Foulds, 1954; Boutwell et al., 1982). However, progression has since come to be considered by some as a third open-ended stage in carcinogenesis following initiation and promotion, a stage characterized by the acquisition of increasing malignancy (Weinstein et al., 1984). The conversion of tumors from benign to malignant is clinically one of the most critical steps in carcinogenesis, and is affected by agents other than tumor promoters (Hennings et al., 1983). Attempts to define these

three sometimes overlapping stages, initiation, promotion, and progression, have led to insights into the mechanisms of carcinogenesis.

1.2 Initiation

Initiation is generally achieved by a single low dose of a carcinogen, insufficient by itself to elicit tumor formation. DMBA and benzo(a)pyrene (BP) are two examples of the polycyclic aromatic hydrocarbon carcinogens, for which there is a good correlation between their tumor-initiating activities and ability to form covalent DNA adducts (Slaga, 1984; Harris, 1987). Initiation is irreversible; the time lapse between application of the initiator and the first application of the promoter can be anywhere between one week and at least one year (Boutwell, 1964). These findings and others have led to the conclusion that initiators act by causing somatic cell mutations (Slaga, 1984). This is supported by the finding that 90% of 175 carcinogens tested were mutagenic in the Salmonella/microsome test (McCann and Ames, 1976).

Many compounds, including DMBA and BP, are carcinogenic only after enzymatic activation (Harris, 1987). BP is activated by the microsomal cytochrome P-450 monooxygenase system to yield benzo(a)pyrene-7,8-diol-9,10-epoxide, which reacts with the 2-amino group of guanine residues present in nucleic acids (Weinstein, 1988). Carcinogen-DNA adducts create conformational changes in the DNA, generally interfering with base pairing and resulting in errors during replication or attempted repair. Initiation appears to be the critical step that leads to the genetic abnormalities characteristic of cancerous cells, i.e. point mutations, gene amplifications, deletions, insertions, and major rearrangements such as

chromosomal translocations (Weinstein et al., 1984). Specific genetic targets will be discussed in section 1.5.

There is both tissue and individual variation (from 10- to 150-fold) in capacity for metabolic activation of procarcinogens (Harris, 1987). This variation, along with differences in deactivation and DNA repair rates, no doubt contributes to individual variations in cancer risk (Yuspa and Poirier, 1988). Several classes of compounds can inhibit tumor initiation. The flavones and antioxidants appear to inhibit the metabolic activation of procarcinogens, while other inhibitors act by inducing metabolic detoxification pathways or by killing or slowing the growth of initiated cells (Slaga, 1984).

Most initiators can act as complete carcinogens when given at a sufficiently high dose. However, there are compounds (e.g. urethane) that can act as mouse skin tumor initiators but not complete carcinogens, requiring the subsequent action of a tumor promoter (Slaga, 1984). The existence of compounds with these properties supports the idea that promotion is a required stage in carcinogenesis.

1.3 Promotion

1.3.1 General Characteristics

The first substance used as a tumor promoter in mouse skin was croton oil, of which the active components are phorbol-12,13-diesters including TPA, which is the most potent (Hecker, 1968). A wide variety of chemical compounds other than TPA possess strong to weak activity as mouse skin tumor promoters. The list includes teleocidin, anthralin, benzo(a)pyrene, benzoyl peroxide, tobacco smoke condensate, certain fatty acids, phenolic compounds, and surface active agents (Slaga, 1984). Promotion is

initially reversible, requires a certain frequency of application of the promoting agent, and appears to lead to an increase in tumor incidence with no change in character or kinetics. The order of events in this model of two-stage carcinogenesis is critical. If a tumor promoter is repetitively applied before the initiating carcinogen, no tumors develop (Slaga, 1984).

Evidence has accumulated demonstrating that promotion itself can be divided into two operationally distinct stages, conversion (Stage I) and propagation (Stage II) (Boutwell, 1964; Slaga et al., 1980). Conversion (Stage I) requires only a single application of the promoter, has a half-life of 10-12 weeks and, surprisingly, can be effected prior to initiation (Kinzel et al., 1986; Ewing et al., 1988b). Stage II requires multiple frequent applications of the promoter following initiation (Slaga et al., 1980). TPA and all of the compounds described previously can act as complete promoters. In SENCAR mice, mezerein and L-α-dioctanoylglycerol are incomplete but potent Stage II promoters, whereas 4-O-methyl-TPA and the calcium ionophore A23187 are incomplete but weak Stage I promoters (Slaga et al., 1980; Ewing et al., 1988b; Verma, 1988a). These studies support the idea that conversion, initiation, and Stage II promotion are mechanistically distinct stages of carcinogenesis.

1.3.2 Mechanisms

Unlike initiators, promoters are thought to act largely at the epigenetic level, since they do not bind to DNA and initially act reversibly. Tissue responses to tumor promoters are pleiotropic and have been best characterized in mouse skin. Induction of inflammation and hyperplasia may be necessary but not sufficient steps (Boutwell, 1964; Slaga et al., 1980). One of the key responses appears to be the induction

of terminal differentiation in normal cells and the blockage of terminal differentiation and induction of cell proliferation in initiated cells (Friedman, 1981; Yuspa, 1985; Harris, 1987). Normal mouse epidermal cells cultured in low Ca^{2+} (which maintains an undifferentiated state) respond heterogeneously to TPA: some (mature cells) are induced to terminally differentiate, while others (stem cells) are induced to proliferate and become transiently refractory to terminal differentiation (Yuspa, 1985). Multiple exposures to TPA select and expand the proliferating population. Cells initiated in vivo or treated with a carcinogen in vitro escape the terminal differentiation-inducing effects of Ca^{2+} or TPA and continue to proliferate (Yuspa, 1985). Such differences in control of growth and differentiation of initiated versus normal cells and selection by tumor promoters could lead to clonal expansion of preneoplastic and neoplastic cells.

Effects of tumor promoters on epidermal cells in vivo include: (1) induction of DNA, RNA and protein synthesis; (2) an increase in the number of dark basal keratinocytes (primitive stem cells) and proteins characteristic of less differentiated cells; (3) induction of ornithine decarboxylase activity followed by an increase in polyamine levels; (4) increased synthesis of prostaglandins; and (5) increased turnover of phosphatidylcholine (Boutwell et al., 1982; Slaga, 1984). These changes correlate well with tumor formation, and agents which block certain of these responses also block tumor formation (Boutwell et al., 1982; Slaga, 1984). Many of these changes may be markers of the altered responses of preneoplastic and neoplastic cells with respect to control of growth and differentiation. For example, TPA increases the levels of mRNA, protein and activity of ornithine decarboxylase (ODC), the rate-limiting enzyme

in the synthesis of the polyamines putrescine, spermidine and spermine (Weeks et al., 1984; Gilmour et al., 1987). The inhibition of ODC activity with α-difluoromethylornithine inhibits tumor promotion (Weeks et al., 1982). ODC induction and elevation of polyamine levels also occurs in many tissues in response to a growth stimulus, and may be required for DNA synthesis and progression through the cell cycle (Boutwell et al., 1982; Denhardt et al., 1986). Thus, ODC and polyamines may be involved in the induction of cell proliferation in initiated cells. Some of these changes, including induction of ODC and hyperplasia, are more pronounced after repeated application of a tumor promoter.

Another property which may be affected by tumor promoters is metabolic cooperation via cell-cell coupling. TPA reduces intercellular communication via gap junctions in many cultured cells, although this does depend on the cell type and culture conditions (Trosko and Chang, 1984). Tumor cells selectively communicated with other tumor cells but not with surrounding normal cells in coculture (Yamazaki and Katoh, 1988). However, patterns of dye spread after intracellular injection of TPA-treated mouse skin showed that functional coupling in vivo was increased, especially between compartments, i.e. epidermis and dermis, which are not normally coupled (Kam and Pitts, 1988). This study suggests that in vitro differences in cell communication may not accurately reflect the in vivo situation.

Tumor promoters may indirectly contribute to the genetic abnormalities characteristic of cancerous cells. TPA treatment leads to increases in the reactive oxygen species superoxide, hydrogen peroxide and hydroxyl radical. This may arise from increases in prostaglandins, eicosanoids and lipoxygenase activity, increased activity of the superoxide-producing

enzyme xanthine oxidase, and decreased activity of the detoxifying enzymes superoxide dismutase and catalase (Reiners et al., 1987, 1988; Fischer et al., 1988). These reactive oxygen species may be responsible for the reported effects of TPA in causing aneuploidy and structural chromosomal aberrations in primary mouse keratinocytes (Petrusevska et al., 1988). However, tumor promoters are not mutagenic in most bacterial or mammalian systems (Yuspa and Poirier, 1988). Genotoxic effects are difficult to reconcile with the initially reversible consequences of tumor promot on, but may contribute to the later irreversible effects.

Certain of these biochemical changes have been associated with either Stage I or Stage II promotion. The synthesis of prostaglandins, the induction of dark cells, and the possible cytogenetic changes appear to be important for Stage I promotion (Ewing et al., 1988b; Petrusevska et al., 1988). Most of the other changes described, including induction of ODC and polyamines, appear to be important for Stage II promotion (Slaga et al., 1980). DNA synthesis may be necessary for both stages (Kinzel et al., 1986; Hirabayashi et al., 1988).

Most agents which inhibit tumor promotion do so by inhibiting one or more of the above changes. Inhibitors of tumor promotion include polyamine and prostaglandin synthesis inhibitors, antioxidants, anti-inflammatory steroids, hydroxyurea, protease inhibitors, phosphodiesterase inhibitors, cyclic nucleotides, and vitamin A derivatives (Boutwell et al., 1982; Slaga, 1984; Kinzel et al., 1986; Petrusevska et al., 1988).

Vitamin A (retinol) and retinoic acid inhibit the TPA-induced increases in ODC mRNA, protein and activity, modify the induction of differentiation, and inhibit TPA-mediated tumor promotion, both Stage I and II (Verma, 1985, 1988b; Yuspa, 1985). However, retinoids do not

inhibit carcinogenesis (or induction of ODC) by multiple applications of DMBA (Boutwell et al., 1982; Weeks et al., 1984). It was hoped that vitamin A might be useful as a preventative measure against carcinogenesis, but effects in experimental animals were mixed, indicating carcinogen and organ specificity (Verma, 1988b).

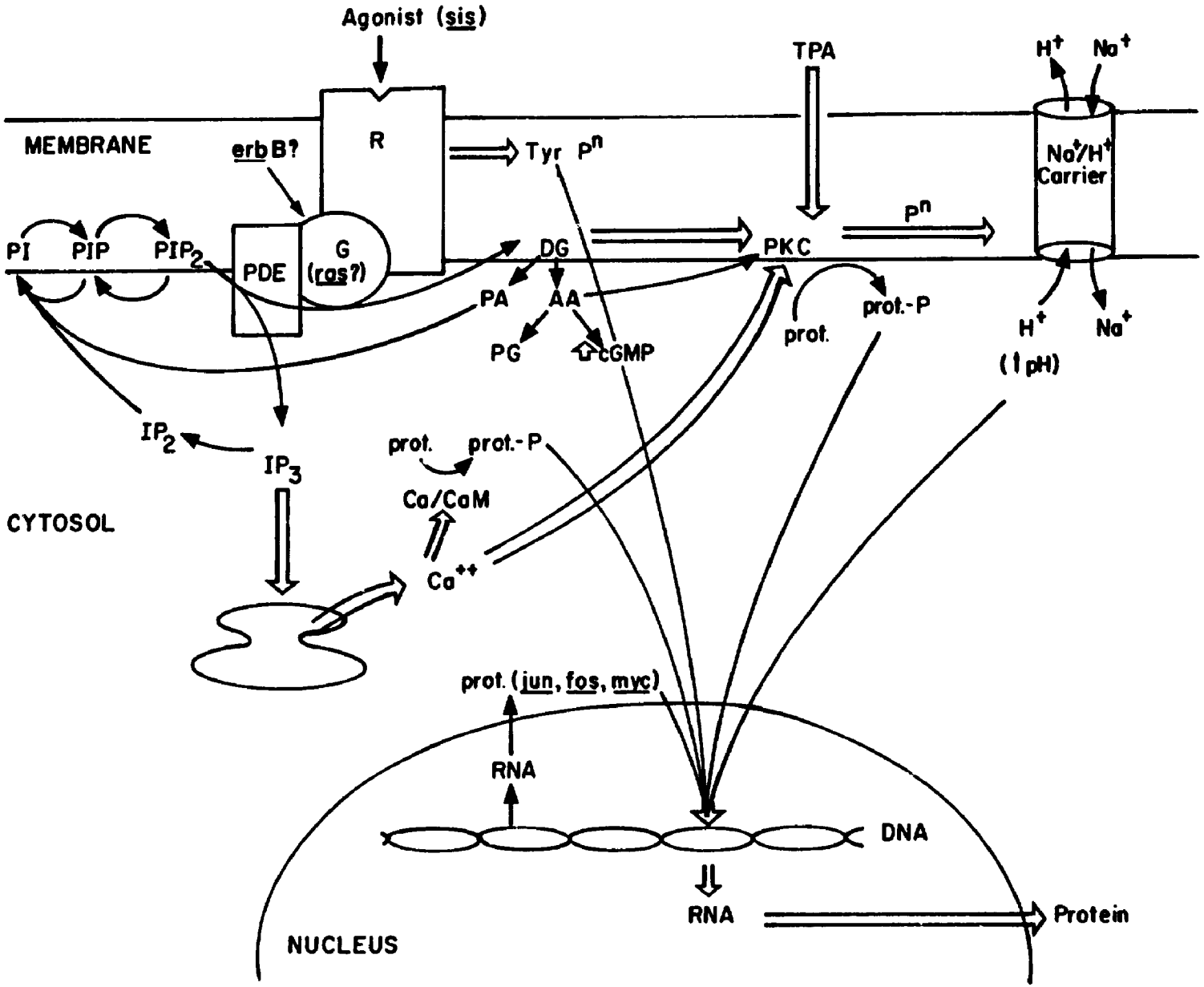
1.3.3 Role of Protein Kinase C

A major breakthrough in understanding the mechanism of tumor promotion was the discovery that the cellular receptor for the phorbol ester tumor promoters is protein kinase C (PKC; Castagna et al., 1982; Blumberg, 1988). PKC, first described in 1977 as a proteolytically activated protein kinase in many tissues (Takai et al., 1977; Inoue et al., 1977), is also functional without proteolysis in a Ca^{2+}-activated phospholipid-dependent manner (Nishizuka, 1984). The enzyme is a ≈77 kDa monomer which phosphorylates serine and threonine (but not tyrosine) residues on a broad range of cellular proteins; there appears to be a requirement for basic residues near the phosphorylation site (Edelman et al., 1987). Activation by diacylglycerol, which is transiently produced from inositol phospholipids in response to extracellular signals, has linked PKC to a major cellular signal transduction mechanism (Nishizuka, 1984). PKC appears to play a crucial role in physiological processes as diverse as cell proliferation, differentiation, tumor promotion, endocrine secretion, neurotransmission, and the sustained neuronal responsiveness associated with memory (Nishizuka, 1986; Berridge, 1986b; Alkon and Rasmussen, 1988).

The signal transduction mechanism involving PKC is outlined in Fig. 1.1. The binding of certain growth factors, neurotransmitters and hormones to their receptors activates a phosphodiesterase (phospholipase

Figure 1.1

Signal transduction pathway involving inositol lipid metabolism, Ca^{2+} mobilization, and activation of protein kinase C. Activation of this pathway by the binding of agonists to their specific receptors can lead to various cellular responses, as discussed in the text. Mitogens which activate this pathway in 3T3 cells include platelet derived growth factor, bombesin, vasopressin, and thrombin (Berridge, 1986); epidermal growth factor may be an activator in some cells (Berridge, 1984). Oncogenes whose products form part of this pathway are indicated. Symbols are: R, receptor; Tyr P^n, tyrosine phosphorylation; G, G-protein; PDE, phosphodiesterase (phospholipase C); PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; Ca/CaM, calcium/calmodulin-dependent protein kinase; prot., protein; prot.-P, phosphorylated protein; DG, diacylglycerol; PA, phosphatidic acid; AA, arachidonic acid; PG, prostaglandin; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; P^n, phosphorylation.


Agonist (sis)
TPA
H+ Na+
MEMBRANE
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Tyr Pn
Na+/H+ Carrier
PI PIP PIP2
PDE
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DG
PKC
Pn
PA
AA
PG
cGMP
prot.
prot.-P
H+ Na+
(↓pH)
IP2
IP3
prot. prot.-P
Ca/CaM
CYTOSOL
Ca++
prot. (jun, fos, myc)
RNA
DNA
RNA
Protein
NUCLEUS

C), likely via a GTP-binding protein, to hydrolyze phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) (Berridge, 1984; Berridge and Irvine, 1984). The rapid transient increases in the two second messengers, IP_3 (soluble) and DG (membrane-bound) leads to Ca^{2+} mobilization from the endoplasmic reticulum, and activation of PKC, respectively (Berridge and Irvine, 1984). IP_3 can also be converted to more highly phosphorylated forms, and DG can be metabolized to arachidonic acid and prostaglandins, which may themselves act as second messengers (Berridge, 1984). Although the later stages along this bifurcating pathway are not yet clear (and are also quite complex, involving interactions with other signal transduction pathways), it is clear that PKC activation and Ca^{2+} mobilization are both essential and often act synergistically to bring about the physiological responses listed above (Nishizuka, 1984; Takai et al., 1985).

The transient increase in intracellular Ca^{2+} activates calmodulin-dependent protein kinases and can also activate PKC. One of the major effects of Ca^{2+} mobilization and PKC activation, largely via protein phosphorylation, is a rearrangement of cytoskeletal systems (Takai et al., 1985; Denhardt et al., 1989). PKC phosphorylates and activates the Na^+/H^+ carrier, causing an increase in intracellular pH, which, along with the increase in Ca^{2+}, is one of the early ionic changes associated with, but not necessarily required for, stimulation of cell proliferation (Berridge, 1984; Berridge and Irvine, 1984; Cook et al., 1988). Other PKC substrates which may be important for growth control include the ribosomal S6 protein, histones, DNA methyl transferase, DNA topoisomerase II, RNA polymerase II, ras p21, the epidermal growth factor receptor, and the insulin receptor (Nishizuka, 1986; Denhardt et al., 1989). These examples

illustrate the central role of protein phosphorylation, by protein kinase C and other kinases, in signal transduction.

Phorbol ester tumor promoters, including TPA, intercalate into the plasma membrane, mimic DG (see Fig. 1.2A), and bind to and directly activate PKC (Castagna et al., 1982), activating one arm of this pathway. Both DG and TPA activate PKC by causing translocation from a cytosolic to a plasma membrane-bound form and concomitantly increasing the affinity for Ca^{2+} (Edelman et al., 1987). PKC is subject to autophosphorylation, which may increase its affinity for both Ca^{2+} and phorbol esters (Edelman et al., 1987).

PKC has not been reproducibly detected in nuclei (Weinstein, 1987). However, Ruff et al. (1989) recently reported that in NIH3T3 cells the distribution of PKC activity changed upon TPA stimulation from 85-95% cytosolic, 5-10% plasma membrane-associated and 1-3% nuclear to 10% cytosolic, 60-80% plasma membrane-associated and 20-40% nuclear. The protein kinase molecule contains a domain similar to the consensus cysteine- and zinc-containing DNA-binding fingers present in many transcriptional regulators, but there is no evidence that PKC binds to DNA (Nishizuka, 1988). It is clear that activation of PKC brings about changes in gene expression, most likely indirectly through a cascade of protein phosphorylation, and that these changes in gene expression are necessary for many of the physiological responses (Nishizuka, 1986; Denhardt et al., 1989). The effect of TPA on the expression of specific genes will be further discussed in section 1.5.

In addition to the positive effects of PKC described above, PKC also exerts negative feedback control over this same and other pathways. This is important in limiting the cellular response to agonists. In the short

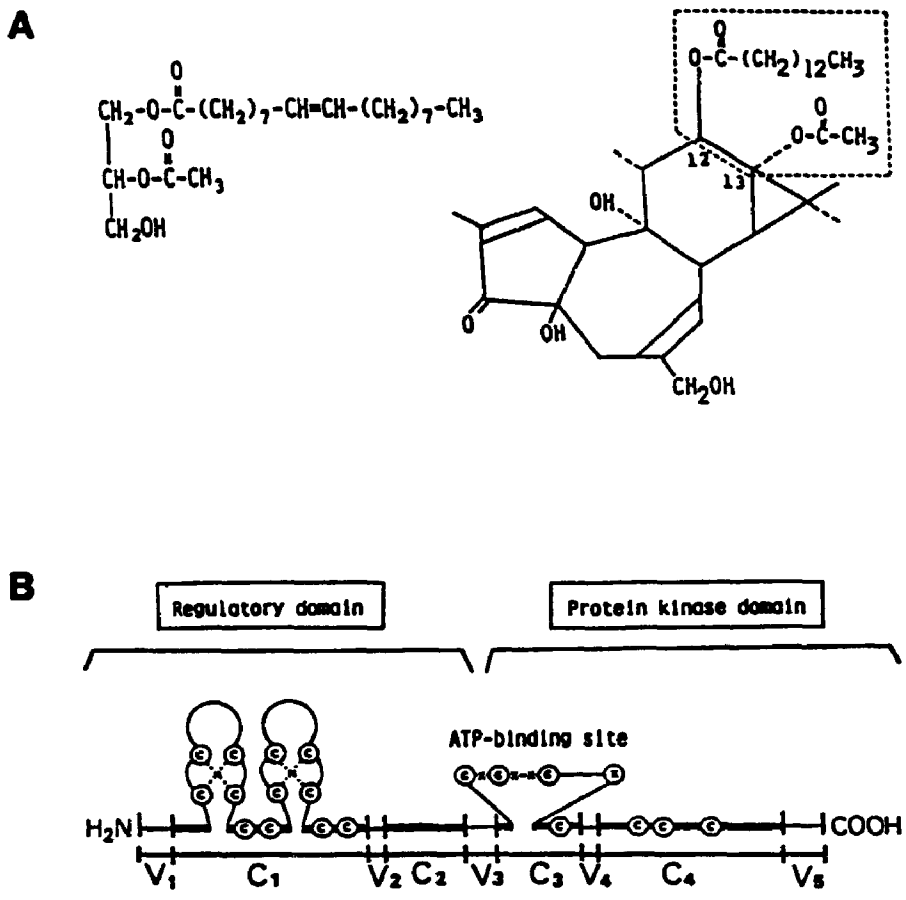


Figure 1.2

Structures of diacylglycerol, phorbol ester tumor promoter, and protein kinase C.

A. Structures of synthetic diacylglycerol (1-oleoyl-2-acetylglycerol; left) and tumor promoting phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate, right). The boxed region indicates the diacylglycerol-like structure present in TPA. From Nishizuka (1984).

B. Common structure of four members of the protein kinase C family (α, β_I, β_{II}, and γ). Four conserved (C_1-C_4) and five variable (V_1-V_5) regions are indicated. C, G, K, X and M represent cysteine, glycine, lysine, any amino acid, and metal ion, respectively. From Nishizuka (1988).

term, PKC appears to be involved in decreasing the IP_3-induced elevation of intracellular Ca^{2+} levels, by decreasing the IP_3 signal and/or stimulating the export of Ca^{2+} (Nishizuka, 1988). Another site of feedback control is the down-regulation of certain cell surface receptors. For example, epidermal growth factor receptor phosphorylation by PKC leads to a decrease in its growth factor-binding and tyrosine-specific protein kinase activities (Nishizuka, 1986).

Until now PKC has been referred to in this thesis as a single entity. However, there are at least seven different forms or subspecies of PKC, which have been described by Nishizuka et al. (1988). The common structure of α, β_I, β_{II} and γ is shown in Figure 1.2B. Subspecies δ and ε lack the C_2 region, and ζ lacks the C_2 region and one of the cysteine-rich sequences in C_1. Most of these species are produced from different genes, although β_I and β_{II} appear to be derived from a single gene by alternate splicing of the primary transcript.

It seems likely that the diverse biological roles of PKC involve activation of different subspecies. While this idea has not been experimentally verified, some important general principles have been determined (Nishizuka, 1988). There are differences in the distribution of the subspecies at the tissue, cellular, and even sub-cellular levels. Most cell types express more than one sub-species of PKC, and most of the subspecies are present in many tissues, with the exception of γ and ε, which appear to be specific to the brain and spinal cord. The subspecies also differ in kinetics and sensitivity to activation by Ca^{2+}, DG, and arachidonic acid (Nishizuka, 1988). There are also likely differences in substrate specificity. An important observation with respect to tumor

promotion is that several subspecies of PKC co-expressed in a single cell type disappeared at different rates upon treatment with TPA (Nishizuka, 1988).

The different physiological responses to TPA versus diacylglycerol may be due to differential effects on individual subspecies of PKC, effects of TPA not mediated through PKC and/or differences in stability. Whereas endogenous or permeable synthetic DGs are rapidly metabolized, TPA is hardly degraded, and its continued presence distorts the temporal sequence of events (Nishizuka, 1986). While a single addition of DG is ineffective at mimicking TPA in inducing differentiation of HL60 cells, bihourly treatments with DG are effective (Ebeling et al., 1985). There is a recent report that phospholipids, Ca^{2+}, and phorbol esters stimulate formation of a membrane-inserted form of PKC whose activity is no longer dependent on Ca^{2+} and phorbol esters, suggesting that sustained activation of PKC could occur (Bazzi and Nelsestuen, 1989). There are also many reports that prolonged TPA treatment leads to a subsequent down-regulation of PKC and a period in which the cells are refractory to further stimulation (Nishizuka, 1986, 1988; Blumberg, 1988). A single application of TPA to mouse epidermis *in vivo* led to complete down-regulation of PKC activity for at least 72 hours (Fournier and Murray, 1987). This may be due to proteolysis, since calpain I cleaves PKC in the presence of phospholipid and DG or TPA (i.e. the activated form), yielding an independently active kinase fragment which is rapidly removed from the cell (Nishizuka, 1988). It has been suggested that the TPA-induced removal of PKC may relieve the negative feedback control exerted by the enzyme and lead to uncontrolled cell proliferation (Nishizuka, 1988).

Thus, TPA and similar compounds may exert their tumor promoting effects largely by subversion of a normal cellular pathway involved in cell proliferation and differentiation. However, the role of PKC activation in tumor promotion is not entirely clear. An obligatory role of PKC has not been proven (Verma, 1988a). At least three chemically distinct classes of mouse skin tumor promoters, phorbol esters, teleocidin, and aplysiatoxin, are potent activators of PKC (Weinstein et al., 1984). Some compounds that activate PKC, namely mezerein and several diacylglycerols, are inactive as complete promoters but active as Stage II mouse skin tumor promoters (Slaga et al., 1980; Verma, 1988a). Single or multiple applications of DG to mouse skin in vivo effectively induced transient increases in ODC activity and DNA synthesis, but not epidermal hyperplasia and inflammation, or increases in dark basal cells (Hirabayashi et al., 1988). It seems unlikely that all agents that act as tumor promoters directly activate PKC, but it is possible that they may indirectly lead to PKC activation. Certain bile acids implicated in colon carcinogenesis can activate PKC (Weinstein, 1988). However, although tumor promoters in different organs appear to act by selective growth stimulation of initiated cells or cytotoxicity to normal cells, they may act by entirely different molecular mechanisms (Yuspa and Poirier, 1988).

Tumor promotion can be viewed as selection and expansion of initiated cells accompanied by further, eventually permanent, changes to these cells. Papillomas resulting from an initiation-promotion protocol can be either independent or dependent on further application of the tumor promoter for growth and survival (Ewing et al., 1988a). The development of promoter-independent papillomas suggests that some kind of permanent change has occurred. This change may involve a second "spontaneous"

mutation (see below), or may be due directly to effects of the tumor promoter, such as relatively stable changes in gene expression, perhaps mediated by changes in DNA methylation or other non-mutagenic events.

1.4 Progression

While a fairly large number of papillomas are formed as a result of mouse skin initiation-promotion, only a small proportion develop into malignant carcinomas. A three-stage model of carcinogenesis was employed to determine the effect of various agents on conversion of papillomas to carcinomas (Hennings et al., 1983). After DMBA initiation and TPA promotion to produce a large number of papillomas, Stage III treatment for weeks 11-40 with TPA had no effect on malignant conversion, but treatment with three different initiators increased the conversion rate from 1.4% to 3.6-4.3% (Hennings et al., 1983). This experiment may be relevant to the mechanism of complete carcinogenesis by repeated doses of DMBA, which produces fewer papillomas but many more carcinomas than the initiation-promotion protocol (Saffiotti and Shubik, 1956). This evidence and more suggests that the induction of malignant tumors requires two or more carcinogen-induced mutations, and that the role of promotion is to enlarge the size of the target cell population available for the second mutation (Hennings et al., 1983).

Consistent with this hypothesis, the nature of all three stages has an effect on tumor progression. The malignant conversion rate and carcinoma incidence depend on the initiator dose and the promoter dose, duration, and type (Ewing et al., 1988a). Generally, as the number of papillomas per mouse increased, due to either high initiating or promoting doses, the percent conversion decreased. While part of this difference

may be due to high tumor burden and TPA toxicity, it does suggest that there are different types or stages of papillomas with different probabilities of progressing to carcinomas (Ewing et al., 1988a). Analysis of individual papillomas at 20-35 weeks of promotion showed sequential development of aneuploidy, keratin modifications and increased γ-glutamyltransferase expression (markers of progression towards malignancy), suggesting that progression probably is occurring in the majority of lesions, at differing rates (Aldaz et al., 1988). This is supported by the observation that, when generally measured at 40-60 weeks of promotion, carcinoma incidence is usually still increasing (Aldaz et al., 1988).

Carcinomas were distinguished from papillomas in the above studies by morphological and histological signs of invasion. Most cancers, including squamous cell carcinomas, are formed from epithelia, and therefore invasion entails penetration of the underlying mesenchyme, i.e. basement membrane and interstitial stroma. The basement membrane is a continuous flexible structure made up of type IV collagen, specific glycoproteins such as laminin and entactin, and heparin sulfate proteoglycans (Liotta, 1986). An early sign of progression is defective basement organization, due to decreased synthesis or abnormal assembly of secreted components and/or increased proteolysis (Liotta, 1986). It has been proposed (Liotta, 1986; Liotta et al., 1988) that invasion of extracellular matrix occurs by a cyclic repetition of three steps: (1) tumor cell attachment via cell surface receptors for laminin (basement membrane) or fibronectin (stroma); (2) local degradation of the matrix by tumor-derived proteases, including type IV collagenase; and (3) tumor cell locomotion into the region of degraded matrix, stimulated by chemotactic factors such as the autocrine motility factor.

Metastasis to other organs involves invasion of surrounding normal host tissue, penetration of blood vessels or lymphatics, release of small clumps or single neoplastic cells, survival against the physical, chemical and immunological forces in the circulation, arrest in the capillary beds of a distant organ, penetration of the vessel wall, invasion of the new tissue, and growth to form a secondary tumor (Hart, 1986; Chambers, 1989). Assays to study this phenomenon are of two general types (Chambers, 1989). "Spontaneous" metastasis assays model the whole process, from implantation of tumor cells in a tissue in a host animal and formation of a primary tumor to detection of tumor growth at distant sites. "Experimental" metastasis assays model the latter half of hematogenous spread, from intravenous injection of tumor cells to detection of tumor growth in distant organs.

These types of assays as well as clinical studies indicate that the sites of metastatic spread depend on the site and type of the primary tumor. The reason for site specificity appears to be due partially to anatomical location and sieving effects which determine distribution, and, perhaps to a larger extent, to differential adhesion, survival, and growth in a favourable or unfavourable metabolic environment (the "seed and soil" hypothesis; Hart, 1986; Doerr et al., 1989; Juacaba, 1989). Factors which appear to facilitate metastatic spread of tumor cells include: enhanced secretion of proteases, such as collagenases, plasminogen activators and cathepsins B and L; decreased secretion of protease inhibitors, such as tissue inhibitor of metalloproteinases; aberrant expression of cell adhesion molecules, substrate adhesion molecules, and their receptors; changes in the expression and organization of cytoskeletal proteins; secretion of autocrine motility factors; enhanced responsiveness to

chemotactic factors; changes in major histocompatibility antigens; and production of autocrine growth factors, growth factor receptors, and tumor angiogenesis factor (Hart, 1986; Nowell, 1986; Denhardt et al., 1987; Prodi et al., 1988; Khokha et al., 1989). These factors contribute to increased invasiveness, cell motility, escape from immune surveillance, or growth at a new location.

Although selection is never 100% efficient due to the random nature of the process, metastatic variants can be selected from a heterogeneous population of cells (Hart, 1986). It is clear from the use of phenotypic and genetic markers that metastatic variants in fact comprise only a small sub-population of cells in a primary tumor (Nowell, 1986; Waghorne et al., 1988). Metastatic cells proliferating at secondary sites again rapidly diversify to a heterogeneous population of cells differing in metastatic efficiency and other properties (Nicolson, 1987). Even clonal isolates of highly metastatic cells often rapidly lose metastatic ability during in vitro growth (Chambers, 1989). Such findings can be accommodated by a model of dynamic heterogeneity, which suggests that metastatic cells are generated and lost rapidly, and the majority of cells in a "metastatic" population do not fully express a metastatic phenotype at any one time (Chambers, 1989). The phenotypic instability in metastatic potential and other properties of tumor cell populations may be due to genetic and/or epigenetic changes in sub-populations of cells.

There is a large body of evidence indicating that tumor cells are more genetically unstable than comparable normal cells (Nowell, 1986; Kendal and Frost, 1988). Increases in chromosomal abnormalities accompany an increasingly malignant (metastatic) phenotype (Nicolson, 1987). There is limited and controversial evidence that genomic instability, measured

by the rate of production of cytogenetic abnormalities and spontaneous mutations, may be further increased in more highly malignant tumors (Cifone and Fidler, 1981; Kendal and Frost, 1988). The enhanced mutability characteristic of tumor cells is likely due to defects in DNA synthetic and repair enzymes and in proteins of the mitotic spindle; initial chromosomal abnormalities can also contribute to further genetic errors (Nowell, 1986).

However, rates of phenotypic variant formation in metastatic ability or other biochemical or immunological properties of tumor cells are on the order of 10^{-3} to 10^{-2} variants/cell/generation, and reversion rates may be even more rapid (Nicolson, 1987). This cannot be explained by genotypic instability and selection alone, since even for highly metastic mouse cells one of the highest spontaneous mutation rates reported is $\approx 7 \times 10^{-5}$ mutations/cell/generation (Cifone and Fidler, 1981). Changes in gene expression modulated by tumor cell microenvironments may be responsible for this rapid phenotypic diversification (Nicolson, 1987). Soluble growth factors, and interactions with infiltrating host cells, extracellular matrix and adjacent tumor cells can affect gene expression and malignant phenotype (Nicolson, 1987).

1.5 Role of Gene Expression in Carcinogenesis

1.5.1 Oncogenes

A genetic contribution to cancer is clear from the fact that mice can be bred for susceptibility or resistance to complete and two-stage carcinogenesis (Boutwell, 1964). As discussed above, mutation of cellular genes by chemical carcinogens or initiators is a key event in carcinogenesis. Analysis of transforming retroviruses and tumor cell DNA

transfection experiments for genes able to dominantly transform cultured mouse NIH3T3 fibroblasts have allowed the identification of some of the cellular loci involved - the cellular oncogenes (Bishop, 1985). When activated by mutation, amplification, or inappropriate expression, these genes contribute to the abnormal behaviour of tumor cells (Weinstein, 1987). There are ≈30 clearly defined oncogenes to date, although the total number has been estimated at several hundred (Weinstein, 1987).

Based on the altered properties of tumor cells, one might expect that the cellular oncogenes normally play a major role in the control of growth and differentiation. Indeed, this is the case: *sis* encodes the platelet derived growth factor (PDGF) B-chain; *fms* encodes the colony stimulating factor-1 receptor, *erbB* encodes a truncated epidermal growth factor (EGF) receptor, and *erbA* is homologous to steroid receptors; *ras* encodes a GTP-binding protein which may function in signal transduction; and *fos*, *jun*, and likely *myc*, encode transcriptional activators (Berridge and Irvine, 1984; Heldin and Westermark, 1984; Weinstein, 1987; Chiu et al., 1988; Sassone-Corsi et al., 1988). The growth factor receptors and related oncogene products (including not only *erbB* and *fms*, but also *src*, *ros*, *abl*, and a number of other oncogenes) are plasma membrane-associated protein-tyrosine kinases (Hunter, 1986). It seems likely that these oncogene products transform through unregulated tyrosine phosphorylation. The *mos* and *mil/raf* oncogenes encode soluble protein-serine kinases (Hunter, 1986).

Some of these oncogene products (*sis*, *erbB*?, *ras*?) act on the same signal transduction pathway as does TPA, acting earlier in the pathway and inducing both Ca^{2+} mobilization and PKC activity (Fig. 1.1). The *fos* and *jun* products act later in this pathway, forming a complex (AP-1) to

enhance transcription of specific genes in response to stimulation by TPA or transformation by ras, src, or mos (Schonthäl et al., 1988; Chiu et al., 1988; Sassone-Corsi et al., 1988). It had been suggested that src and ros catalyze the phosphorylation of phosphatidylinositol (Berridge and Irvine, 1984), but subsequent studies with the purified oncogene products showed that they were lacking inositol lipid kinase activity (Berridge, 1986a). Thus, the data support the idea that oncogene products participate in pathways controlling cell proliferation, and that protein phosphorylation and activation of gene transcription are two key processes.

While a single oncogene can transform immortal NIH3T3 cells, transformation of primary rat embryo fibroblasts requires two cooperating oncogenes (Land et al., 1983). Two complementation groups have been identified: myc, polyoma large-T, or adenovirus E1a, which all encode nuclear proteins, can induce in vitro establishment, and can cooperate with either ras or polyoma middle-T, whose gene products are localized to the plasma membrane and implicated in morphologic alterations and anchorage independence (Land et al., 1983). Depending on the cell line, transfection with a single oncogene can bring about some or all of the markers of the transformed phenotype, such as immortalization, altered morphology, production of foci, abrogated requirement for growth factors, anchorage independent growth, and production of tumors in syngeneic or immune deficient hosts. Transfection with oncogenes can also lead to abnormal states of differentiation (Buckley, 1988). The idea of cooperation between oncogenes in effecting in vitro transformation does nicely support the multistage nature of in vivo carcinogenesis described above, and in particular the proposal that at least two mutational events are required.

The importance of activation of these oncogenes in spontaneous cancers is not clear (Weinstein, 1988). Nineteen of 26 human cellular oncogenes have been mapped to chromosomal regions associated with cancer-specific rearrangements (Heim and Mitelman, 1986). Mutations at codon 12 or 61 of ras, and overexpression of ras and myc are frequently found in spontaneous tumors, whereas src activation has not been reported (Weinstein, 1987). Activation of other oncogenes appears to be associated with specific cancers, e.g. sis with fibrosarcoma and glioma, and erbB with squamous cell carcinoma and glioblastoma (Chambers and Tuck, 1989). These observations suggest that some oncogenes are more easily activated than others, and that cell and tissue specificity exists in susceptibility to effects of specific oncogene activation.

Oncogene activation can contribute to both initiation and progression, and possibly even promotion; there appears to be no obligatory sequence of gene activation (Colburn and Smith, 1987; Harris, 1987). Introduction of viral ras into mouse epidermal cells in vivo can replace chemical carcinogens as an initiating agent, causing tumor formation after TPA promotion (Brown et al., 1986). Over 90% of DMBA-initiated mouse skin tumors had an A-T transversion at codon 61 of H-ras, consistent with the specificity of DMBA in producing DNA adducts at deoxyadenosine residues (Quintanilla et al., 1988). Carcinogen-specific mutations with other agents have been reported, although this is not always the case (Quintanilla et al., 1988; Weinstein, 1988). Seven of nine papillomas and carcinomas induced by tumor promotion of uninitiated mouse skin also had mutations at codon 61 of H-ras; this may be due to clastogenic effects of tumor promoters or, more likely, spontaneous mutations (Pelling et al., 1988). Transfection of mouse epidermal cell lines derived from

carcinogen-induced papillomas with activated H-ras induced malignant progression (Harper et al., 1986), and high levels of activated H-ras expression are associated with mouse skin carcinomas (Quintanilla et al., 1988). Levels of activated H-ras expression correlated quantitatively with experimental metastatic ability of transfected NIH3T3 cells (Hill et al., 1988).

1.5.2 TPA-Inducible Genes

In view of the variety of effects of TPA stimulation and PKC induction on growth, differentiation, and neuromodulation in different cell types, it is not surprising that TPA changes the expression of a large number of genes. These changes are cell-type specific and not necessarily mediated by PKC. Only those changes which appear to be most relevant to tumor promotion will be discussed here. As one might expect, there is considerable overlap between the sets of genes induced by TPA, growth factors, and oncogenic transformation. However, a direct involvement of specific gene expression in tumor promotion has rarely been demonstrated, and effects of non-phorbol ester tumor promoters on gene expression have not been well studied.

A number of genes or proteins whose expression is affected by TPA are listed in Table 1.1. Some of these changes are associated with the mitogenic effect of TPA on cultured fibroblasts, others with the differentiation-inducing effect of TPA on erythroid cells, lymphocytes, or other cell lines. Some of the proteins whose expression is induced by TPA are also substrates of PKC, including the glucose transporter, the epidermal growth factor receptor, and the interleukin-2 receptor (Nishizuka, 1986). The products of two of these genes, fos and jun, which are rapidly and transiently induced by TPA in fibroblasts, form transcrip-

TABLE 1.1

GENES OR PROTEINS INDUCED BY TPA[1]	
fos	growth hormone[7]
jun[2]	prolactin
myc	proliferin/MRP
sis	transforming growth factor-ß[8]
fms	
pro-1	EGF receptor
	IL-2 receptor
ornithine decarboxylase	
	interleukin-2
keratins[3]	γ-interferon
epidermal transglutaminase[4]	$ß_2$-interferon
	oncostatin M
metallothionein IIA	
	proenkephalin
vimentin	
actin	lipid-binding protein[9]
collagenase	glucose transporter
cathepsin L/MEP	
transin[5]	ubiquitin[9]
plasminogen activator	
	VL30
TIMP	Epstein Bar Virus BamHI L
	Adenovirus E1A & E1B
calcitonin[6]	Polyoma Virus
	SV40
histocompatibility H-2K[b7]	Bovine Papilloma Virus

GENES OR PROTEINS REPRESSED BY TPA	
procollagen	glycophorin
keratins[3]	

[1]From Denhardt et al. (1989) unless otherwise specified.
[2]Lamph et al. (1988).
[3]Molloy and Laskin (1987).
[4]Lichti and Yuspa (1988).
[5]Matrisian et al. (1986).
[6]De Bustros et al. (1985).
[7]Imagawa et al. (1987).
[8]Akhurst et al. (1988).
[9]Krieg et al. (1988); and G.T. Bowden, personal communication.

tion factor AP-1, which mediates induction of a number of these other genes (Greenberg and Ziff, 1984; Lamph et al., 1988; Chiu et al., 1988; Sassone-Corsi et al., 1988). The SV40 genome, and the cellular genes encoding metallothionein IIA, collagenase, transin and interleukin-2 contain specific regulatory sequences which bind AP-1 and lead to enhanced transcription in response to TPA treatment (Angel et al., 1987b; Kerr et al., 1988). There are a number of other factors which activate transcription in response to TPA. Transcription factor AP-2 is particularly interesting because it mediates induction independently by two different signal transduction pathways, one involving TPA or DG and PKC, and the other involving cAMP and protein kinase A (Imagawa et al., 1987). Genes containing recognition elements for AP-2 include myc, metallothionein IIA, plasminogen activator, H-2K^b, growth hormone, prolactin, proenkephalin, SV40, and bovine papilloma virus (Imagawa et al., 1987; Roesler et al., 1988). Mechanisms of gene activation by TPA will be further discussed in Chapter 5.

While many genes are induced by TPA in various cell lines, induction in vivo has been demonstrated for relatively few. In mouse epidermis, TPA induces the mRNAs corresponding to fos, myc, transforming growth factor-ß (TGFß), ODC, the secreted protease transin, and three tumor-associated "mal" genes recently identified as ß-actin, ubiquitin, and a lipid-binding protein (Matrisian et al., 1986; Gilmour et al., 1986; Akhurst et al., 1988; Krieg et al., 1988; Rose-John et al., 1988; and G.T. Bowden, personal communication). In each case induction was transient, occurring early after TPA treatment (30 min - 2 h) for fos and then myc, later (3-6 h) for ODC and TGFß, and later yet (4-24 h) for transin, ß-actin, ubiquitin, and the lipid-binding protein. ODC mRNA induction occurred in

response to DG, was inhibited by retinoic acid, preceeded and was proportional to the induction of ODC activity, and appeared to occur at the transcriptional level in cultured primary mouse epidermal cells (Verma et al., 1986). ODC induction occurred predominantly in the suprabasal cells lining hair follicles (Gilmour et al., 1986). TGFß mRNA induction occurred in the upper areas of hair follicles, as well as in the highly differentiated cells of the upper epidermal layers, prompting the suggestion that it may act as an endogenous inducer of cell differentiation (Akhurst et al., 1988). Transin mRNA induction only occurred in basal cells, while ß-actin, ubiquitin, and the lipid-binding mRNAs were induced in both basal and differentiated cells (Krieg et al., 1988). The relevance of fos, myc, and TGFß to carcinogenesis has been well demonstrated (Levine et al., 1984; Vande Woude et al., 1984). The relevance of these other genes is suggested by constitutively high expression in epidermal carcinomas (Matrisian et al., 1986; Melber et al., 1986; Gilmour et al., 1987).

The genes encoding cathepsin L (MEP) and tissue inhibitor of metalloproteinase, under study in this laboratory because their level of expression is related to metastatic ability (Denhardt et al., 1987; Khokha et al., 1989), are also transiently induced by TPA in mouse epidermis (Gottesman and Yuspa, 1981; D.T. Denhardt, personal communication). The biosynthesis of epidermal transglutaminase and of specific keratins is changed after exposure to TPA, and in epidermal papillomas and carcinomas, indicative of the changes in epidermal differentiation and proliferation (Molloy and Laskin, 1987; Lichti and Yuspa, 1988; Roop et al., 1988). At least in cultured cells, the TPA-induced transcription of some of the early genes (fos, myc) is independent of protein synthesis, whereas

transcription of some of the later genes (ODC, many of the secreted proteins) depends on prior protein synthesis (Denhardt et al., 1989). It remains to be determined whether induction of these genes in vivo occurs transcriptionally, perhaps depending on AP-1, AP-2, or other factors, and what is the exact significance of each gene to tumor promotion.

1.5.3 Other Genes Relevant to Carcinogenesis

Only about 20% of the human tumors examined yielded DNA able to transform NIH3T3 cells, suggesting that most human tumors may result from recessive changes or complex multigene interactions (Weinstein et al., 1984). Deletions of specific loci are associated with retinoblastoma and osteosarcoma (chromosome 11), Wilms' tumor (chromosome 13), and also lung cancer, bladder cancer and colon cancer (Harris, 1987; Weinstein, 1988). These tumor suppressor genes, or "anti-oncogenes", are likely involved in inhibiting growth and/or inducing terminal differentiation (Weinstein, 1988). Recessive mutations in over 20 loci in Drosophila predispose to developmental abnormalities and to tumors of various types (Weinstein, 1987). The biochemical function of most of the tumor suppressor genes is not yet known, but it has been suggested that they may have effects opposing those of PKC and tyrosine protein kinases (Weinstein, 1987). The DNA segment deleted or mutated in retinoblastomas encodes a 105 kDa nuclear DNA-binding phosphoprotein; the wild-type but not a mutant protein forms complexes with adenovirus E1A, papilloma virus-16 E7 and SV40 large T oncoproteins, suggesting that these viral proteins cause transformation by inactivating the RB protein (Dyson et al., 1989; Horowitz et al., 1989). Further evidence for the existence of tumor suppressor genes derives from cell fusion experiments, in which normal human fibroblasts

can suppress the malignant phenotype, even in hybrids with cells expressing an activated ras oncogene (Harris, 1987).

Another class of genes which may be important in carcinogenesis are endogenous retrovirus-like elements, which contain cis-acting promoter and enhancer elements. Increased expression and transposition of these elements has been found in several chemically-induced murine and rat tumors (Weinstein, 1988). Carcinogen treatment can also induce a trans-acting factor capable of inducing asynchronous replication of integrated viral DNA (Weinstein, 1988). Whether such factors act to amplify cellular genes is not clear, but this may explain the ability of chemical carcinogens to act synergistically with viruses in transformation.

The above discussion has focussed on genes involved in tumor formation. There appears to be an overlapping set of genes involved in tumor progression. Genes contributing specifically to invasion and metastasis include the genes coding for specific proteases, protease inhibitors, adhesion molecules, motility factors, histocompatibility antigens, and growth and angiogenesis factors described in section 1.4. It has been suggested that oncogenes such as ras contribute to tumor progression by inducing such a set of "metastasis-relevant" genes (Chambers and Tuck, 1989). Changes in DNA methylation may be involved (Weinstein, 1988). Other strategies for identifying metastasis-associated genes include differential hybridization of DNA libraries (Steeg et al., 1985) and DNA transfection experiments (Chambers, 1989).

1.6 The TPA-Inducible Gene Designated 2ar

A project was begun in this laboratory to attempt to clone genes whose expression might be critical to tumor promotion. The system chosen

was an in vitro model of multistage carcinogenesis developed by Colburn et al. (1979; 1988). Clones of JB6 mouse epidermal cells derived from Balb/c primary cultures differ in their ability to irreversibly acquire anchorage independence and tumorigenicity in nude mice in response to tumor promoter treatment. These clones have been called promotable (P^+) or non-promotable (P^-), although it might be argued that this system models progression, i.e. malignant conversion, rather than promotion. Transformation of P^+ lines can be induced by Stage II but not Stage I promoters, and also by serum, epidermal growth factor, and transforming growth factors (Colburn et al., 1979; Lerman et al., 1986).

A cDNA library from TPA-treated JB6 P^+ (Cl22) cells was hybridized with labeled cDNA from either TPA-treated P^+ (Cl22) or P^- (Cl30) cells; a clone which was more highly represented in the P^+ population was selected and designated 2ar (Smith and Denhardt, 1987). This clone represents a 1.6 kb mRNA. Further analysis of the level of 2ar mRNA in several TPA-treated P^+ or P^- cell lines revealed that the initial screen had been misleading, and there was no correlation of 2ar expression with "promotability" of JB6 clones. However, 2ar mRNA expression was highly inducible by TPA in all of these cell lines, supporting the original idea that expression might be related to tumor promotion. Induction by TPA was biphasic; exposure of subconfluent cells to TPA resulted in a transient induction of 2ar mRNA, with a peak at 6 h, and continued exposure to TPA resulted in stable re-induction of the message as the cells became confluent (Smith and Denhardt, 1987). 2ar mRNA was also induced in quiescent 3T3 fibroblasts stimulated with serum, platelet derived growth factor, or epidermal growth factor (Smith and Denhardt, 1987).

The aim of this thesis is to explore further this unknown gene, and to attempt to answer the following questions. (1) What is the biochemical function and physiological role of the 2ar gene product? (2) What is the relevance of 2ar to carcinogenesis? (3) What are the mechanisms of regulation of 2ar expression? These questions are addressed in chapters 3, 4, and 5, respectively. Based on some of the work presented here, this gene has been given the formal designation spp and the name "secreted phosphoprotein 1", although it will still be referred to as 2ar throughout most of this thesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains, Plasmids and Phages

The Escherichia coli strains used were: RR1 for maintenance of plasmids; JM103 for growth of M13 phages; LE392 for growth of lambda phages; and NF1 for expression of ß-galactosidase fusion proteins. RR1 (Bolivar et al., 1977) has the genotype F^-, hsdS20 (r_B^-,m_B^-), ara-14, proA2, lacY1, galK2, rpsL20 (Sm^r), xyl-5, mtl-1, supE44, λ^- and can be transformed efficiently with plasmid DNA. JM103 (Messing et al., 1981), a host for M13 phages, has the genotype Δ (lac pro), thi, strA, supE, endA sbcB, $hsdR^-$, F'traD36, proAB, $lacI^q$, Z ΔM15. LE392 (Maniatis et al., 1982) has the genotype F^-, hsdR514 (r_K^-,m_K^+), supE44, supF58, lacY1 or Δ(lacIZY)6, galK2, galT22, metB1, trpR55, λ^- and is an appropriate strain for propagation of the bacteriophage λ Charon 4A. NF1, also known as K12ΔH1Δtrp, (Bernard et al., 1979; obtained from K. Stanley) is a $lacZ^-$ am strain carrying the defective λ prophage λ Nam7 Nam53 cI857 ΔH1. JM103 was grown in YT medium (0.8% Bacto-trytone, 0.5% Bacto-yeast extract, 0.5% NaCl). All other strains were grown in Luria broth (LB) (Miller, 1972), with the addition of 0.2% maltose for LE392. Stocks were maintained in media containing 15% glycerol at -70°C.

Plasmids and phages used are listed in Table 2.1. Plasmids were selected for in media containing 100 µg/ml carbenicillin (amp^r) or 12.5 µg/ml tetracycline (tet^r). The λ9090 mouse genomic library was made from Balb/c embryo DNA partially restricted with HaeIII and cloned with linkers into the EcoRI site of λ phage Charon 4A (J. Sideman, Harvard; obtained from V. Morris). Lambda phage stocks were grown and titered by the method

TABLE 2.1

PLASMIDS AND PHAGE

Plasmid/Phage	Features	Drug^r [1]	Source	Reference
pBR322	cloning vector	Amp^r, Tet^r	-	Bolivar et al., 1977
pSP64, pSP65, pGEM3, pGEM4	RNA transcription from SP6 and T7 promoters	Amp^r	Promega	Melton et al., 1984
pEX2	expression of ß-galactosidase fusion proteins from λP_R promoter	Amp^r	K. Stanley	Stanley and Luzio, 1984
pSV0CAT	chloramphenicol acetyltransferase (CAT) gene under inserted promoter; SmaI cloning site	Amp^r	A. Ridgeway	Gorman et al., 1982
pSV2CAT	SV40 promoter-CAT gene	Amp^r	A. Ridgeway	Gorman et al., 1982
pNMT	metallothionein promoter for insert expression in mammalian cells	Amp^r, Neo^r	R. Khokha	Khokha and Denhardt, 1987
pCGH	cytomegalovirus promoter for insert expression in mammalian cells	Amp^r, Neo^r	P. Waterhouse	-
pM-GAP	mouse glyceraldehdye-3-phosphate dehydrogenase cDNA	Amp^r	P. Curtis via D. Edwards	-
pODC16, pODC74	mouse ornithine decarboxylase cDNAs	Amp^r	O. Jänne	Hickok et al., 1986
pAL41	mouse non-muscle ß-actin cDNA	Amp^r	M. Buckingham	Minty et al., 1983
HB11	v-H-ras gene from Harvey murine sarcoma virus in pBR322	Amp^r	ATCC 41013	Ellis et al., 1981
2ar	original 2ar cDNA in pSS24	Amp^r	J. Smith	Smith and Denhardt, 1987
M13mp18, M13mp19	sequencing vectors	-	-	Yanisch-Perron et al., 1985

[1]Abbreviations used are: Amp^r, ampicillin resistance; Tet^r, tetracycline resistance; Neo^r, neomycin resistance.

TABLE 2.2

MAMMALIAN CELL LINES

Cell Line	Description	Culture Medium[1]	Source	Reference
JB6 Cl22	mouse epidermal, promotable	MEM + 8% FBS	N. Colburn	Colburn et al., 1979
MEF	primary Swiss mouse embryo fibroblast	DMEM + 10% FBS	this lab	-
Swiss3T3	mouse fibroblast	DMEM + 10% CS	ATCC (CCL 92)	Todaro and Green, 1963
NIH3T3	mouse fibroblast	DMEM + 10% CS	A. Chambers	Jainchill et al., 1969
PAP2	T24 H-*ras*-transformed NIH3T3	DMEM + 10% CS	A. Chambers	Hill et al., 1988
Balb/c3T3	mouse fibroblast, subcloned for flat morphology	DMEM + 10% CS	C. Parfett	-
KA31	Kirsten murine sarcoma virus-transformed Balb/c3T3	DMEM + 10% CS	B. Mukherjee	Aaronson and Weaver, 1971
DMBA 50	DMBA-transformed Balb/c3T3	DMEM + 10% CS	C. Parfett	-
NRK	normal rat kidney	DMEM + 10% FBS	A. Chambers	Duc-Nguyen et al., 1966
NRK-49F	fibroblastic subclone of NRK	DMEM + 10% CS	B. Mukherjee	DeLarco et al., 1978
KNRK	Kirsten murine sarcoma virus-transformed NRK	DMEM + 10% CS	B. Mukherjee	Aaronson and Weaver, 1971
LA23-NRK	*ts* Rous sarcoma virus-transfromed NRK	DMEM + 10% FBS	A. Chambers	Chen et al., 1977
HOS	human osteogenic sarcoma	MEM + 10% FBS	ATCC (CRL 1543)	Rhim et al., 1975a
MNNG-HOS	MNNG-transformed HOS	MEM + 10% FBS	ATCC (CRL 1547)	Rhim et al., 1975b
IMR-90	human diploid fibroblast	MEM + 10% FBS	ATCC (CCL 186)	Nichols et al., 1977

[1]Abbreviations used are: MEM, minimum Eagle's medium with non-essential amino acids; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CS, calf serum; ATCC, American Type Culture Collection (Rockville, MD). Media and sera were obtained from Gibco.

of Schlief and Wensink (1981) and stored at 4°C in SM buffer (50 mM $MgSO_4$, 100 mM NaCl, 50 mM TrisHCl, pH 7.5, 0.01% gelatin). Stocks of M13 phages ($\approx 10^{11}$ p.f.u./ml) were prepared by incubation of a plug of agar containing the M13 plaque with JM103 ($\approx 10^8$) for 18 h at 37°C. Cells were removed by centrifugation (10,000 X g for 5 min) and the supernatant was heated at 75°C for 10 min and then stored at 4°C.

2.2 Mammalian Cell Culture

Mammalian cell lines used are listed in Table 2.2. All cells were grown at 37°C in an atmosphere of 5% CO_2 at subconfluent densities and passaged by trypsinization (Freshney, 1983) approximately every 6 days, depending on the growth rate. For long-term maintenance, cells were stored in media containing 8% dimethyl sulfoxide (DMSO) in liquid nitrogen. Where indicated, NRK and LA23-NRK cells were grown at 36°C or 39°C for 36-48 h prior to and during metabolic radiolabeling and RNA isolation. TPA (LC Services Corp., Woburn, MA) dissolved in DMSO was added to 10 ng/ml (unless otherwise indicated) with a final DMSO concentration of 0.1%. Actinomycin D was added to 5 μg/ml from a 5 mg/ml stock in ethanol.

2.3 Nucleic Acid Purification

2.3.1 Purification of Plasmid and Phage DNA

For large scale plasmid preparations, cells were resuspended in 25% sucrose, 50 mM TrisHCl, pH 8.0 (1 ml/100 ml overnight culture), and lysed by the sequential addition of lysozyme to 1.3 mg/ml, EDTA to 125 mM, Brij 58 to 0.55%, and sodium deoxycholate to 0.22%. Cell debris was removed by centrifugation at 50,000 X g and plasmid DNA was isolated by centrifugation in cesium chloride (CsCl)-ethidium bromide gradients (Maniatis

et al., 1982) followed by extraction with n-butanol, ethanol precipitation (by addition of ammonium acetate to 2.5 M and 2.5 volumes of ethanol), phenol-chloroform extraction, and a second ethanol precipitation. In some cases, the DNA was further purified by spermine precipitation (Hoopes and McClure, 1981). Small scale plasmid preparations were obtained by the boiling method of Holmes and Quigley (1981) except that a phenol-chloroform extraction was done prior to ethanol precipitation. M13 RFI DNA was prepared by the same procedures after infection of JM103 at a density of 2 X 10^8 cells/ml with high titre phage stock (≈7 µl/ml, for m.o.i.≈10) and a further 3 h incubation at 37°C.

Bacteriophage λ was prepared from either liquid lysates or plate lysates by the Yamamoto purification scheme as described by Maniatis et al. (1982); in some cases the CsCl step gradients were replaced with a uniform density (1.50) suspension. Liquid lysates were prepared by addition of $MgSO_4$ to 10 mM and phage to a m.o.i. of 1 to cells at a density of 3 X 10^8/ml, followed by incubation at 37°C for 4 h and completion of lysis by addition of chloroform. DNA was prepared from the isolated lambda phage either by the method of Maniatis et al. (1982) or by addition of 1 ml of 55 mM TrisHCl, pH 8.0, 10 mM EDTA, 0.1% SDS, and 50 µg of proteinase K, followed by incubation at 37°C for 1 h, extraction with phenol-chloroform, and ethanol precipitation.

Single-stranded M13 phage was prepared as reported by Messing (1983) but with the modifications of G. Mackie (personal communication). JM103 cells (2 X 10^8 in 1.5 ml) were infected with M13 phage (m.o.i≈50) for 5-7 h at 37°C. Cells were removed by centrifugation (10,000 X g for 5 min) and phage were precipitated by addition of 0.3 ml of 25% polyethylene

glycol (PEG) 8000, 2.5 M NaCl, incubation at 25°C for 15 min then 4°C for 5 min, and centrifugation at 10,000 X g for 15 min. The pellet was resuspended in 0.8 ml of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 0.5% sarkosyl, reprecipitated with 0.2 ml of the PEG solution as above, and resuspended in 0.2 ml of TE. After extraction with phenol and phenol-chloroform, the DNA was precipitated with ethanol, and further purified by spermine precipitation.

2.3.2 Purification of DNA and RNA from Cultured Mammalian Cells

For DNA isolation, cells were homogenized in a buffer containing 30 mM TrisHCl, pH 7.6, 150 mM NaCl, 1.5 mM $MgCl_2$, and 0.2% Nonidet P-40, and centrifuged at 800 X g for 2 min to collect the nuclei. DNA was then isolated as described by Bondy and Denhardt (1983). Cytoplasmic RNA was prepared as reported by Edwards and Denhardt (1985).

2.3.3 Tissue RNA Isolation

The organs of interest were dissected from adult CD1 mice and immediately placed into liquid nitrogen. Epidermis was obtained by scraping skin that was maintained at liquid nitrogen temperatures. Total RNA was isolated from frozen pulverized mouse organs using a combination of the guanidinium isothiocyanate/hot phenol and CsCl methods (Maniatis et al., 1982). For TPA-treatment of skin, the backs of mice were shaved one day prior to treatment with the tumor promoter. The shaved area, approximately 5 cm^2, was painted with 200 µl of acetone containing 5 µg of TPA; controls were exposed only to acetone.

2.4 Gel Electrophoresis

DNA, in TE with 7% sucrose, 0.03% bromophenol blue and 0.03% xylene cyanol, was analyzed on 0.6-2.0% agarose (Seakem) gels. The running

buffer contained 50 mM TrisHCl, pH 8.0, 5 mM sodium acetate, 0.5 mM EDTA, and 0.5 μg/ml ethidium bromide. DNA was visualized and photographed under short wave ultraviolet light. For isolation of DNA from agarose gels, high purity low temperature agarose (BioRad) was used, and the DNA monitored with a long wave ultraviolet light. The fragment of interest was eluted into a trough protected by dialysis membrane, and recovered by phenol-chloroform extraction and ethanol precipitation. For isolation of several closely spaced fragments, the gel was melted and extracted with phenol and chloroform (Maniatis et al., 1982), and the DNA recovered by spermine precipitation.

For analysis or isolation of DNA fragments smaller than ≈0.7 kb, polyacrylamide gels (5%) run in Tris-borate buffer were used (Maniatis et al., 1982). DNA fragments were isolated by the method of Maxam and Gilbert (1977). Oligonucleotides (25-mers) were isolated from 12% polyacrylamide gels containing 7 M urea.

RNA was analyzed on 1.1% agarose gels containing formaldehyde (Maniatis et al., 1982). Molecular weight markers on DNA and RNA gels were restriction fragments of either pBR322 or lambda DNA, and are given in kb, unless otherwise indicated.

Denaturing SDS-polyacrylamide gel electrophoresis of proteins was performed according to Laemmli (1970), using 4.5% stacking gels and 7.5-12.5% running gels. Gels containing ^{35}S were impregnated with 2,5-diphenyloxazole according to Bonner and Laskey (1974) and fluorographed at -70°C using pre-flashed Kodak XAR film. Molecular weight markers used were phosphorylase B (92,500), bovine serum albumin (69,000), ovalbumin (46,000) and carbonic anhydrase (30,000) ([methyl-^{14}C] methylated; DuPont-NEN); sizes on figures are indicated in kDa. For analysis of ß-galacto-

sidase fusion proteins, E. coli RNA polymerase subunits were used as additional molecular weight markers corresponding to 160,000 (β'), 150,000 (β) and 86,000 (σ) (Chamberlin, 1976).

2.5 In Vitro DNA Manipulations

Restriction endonucleases and other DNA modifying enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, Pharmacia LKB Biotechnology, Boehringer-Mannheim, or Promega Biotech, and were used according to supplier's instructions unless otherwise indicated.

For filling in recessed 3' ends, restricted DNA (≈1 μg) was incubated with Klenow fragment of DNA polymerase I (1-5 U) and 0.2 mM all four deoxynucleoside triphosphates (dNTPs) in a 25 μl reaction containing 50 mM TrisHCl, pH 7.6, 10 mM $MgCl_2$ at 25°C for 15 min (Maniatis et al., 1982). Recessed 5' ends were rendered blunt by the removal of the 3' overhang with T4 DNA polymerase (5 U) and 50 μM all four dNTPs in 70 mM TrisHCl, pH 7.4, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT) in a 25 μl reaction at 37°C for 15 min (Davis et al., 1986). Alternatively, 3' or 5' overhangs were removed by treatment with either S1 nuclease or mung bean nuclease (50 μl reactions). S1 nuclease (6 U) was used in a buffer containing 30 mM sodium acetate, pH 4.5, 3 mM $ZnCl_2$, 0.3 mM NaCl at 25 °C for 15 min. Mung bean nuclease (50 U) was used in a buffer containing 30 mM sodium acetate, pH 4.6, 50 mM NaCl, 0.5 mM $ZnSO_4$, 5% glycerol at 30°C for 20 min. Reactions were terminated by addition of EDTA to 25 mM and/or heating to 65°C for 10 min, followed by a phenol-chloroform extraction and ethanol precipitation.

Vectors for subcloning were routinely dephosphorylated to prevent self-ligation. Dephosphorylation of 1-5 μg of DNA was achieved by treat-

ment with calf intestinal phosphatase (2 aliquots of 0.5 U) in 50 µl of TrisHCl, pH 8.0 at 37°C for 30 min and then 55°C for 30 min. The enzyme was removed by three phenol-chloroform extractions and an ethanol precipitation.

Ligations were performed with T4 DNA ligase (10 U) in a 10 µl reaction in 50 mM TrisHCl, pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermidine, 1 mM ATP, 0.1 mg/ml BSA for 18 h at 15°C. Typically, ≈200 ng of dephosphorylated vector and a 5- to 10-fold molar excess of insert were used.

2.6 Transformation of E. coli with DNA

DNA was introduced into E. coli by the method of Hanahan (1983). Frozen competent cells were sometimes used for transformation into RR1, but were inefficient for transformation into JM103. Transformed cells were selected by growth in the presence of an antibiotic to which resistance was conferred by the introduced plasmid. For transformation of M13 into JM103, the procedure was modified: after the heat shock, aliquots of the transformation mixture were plated in 2.5 ml of molten YT top agar (0.7%) containing ≈10^7 JM103 cells, 0.5 mM isopropyl-ß-D-thiogalactopyranoside (IPTG), and 0.03% 5-brom-4-chlor-3-indolyl-ß-D-galactopyranoside (Xgal). In the presence of the inducer IPTG and the substrate Xgal, expression of ß-galacosidase from the lacZ gene in non-recombinant M13 produces blue plaques. Thus, recombinant phage were identified as colourless plaques, due to disruption of the lacZ gene.

Specific plasmid and phage constructs were identified by restriction enzyme analysis of DNA produced by the rapid boiling method. Colony or plaque hybridization was also used occasionally. The relative orientations of inserts in M13 phages were determined by complementation tests.

The two phage stocks (30 µl each) were adjusted to 25 mM EDTA and 0.25% SDS, heated to 75°C for 1 h, and then left to anneal at 25°C for 1 h. Phage mixtures with complementary inserts were identified by reduced mobility on agarose gels (Messing, 1983).

2.7 Preparation of Radiolabeled DNA/RNA

Most of the ^{32}P-labeled DNA probes were made by a nick-translation procedure modified from Rigby et al. (1977). The 10 µl reaction mixture contained the DNA (100 ng), 50 mM TrisHCl, pH 8.0, 5 mM $MgCl_2$, and all four dNTPs; usually 2,3,or 4 [α-^{32}P]dNTPs were used (2.5-6 µM; 800-3000 Ci/mmol; DuPont-NEN or ICN Radiochemicals), supplemented with the complementary unlabeled dNTPs (8 µM). This mixture was incubated with DNase I (2.5 ng/ml; Worthington) for 3 min at 25°C and then E. coli DNA polymerase I (1-2 U) was added and incubation continued for 2 h at 15°C. After addition of EDTA to 20 mM, the labeled DNA was separated from unincorporated dNTPs by passage through a Sephadex G-50 column (Pharmacia) in TE buffer (Maniatis et al., 1982). Specific activity of probes, monitored by trichloroacetic acid precipitation of column fractions, was usually $\approx 10^8$ cpm/µg. For labeling linear DNA fragments, oligonucleotide-primed labeling as described by Feinberg and Vogelstein (1983, 1984) was also used; this technique produced probes of slightly higher specific activity.

End-labeled DNA fragments were used as markers for Southern blots and S1 mapping and primer extension experiments. DNA with 3' recessed ends was labeled by filling in the ends with Klenow fragment as described in section 2.5, except that one of the required unlabeled dNTPs was replaced with [α^{32}P]dNTP (1.5 µM). DNA with blunt or 5' recessed ends was

dephosphorylated and then ^{32}P-labeled by T4 polynucleotide kinase as described in section 2.11.

RNA probes were occasionally used, and were synthesized from the T7 or SP6 promoters of pSP64, pSP65, pGEM3, or pGEM4 across the insert as recommended by Promega LKB Biotechnology (from Melton et al., 1984).

2.8 Hybridizations

2.8.1 DNA and RNA Blot Analysis

DNA or RNA was transferred from agarose gels to either nitrocellulose (0.45 μm, BA-85, Schleicher and Schuell) or GeneScreen Plus (DuPont-NEN) filters. The gel was soaked in 0.25 M HCl for 10 min to hydrolyze large fragments of DNA, rinsed in water, and then the DNA was denatured in 0.5 M NaOH, 1.5 M NaCl for 30 min, followed by neutralization in 0.5 M TrisHCl, pH 7.5, 1.5 M NaCl for 30 min. Transfer was effected by the method of Southern (1975) in 20X SSC (nitrocellulose) or 10X SSC (GeneScreen Plus) (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate). Filters were baked in vacuo at 80°C for 2 h; GeneScreen Plus filters were also exposed to ultraviolet light to crosslink the DNA to the filter. Hybridization of nitrocellulose filters was performed as described by Edwards and Denhardt (1985), except that poly(rA) was omitted from the hybridization solution. Final washes were in 0.2X SSC, 0.1% SDS at either 42°C or 65°C, depending on the stringency desired. Probes were removed by washing in TE, 0.1% SDS at 90°C. GeneScreen Plus filters were hybridized in 50% formamide, 10% dextran sulfate, 1% SDS, 1 M NaCl, 100 μg/ml denatured salmon sperm DNA, and denatured probe (1-5 X 10^5 cpm/ml). Blots were washed and probes were removed as described (Denhardt et al., 1987;

Khandjian, 1987). Slot blot analysis was performed on nitrocellulose filters with the Schleicher and Schuell Minifold II apparatus as recommended. Blots were autoradiographed at -70°C with a fluorescent screen. Hybridization was quantified by scanning autoradiograms with an LKB UltroScan XL densitometer.

2.8.2 Plaque Hybridization

For screening of the λ 9090 library, phage were plated in tryptone top agarose (1% tryptone, 0.5% NaCl, 0.7% agarose and 10 mM $MgSO_4$) on LB plates (1.5% agar) with 0.2% glucose. Phage DNA was transferred to nitrocellulose and hybridized as described by Maniatis et al. (1982). Initial screens contained 40,000 phage on a 150 mm plate; plaques in the region which produced a signal were picked into SM buffer and replated at ≤20,000/150 mm plate. This method was continued until the positive clone was purified. M13 plaques were sometimes screened for the desired insert by growth on a lawn of JM103 on YT plates, transfer to nitrocellulose, and hybridization as described above.

2.8.3 Colony Hybridization

Colony hybridization was performed using a filtration device (Schleicher and Schuell) according to Grunstein and Hogness (1975), except that an additional 3 min lysis in 10% SDS was added prior to the alkali denaturation step, and the proteinase K wash was omitted.

2.9 DNA Sequencing and Sequence Analysis

DNA sequence was obtained by the dideoxynucleotide chain termination method of Sanger et al. (1977), using [α^{35}S]dATP and either Klenow, Moloney murine leukemia virus reverse transcriptase or Sequenase.

Sequenase (United States Biochemical Corporation, Cleveland, OH; Tabor and Richardson, 1987) was used when available. Sequence was usually determined from single-stranded M13 clones, but direct sequencing of double-stranded plasmids (Zhang et al., 1988) was used in two instances. The primer used for cloning the 5' 2ar cDNA (section 2.10) was used to prime sequencing reactions, allowing a partial determination of the 5' 2ar cDNA sequence. Another specific primer corresponding to part of the CAT gene was used to check the junctions of the 2ar promoter-CAT gene expression constructs (section 2.18). The deletion derivatives used for sequencing the 2ar promoter region were made from M13 subclones of the 1-kb PstI fragment using the Cyclone System (International Biotechnology Inc.). This method involved annealing of a primer to the M13 polylinker, cleavage, limited 3' to 5' exonuclease digestion of the insert with T4 DNA polymerase, tailing and religation (Dale et al., 1985).

Products of the sequencing reactions were analyzed on 6% or 8% polyacrylamide gels containing 7 M urea, in a buffer composed of 50 mM Tris-borate, pH 8.3, 1 mM EDTA. Wedge gels (0.2-0.4 mm) were often used. Gels were fixed in 7.5% acetic acid, 5% methanol, dried onto Whatman 3MM paper, and exposed to XAR-5 film. Sequence determination was aided with an IBI Gel Reader and the computer programs of Pustell (International Biotechnology Inc., New Haven, CT). The software of Lagrimini et al. (1984), Mount and Conrad (1986), and the PC Gene Programs (IntelliGenetics Inc.) were used for sequence analysis.

2.10 Primer Extension and cDNA Library Synthesis

To obtain a clone representing the 5' end of the 2ar message, a primer-extended library was made from poly(A^+) RNA isolated from TPA-

treated JB6 cells. The RNA was isolated as described (Edwards and Denhardt, 1985) and size-selected on a 7-17% sucrose gradient containing 75% formamide in 25 mM TrisHCl, pH 7.2, 2.5 mM EDTA, by centrifugation in a Beckman SW40 rotor at 35000 rpm for 60 h. Fractions were assayed by slot blot analysis to select a population abundant in 2ar mRNA. First strand cDNA synthesis was specifically primed with a synthetic 25-mer (ACCAGCCATGTGGCTATAGGATCTG; synthesized by G. Mackie) corresponding to position 15-40 of the original cDNA. ^{32}P-labeled oligonucleotide was used to prime a test reaction for analysis of extended products. The 25-mer (100 pmole, 1 μg) was labeled with T4 polynucleotide kinase (5 U) and [γ^{32}P]ATP (5 μCi, 2000 Ci/mmol; DuPont-NEN) at 37°C for 30 min in kinase buffer (70 mM TrisHCl, pH7.6, 10 mM $MgCl_2$, 5 mM DTT, 66 μM unlabeled ATP, 1 mM spermidine). The labeled 25-mer was separated from ATP by passage through Sephadex G50 superfine in 10 mM triethylammonium bicarbonate, pH 7.0. The 25-mer (0.5 μg; ≈2 X 10^5 cpm for the test reaction) was annealed to the RNA (1 μg) in 2.5 μl of a buffer containing 10 mM TrisHCl, pH 8.0, 0.2 mM EDTA, and 200 mM NaCl by heating at 85°C for 5 min and slowly cooling to 42°C in a sealed glass capillary. First strand cDNA synthesis (Okayama and Berg, 1982) from the specific primer was performed with avian myeloblastosis virus reverse transcriptase (Pharmacia). Primer extended products of the test reaction were analyzed on a 6% polyacrylamide gel containing 7 M urea. For cloning the extended product, second strand synthesis was performed with RNase H, DNA polymerase I, and E. coli DNA ligase (Gubler and Hoffman, 1983). The resulting duplex was treated with S1 nuclease and cloned by dC-dG homopolymer tailing (Michelson and Orkin, 1982) into pBR322 restricted with PstI. Clones were screened with a probe containing exons 1-3 from the 2ar genomic clone.

2.11 S1 Mapping

Nuclease protection assays were done with 5' end-labeled probe, synthesized by a protocol modified from Barker et al. (1983). For example, for determination of the transcriptional start site of 2ar, the 1-kb StyI fragment from the genomic clone was dephosphorylated and then labeled with ^{32}P by T4 polynucleotide kinase. The DNA (10 µg) was heated at 70°C for 5 min in 30 µl denaturation buffer (10 mM TrisHCl, pH 9.5, 0.5 mM spermidine, 0.05 mM EDTA). The buffer was adjusted to 50 mM TrisHCl, pH 9.5, 10 mM $MgCl_2$, 5 mM DTT, 5% glycerol, and [$\gamma^{32}P$]ATP (100 µCi, 2000 Ci/mmol; DuPont-NEN) and T4 polynucleotide kinase (10 U) were added. After 30 min at 37°C, the reaction was stopped by addition of ammonium acetate to 0.5 M, and the DNA was purified away from ATP by passage through a Sephadex G50 coarse "spun" column (Maniatis et al., 1982). The DNA was then restricted with the second enzyme (in this case, PstI) and the desired fragment, 5'-labeled at only one end, was purified by gel elution.

The ^{32}P-labeled DNA probe (2 X 10^5 cpm) was mixed with 10 µg of poly(A^+) RNA from TPA-treated JB6 cells in 30 µl of 80% formamide, 0.4 M NaCl, 40 mM piperazine-N,N'-bis(2 ethanesulfonic acid) (PIPES), pH 6.5, 1 mM EDTA. The mixture was incubated in a sealed glass capillary for 15 min at 80°C and then for 18 h at 37°C to promote DNA-RNA hybridization. After treatment with S1 nuclease (40 U) in 400 µl of buffer (30 mM sodium acetate, pH 4.5, 0.25 M NaCl, 1 mM $ZnSO_4$, 5% glycerol, 20 µg/ml denatured calf thymus DNA) for 60 min at 37°C, the DNA was ethanol precipitated with 5 µg of carrier tRNA. The protected species were resuspended in formamide buffer (95% formamide, 10 mM EDTA, 0.5% bromophenol blue and 0.5% xylene cyanol) and analyzed on an 8% polyacrylamide-7 M urea gel.

2.12 Production of ß-Galactosidase-2ar Fusion Proteins

Two restriction fragments of the 2ar cDNA were cloned into the pEX2 expression vector (Stanley and Luzio, 1984; obtained from K. Stanley) such that transcription would proceed from the cro-ß-galactosidase coding region through the 2ar insert in the reading frame of the predicted polypeptide. Thus, an internal XmnI-PvuII restriction fragment containing 2ar coding sequence was cloned into the SmaI site of pEX2 (see Fig. 3.5A). The PvuII-HindIII fragment representing the C-terminal region was cloned into pEX2 at the BamHI site, after filling the 3'-recessed ends with Klenow. Restriction fragments spanning the cloning junctions were subcloned into M13 phages and sequenced to ensure that the correct reading frame would be used through the 2ar insert.

The expression constructs were transformed into E. coli strain NF1, which carries the lambda cIts gene to allow temperature-dependent expression from the from the λP_R promoter of the expression vector. Plasmids were amplified by cell growth at 30°C followed by induction of fusion protein synthesis at 42°C for 90 min. Cells were lysed with 1 mg/ml lysozyme and 0.5% Triton X-100 in a buffer containing 50 mM TrisHCl, pH 8.0, 50 mM EDTA, and 25% (w/v) sucrose. After addition of an equal volume of RIPA buffer (25 mM TrisHCl, pH 7.2, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% SDS, 1% sodium deoxycholate), insoluble matter was pelleted at 13,000 X g and washed again in 50% RIPA buffer. This procedure yielded pellets highly enriched in the poorly soluble cro-ß-galactosidase fusion proteins (Stanley and Luzio, 1984; J. Bechberger, personal communication). Fusion proteins were further purified by electroelution from SDS-polyacrylamide gels (7.5%).

2.13 Production and Purification of Antisera

Each of the three gel-purified proteins (shown in Fig. 3.5C) was injected subcutaneously into New Zealand White male rabbits (180-250 μg/injection) on days 0, 20, 35, 70, and 91. RIBI adjuvant (monophosphoryl lipid A plus trehalose dimycolate; RIBI ImmunoChem Research, Hamilton, MT) was used for the first three injections, and Freund's adjuvant for the last two. Immune sera were collected on days 40, 75, and 99; day 99 sera (cardiac puncture) appeared to have the highest titer and were used for the experiments shown here, unless otherwise indicated.

Antibodies were purified by passage of the serum through a protein A-Sepharose column (Pharmacia). All solutions for antibody purification contained 0.02% sodium azide. After washing with 0.1 M phosphate, pH 8, antibodies (predominantly IgG) were eluted with 0.1 M glycine, pH 3, into an equal volume of 0.1 M TrisHCl, pH 8. The fractions containing IgG were pooled and loaded onto fusion protein affinity columns.

Affinity columns were made by coupling cro-ß-galactosidase and derivatives, isolated as above, to cyanogen bromide (CnBr)-activated Sepharose 4B (Pharmacia). For a 1 ml column, the protein (12 mg) was mixed with the gel matrix (0.32 g) with slow rotation for 2 h at 25°C in 30 ml of 0.1 M $NaHCO_3$, pH 8.3, 0.025% SDS. The gel was then incubated in 0.2 M glycine, pH 8, for 18 h at 4°C to block remaining active groups, and washed alternately in 0.1 M $NaHCO_3$, pH 8, and 0.1 M sodium acetate, pH 4. Columns were loaded and washed in 0.1 M glycine, 0.1 M TrisHCl, pH 7.7, so that fractions from the protein A-Sepharose columns could be loaded directly. Bound material was again eluted with 0.1 M glycine, pH 3, into an equal volume of 0.1 M TrisHCl, pH 8. Antibodies which did not bind to the cro-ß-galactosidase column and which did then bind to the appropriate

2ar fusion protein column were concentrated in collodion bags (Schleicher and Schuell). Final yields from 15 ml sera were ≈1.4 mg (in 1 ml) affinity purified antibodies.

2.14 Western Blot Analysis

Electroblotting of proteins from SDS-polyacrylamide gels onto nitrocellulose was performed in a BioRad Mini Trans-Blot Cell as recommended (after Towbin et al., 1979). Marker lanes were stained with Amido Black (0.1% in 50% methanol, 10% acetic acid). Other lanes were processed for immunodetection either by immunogold staining and silver enhancement with an Auroprobe detection kit (Janssen Life Sciences, Beerse, Belgium; used as recommended) or with a peroxidase-linked secondary antibody. The latter method involved incubation of the nitrocellulose in TBS (20 mM TrisHCl, pH 7.4, 146 mM NaCl) for 5 min and then in TBS with 0.05% Tween 20 for 15 min to block non-specific adsorption. Primary antibody was used at a dilution of 1/500 in TBS plus Tween 20 for 30 min. After washing in several changes of TBS and re-blocking in TBS with Tween 20, the blots were incubated for 2 h in the secondary antibody, horseradish peroxidase-linked goat anti-rabbit IgG, at a dilution of 1/1000 in TBS plus Tween 20. The blots were washed in TBS and the presence of immunoreactive material visualized by reaction with peroxidase substrate (0.7 mg/ml 4-chloro-1-napthol (from a 3 mg/ml stock in methanol) and 0.02% H_2O_2 in TBS).

2.15 Metabolic Radiolabeling of Mammalian Cell Proteins

Subconfluent actively growing cells were labeled 24-48 h after passaging. For labeling of phosphoproteins, cells were washed twice and

starved for 0.5 h in serum- and phosphate-free modified Eagle's medium (Flow Laboratories, or donated by J. Bechberger). Labeling was accomplished in the same medium containing 400 μCi/ml of carrier-free [^{32}P]orthophosphate (ICN Biomedicals, or Amersham), usually for 3 or 4 h. Cells on 35 mm plates were labeled in 0.5 ml of medium. Cells were labeled for 4 h with [^{35}S]methionine (300 μCi/ml of Tran^{35}S-label, ICN Biomedicals) in methionine-free Dulbecco's modified Eagle's medium (Gibco) containing 3% dialyzed serum, after a 1 h starvation for methionine and serum. Conditioned media were adjusted to 5 mM phenylmethylsulfonylfluoride, centrifuged at 10,000 X g for 15 min to remove detached cells and cell debris, and stored at -20°C until use.

2.16 In Vitro Translation

RNA was denatured with 2 mM methyl mercury and then translated in vitro using [^{35}S]methionine and a rabbit reticulocyte lysate (DuPont-NEN, after Pelham and Jackson, 1976). 2ar mRNA that had been hybrid-selected with the 2ar cDNA clone fixed to aminophenylthioether paper (Maniatis et al., 1982) was obtained from D.T. Denhardt.

2.17 Immunoprecipitation

Immunoprecipitations from in vitro translation lysates or from cell culture media were performed as described by Denhardt et al. (1987). Cell culture media were normalized for immunoprecipitation either by volume or by amount of acid-precipitable ^{35}S-radioactivity. For immunoprecipitation of [^{35}S]methionine-labeled proteins, an initial pre-adsorption step with preimmune serum and protein A-Sepharose was added.

2.18 Transient Transfection Assays

For assay of promoter activity, portions of the 2ar promoter differing in the 5' endpoint and all extending to the StyI site at the end of exon 1 (filled in with Klenow to +79) were cloned next to the chloramphenicol acetyltransferase (CAT) reporter gene. The 5' endpoints were at -777 (PvuII), -543 (NheI and filled in with Klenow), and -253 (BamHI and filled in with Klenow) of the 2ar gene. The isolated restriction fragments were cloned into the SmaI site of pSV0CAT. Constructs were identified by restriction patterns, and the sequence of the 2ar promoter-CAT gene junction was verified by direct plasmid sequencing with a primer specific for the CAT gene.

Plasmids were transfected into mammalian cells by the calcium phosphate coprecipitation method of Graham and van der Eb (1973). The $CaCl_2$-DNA solution was added dropwise to the Hepes-buffered saline while bubbling through inert gas to facilitate formation of a fine precipitate. Cells were plated at low density (2 X 10^5 cells/100 mm plate for JB6) 18-24 h prior to transfection with 1-10 µg of DNA. The 1 ml of calcium phosphate precipitate was added to the 10 ml of medium on each plate. After a 6-10 h incubation, the media was replaced with fresh media. Cells were narvested 40-48 h after addition of the DNA. Where used, TPA and DMSO were added to the cells 12 h prior to harvest.

Washed, pelleted cells were resuspended in 100 µl of 0.25 M TrisHCl, pH 7.8, and lysed by three cycles of quick freezing and thawing. Cell debris was removed by centrifugation at 11,000 X g for 15 min at 4°C. Protein concentrations of the cell lysates were determined as described by Bradford (1976) using a Bio-Rad Protein Assay Kit. Assays for CAT

activity, using ≈40 µg of cell protein, were performed for 2 h at 37°C as described by Gorman et al. (1982).

2.19 In Situ Hybridization

2ar sense and antisense ^{35}-labeled RNA probes were prepared from the linearized pGEM3-2ar vectors by the protocol recommended by Promega, and then hydrolyzed to ≈150 nt fragments (Cox et al., 1984). Kidneys were dissected from adult CD1 mice immediately after sacrifice, frozen in isopentane/dry ice and stored at -70°C. Cryostat sections (10 µm) were cut at -17°C and collected onto slides coated with BSA-Denhardt's solution (Lum, 1986). Cut sections were immediately fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min, dehydrated in a graded ethanol series containing 0.33 M sodium acetate, and stored at -70°C.

The hybridization protocol was obtained from C. Naus (personal communication). Sections were rehydrated in PBS, treated with 25 µg/ml proteinase K in 50 mM TrisHCl, pH 7.6, 5 mM EDTA for 20 min at 25°C, and washed in PBS. Tissues were postfixed in 4% paraformaldehyde in PBS for 5 min, again dehydrated in a graded ethanol series, and air dried. Prehybridization was performed in 50% formamide, 5X Denhardt's solution (0.1 % Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA), 25 mM PIPES, 25 mM EDTA, 750 mM NaCl, 0.2% SDS, 40 mM DTT, 250 µg/ml sheared salmon sperm DNA, and 250 µg/ml yeast tRNA for 2-3 h at 45-50°C in a humidified chamber. Hybridization was performed in the same buffer with the addition of 10% dextran sulfate and 0.1-1 ng/ml radiolabeled RNA probe for 12 h at 45-50°C; to prevent dehydration, slides were sealed with a coverslip and contact cement. Slides were washed in large volumes of 4X SSC, 10 mM ß-mercaptoethanol for 5 min, and twice in 4X SSC for 10 min each.

Sections were treated with RNase A (20 µg/ml in 0.5 M NaCl, 10 mM TrisHCl, pH 8.0, 1 mM EDTA) for 30 min at 37°C to degrade unhybridized probe. Washing was continued in 0.5 M NaCl, 10 mM TrisHCl, pH 8.0, 1 mM EDTA for 30 min at 37°C, 2X SSC for 30 min at 25°C, and 0.1X SSC for 30 min at 45°C.

Sections were coated with autoradiographic emulsion (Kodak NTB-2), exposed 5-7 days, and developed. Some slides were then counterstained with Harris's haematoxylin and eosin (Bancroft and Cook, 1984). Tissues were examined and photographed with a Zeiss Photomicroscope III.

CHAPTER 3

BIOCHEMICAL FUNCTION AND PHYSIOLOGICAL ROLE OF 2AR

3.1 INTRODUCTION

The starting point for this thesis was the cloning of a cDNA, named 2ar, representing an mRNA that is regulated in an interesting manner. Induction by TPA suggests a role for this gene product in carcinogenesis; this suggestion is further explored in Chapter 4. In order to understand any role in carcinogenesis, it is necessary to answer a few fundamental questions. What does the 2ar gene product do? What is its function at the molecular level? What is its normal physiological role? The work described in this chapter is an attempt to answer these questions.

Similar questions have been asked for other genes cloned on the basis of regulation or phenotypic changes. The oncogenes, cloned on the basis of a dominant contribution to transformation, presented one of the first such challenges in the search for the molecular function and biological role of an unknown gene product, one which in many cases is still open. Even when the purified protein is available, it is not practical to test randomly for all possible activities. Thus, the strategy used here was simply to gain clues to the function of 2ar using every readily available technique: cDNA sequence analysis, production of bacterial fusion proteins and antisera, immunoprecipitation of the protein, and tissue localization of the protein and mRNA.

While this work was in progress, the developmental expression of 2ar mRNA during mouse embryogenesis was reported (Nomura et al., 1988; Swanson et al., 1989). In situ hybridization revealed high levels of 2ar mRNA in developing limb bones and calvaria, granulated metrial gland cells of the deciduum and placenta, kidney tubules, uterine epithelium, and sensory

epithelium of the inner ear (Nomura et al., 1988). Expression of 2ar mRNA in developing bone began at 14.5 days postcoitum in the central marrow region where endochondral ossification is initiated after the vascular bud breaks through the ossification collar. The granulated metrial gland cells that express 2ar are derived from maternal bone marrow, expanding between 6.5 and 10.5 days postcoitum. Their function is not clear, although they may play a role in breaking down and remodeling the stroma of the uterus and maternal blood vessels (Nomura et al., 1988). 2ar was also expressed in an oil-induced deciduoma in a pseudopregnant female. The uterine epithelium adjacent to the deciduoma expressed high levels of 2ar, while the contralateral horn did not (Nomura et al., 1988).

In the inner ear, 2ar mRNA was expressed beginning from 16.5 days postcoitum in the maculae (sensory epithelia), which is overlain by the otoconial membrane, an extracellular matrix containing calcium carbonate crystals (Swanson et al., 1989). 2ar was also expressed by the secretory cells of the stria vascularis of the cochlea, epithelium of the endolymphatic sac, patches of epithelia lining the semicircular canals, and both the auditory and vestibular ganglia and nerves; expression at these sites may be restricted to certain stages of development (Swanson et al., 1989). Expression of 2ar in kidney began at 16.5 days postcoitum and in newborns was detected in the cortex in proximal and distal tubules and loops of Henle, but not glomeruli or collecting ducts (Nomura et al., 1988). 2ar mRNA was detected in the adrenal gland of a pregnant female, and in individual cells scattered throughout various tissues of the embryonic mouse head, but not in most other adult mouse tissues (e.g. lung, ovary, liver; Nomura et al., 1988; Swanson et al., 1989).

The localization of the 2ar gene to mouse chromosome 5 was also reported during the course of this work (Fet et al., 1989). The localization is based on the observation that genomic DNA restricted with StuI and probed with the 2ar cDNA yielded fragments of 18 kb from C57Bl/6J mice and 16 kb from DBA/2J mice. The strain distribution pattern of this restriction fragment length polymorphism in 25 BXD recombinant inbred lines was identical with that reported for the mouse gene Ric (Rickettsia resistance). This placed 2ar on mouse chromosome 5 with a 95% confidence limit of being within 4.32cM of Ric (Fet et al., 1989). Mice with the Ric^r allele are resistant to infection with the obligate intracellular bacterium Rickettsia tsutsugamushi (scrub typhus). Although the molecular basis for resistance is not known, the peritoneal macrophages from susceptible mice are killed by the bacterium while those from resistant mice are not. The possibility of allelism between 2ar and Ric has been suggested (Fet et al., 1989).

Thus, 2ar mRNA is expressed in a limited number of diverse tissues. The protein presumably encoded by this message appears to play a role in bone development as well as other physiological processes, perhaps including bacterial resistance. A biochemical function for this protein is not obvious from the tissue and chromosomal localization. Sequence analysis of the original cDNA indicated the presence of a large open reading frame; an initial search of the 1985 Genbank and NBRF protein data banks revealed no significant homologies with the 2ar sequence determined (Smith and Denhardt, 1987).

3.2 RESULTS

3.2.1 Cloning and Sequence Analysis of the 2ar cDNA

The original 2ar cDNA was about 200 nucleotides shorter than the ≈1600 nt message (Smith and Denhardt, 1987). The 5' sequence of this cDNA was already within a large open reading frame, indicating that this clone was lacking the 5' coding sequence (Denhardt et al., 1989). A number of attempts were made to obtain the complete sequence of the 2ar mRNA.

As will be described in section 3.2.2, the original 2ar cDNA appeared to be homologous to a rat osteopontin cDNA. An antisense oligodeoxynucleotide corresponding to position 1-25 in the 5' untranslated region of the rat cDNA was used to probe a Northern blot of mouse RNA relatively abundant in 2ar, and to probe Southern blots of a mouse 2ar genomic clone (see Chapter 5). No hybridization was observed, even under non-stringent conditions. Thus, this short osteopontin probe was not useful in obtaining a longer 2ar cDNA or in locating the first exon in the mouse genomic clone.

The 2ar genomic clone itself was used to obtain more sequence of the 2ar mRNA. The 5' end of the original cDNA appeared to map to the 5' boundary of an exon (later defined as exon 4 in Fig. 5.2), as deduced by comparison with consensus intron/exon sequences and loss of the open reading frame. Restriction fragments upstream of this exon were labeled and used to probe Northern blots containing 2ar mRNA. Three fragments chosen (3.1 kb SphI, 1.6 kb PstI, and 0.5 kb EcoRI-PvuII) hybridized to a band the size of 2ar mRNA on the Northern blot. The smallest fragment was subcloned into M13 phages and sequenced. By comparison with consensus intron/exon and exon/intron boundary sequences, this fragment appeared to contain a 36 bp upstream exon (later confirmed as exon 3 in Fig. 5.2) 5'

to those in the original cDNA. Thus, a strategy of "working backwards" through the genomic clone to locate the first exon did not appear to be the most efficient way to proceed, but a probe of an upstream exon had been isolated.

The upstream exon probe (3.1 kb SphI fragment, which contained the sequenced exon and possibly more) was used to re-screen the original JB6 Cl22 cDNA library. Three positive clones were obtained, but only one of these (called 2ar*) gave a positive signal when hybridized to the 5' 551 bp of the original cDNA. A Northern blot carrying RNA from JB6 cells treated with TPA, PDGF, or TPA plus inhibitors of PKC probed with the 2ar* clone gave a signal corresponding to a 1600 nt mRNA with the same pattern of induction as 2ar. The possibility that the 2ar* clone might be a longer 2ar cDNA was pursued by sequence analysis of the 2ar* clone, sequence analysis of the region of the genomic clone containing 2ar*-hybridizable sequence, and S1 protection analysis of the 5' end of the 2ar* clone. The results can be summarized as follows: the 3' end of the 2ar* clone was identical to the 3' 2/3rds of the 2ar cDNA, but diverged at the 5' end, and did not contain a continuous open reading frame; the 5' sequence of the 2ar* clone was present as "exons" on the opposite strand and in the opposite direction to the exons of the original 2ar cDNA; and only the 3' 2/3rds of the 2ar* probe was protected from S1 nuclease by poly(A^+) RNA from TPA-treated JB6 cells. As a control in the S1 mapping experiment, the complete probe from the original 2ar cDNA (nt 89-642 in Fig. 3.2) was protected (a minor species corresponding to protection only of the region from nt ≈222-642 was also observed). The conclusion reached was that the 2ar* clone was not a longer 2ar cDNA or

an alternately spliced product, but was a cloning artifact. The original JB6 cDNA library did not appear to contain a full-length 2ar cDNA.

Primer extension and cloning of the extended product (depicted schematically in Fig. 3.1A) was then used to obtain a cDNA representing the 5' end of the 2ar message. The specific primer was an oligonucleotide corresponding to position 15-40 of the original cDNA (nucleotides 107-131 in Fig. 3.2). This primer contains a unique restriction enzyme site for BstXI, which would allow the original and 5' cDNAs to be recombined to produce a complete cDNA. Primer extension was performed on size-selected poly(A^+) RNA from TPA-treated JB6 Cl22 cells. The extension products of test reactions with end-labeled primer were analyzed on denaturing polyacrylamide gels, revealing an extended product of ≈240 nt and several smaller species (Fig. 3.1B). The small primer-extended library (136 clones) was screened with the 0.5-kb EcoRI-PvuII genomic clone fragment containing the upstream exon, yielding two positive clones. Partial sequence obtained by double-stranded sequencing using the 2ar 25-mer as a primer revealed that the two inserts (≈230 bp and 180 bp) were indeed 5' 2ar cDNAs. The longest 5' cDNA and the junction fragment after recombination with the original cDNA were subcloned into M13 phages and unambiguous sequence was obtained.

The sequencing strategy and sequence of the complete 2ar cDNA is shown in Fig. 3.2. [A major portion of the original cDNA sequence is from J.H. Smith and D.T. Denhardt (Denhardt et al., 1989)]. The longest open reading frame in the 2ar cDNA encodes a protein of M_r 32,350 (294 amino acids). The sequence at the translation initiation site, **CGACCAUGG**, is very similar to the optimal consensus sequence for initiation of eukaryotic translation, (CC)ACCAUGG (Kozak, 1986). The encoded polypeptide

Figure 3.1

Primer extension and cloning of a 5' 2ar cDNA.

A. Strategy for cloning a 5' 2ar cDNA using a specific primer corresponding to the 5' end of the original cDNA. Second strand synthesis catalyzed by DNA polymerase I in the presence of RNase H would presumably result in loss of some information at the 5' mRNA terminus, due to removal of the RNA primer and hydrolysis of the corresponding single-stranded first strand cDNA. Molecules resulting from hairpin-primed second strand synthesis and S1 nuclease cleavage would also be lacking some information at the 5' mRNA terminus. The information lost in either case is usually ≈20 nucleotides or less (D'Alessio and Gerard, 1988).

B. Denaturing polyacrylamide gel analysis of first strand primer extension products, using 5' end-labeled 2ar-specific primer: lane 1, primer alone; lane 2, products of control reaction lacking reverse transcriptase, 25°C; lane 3, extension products, 25°C; lane 4, extension products, 37°C; lane 5, extension products, 47°C. Marker sizes are given in nucleotides.

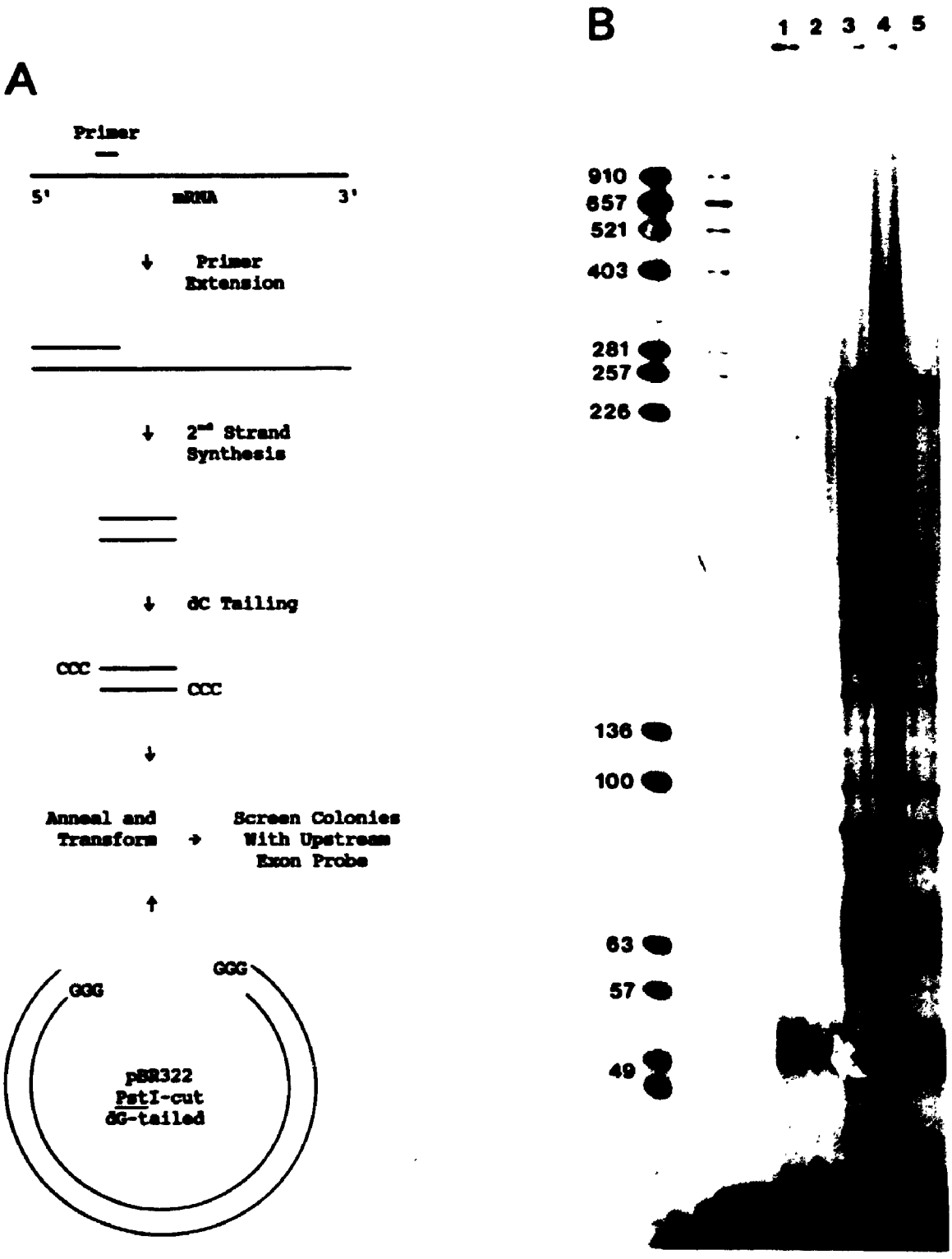
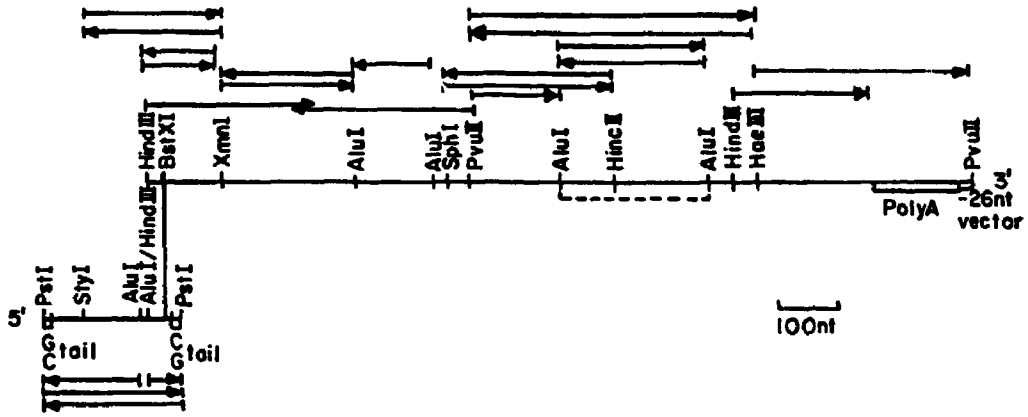

A
Primer
5'
mRNA
3'
Primer Extension
2nd Strand Synthesis
dC Tailing
CCC
CCC
Anneal and Transform
Screen Colonies With Upstream Exon Probe
GGG
GGG
pBR322
PstI-cut
dG-tailed
B
1 2 3 4 5
910
657
521
403
281
257
226
136
100
63
57
49

Figure 3.2

Nucleotide sequence of the 2ar cDNA and deduced primary structure of the peptide.

Top: sequencing strategy. The indicated restriction fragments from the two separate cDNA clones, or from the complete cDNA recombined at the BstXI site, were subcloned into M13 vectors and sequenced by the dideoxy-nucleotide chain termination method. The dashed line indicates a region that was sequenced by the technique of Maxam and Gilbert. Only restriction sites that were used are shown. A large portion of this sequence was determined by J.H. Smith and D.T. Denhardt.

Bottom: nucleotide sequence. Numbers above the line indicate the nucleotide position relative to the predicted initiation site for translation. The stop codon is indicated by asterisks, and the putative polyadenylation signal is underlined in the nucleotide sequence. Sites distinguished in the predicted amino acid sequence are: proposed site of signal peptide cleavage (open arrow), potential N-linked glycosylation site (single underline), 10 consecutive aspartate residues (double underline), potential cell adhesion site (bold), possible sites of cleavage by thrombin (closed arrows), and potential E-F hand Ca^{2+} binding loop (dashed underline).




```
-69                                                                -1
  CTTGCTTGGGTTTGCAGTCTTCTGCGGCAGGCATTCTCGGAGGAAACCAGCCAAGGACTAACAACGACC
                                                                   +72
ATGAGATTGGCAGTGATTTGCTTTTGCCTGTTTGGCATTGCCTCCTCCCTCCCGGTGAAAGTGACTGATTCT
MetArgLeuAlaValIleCysPheCysLeuPheGlyIleAlaSerSerLeuProValLysValThrAspSer
                                                                  144
GGCAGCTCAGAGGAGAAGCTTTACACCCTGCACCCAGATCCTATAGCCACATGGCTGGTGCCTGACCCATCT
GlySerSerGluGluLysLeuTyrSerLeuHisProAspProIleAlaThrTrpLeuValProAspProSer
                                                                  216
CAGAAGCAGAATCTCCTTGCGCCACAGAATGCTGTGTCCTCTGAAGAAAAGGATGACTTTAAGCAAGAAACT
GlnLysGlnAsnLeuLeuAlaProGlnAsnAlaValSerSerGluGluLysAspAspPheLysGlnGluThr
                                                                  288
CTTCCAAGCAATTCCAATGAAAGCCATGACCACATGGACGACGATGATGACGATGATGATGACGATGGAGAC
LeuProSerAsnSerAsnGluSerHisAspHisMetAspAspAspAspAspAspAspAspAspGlyAsp
                                                                  360
CATGCAGGGAGCGAGGATTCTGTGGACTCGGATGAATCTGACGAATCTCACCATTCGGATGAGTCTGATGAG
HisAlaGlySerGluAspSerValAspSerAspGluSerAspGluSerHisHisSerAspGluSerAspGlu
                                                                  432
ACCGTCACTGCTAGTACACAAGCAGACACTTTCACTCCAATCGTCCCTACAGTCGATGTCCCCAACGGCCGA
ThrValThrAlaSerThrGlnAlaAspThrPheThrProIleValProThrValAspValProAsnGlyArg
                                                                  504
GGTGATAGCTTGGCTTATGGACTGAGGTCAAAGTCTAGGAGTTTCCAGGTTTCTGATGAACAGTATCCTGAT
GlyAspSerLeuAlaTyrGlyLeuArgSerLysSerArgSerPheGlnValSerAspGluGlnTyrProAsp
                                                                  576
GCCACAGATGAGGACCTCACCTCTCACATGAAGAGCGGTGAGTCTAAGGAGTCCCTCGATGTCATCCCTGTT
AlaThrAspGluAspLeuThrSerHisMetLysSerGlyGluSerLysGluSerLeuAspValIleProVal
                                                                  648
GCCCAATTCTGAGCATGCCCTCTGATCAAGACAACAACGGAAAGGGCAGCCATGAGTCAAGTCAGCTGGAT
AlaGlnLeuLeuSerMetProSerAspGlnAspAsnAsnGlyLysGlySerHisGluSerSerGlnLeuAsp
                                                                  720
GAACCAAGTCTGGAAACACACAGACTTGAGCATTCCAAAGAGAGCCAGGAGAGTGCCGATCAGTCGGATGTG
GluProSerLeuGluThrHisArgLeuGluHisSerLysGluSerGlnGluSerAlaAspGlnSerAspVal
                                                                  792
ATCGATAGTCAAGCAAGTTCCAAAGCCAGCCTGGAACATCAGAGCCACAAGTTTCACAGCCACAAGGACAAG
IleAspSerGlnAlaSerSerLysAlaSerLeuGluHisGlnSerHisLysPheHisSerHisLysAspLys
                                                                  864
CTAGTCCTAGACCCTAAGAGTAAGGAAGATGATAGGTATCTGAAATTCCGAATTTCTCATGAATTAGAGAGT
LeuValLeuAspProLysSerLysGluAspAspArgTyrLeuLysPheArgIleSerHisGluLeuGluSer
                                                                  936
TCATCTTCTGAGGTCAACTAAAGAAGAGGCAAAAACACAGTTCCTTACTTTGCATTTAGTAAAAACAAGAAA
SerSerSerGluValAsn***
                                                                 1008
AAGTGTTAGTGAGGATTAAGCAGGAATACTAACTGCTCATTTCTCAGTTCAGTGGATATATGTATGTAGAGA
                                                                 1080
AAGAGAGGTAATATTTTGGGCTCTTAGCTTAGTCTGTTGTTTCATGCAAACAACCGTTGTAACCAAAAGCTT
                                                                 1152
CTGCACTTTGCTTCTGTTCTTCCTGTACAAGAAATGCAAACGGCCACTGCATTTTAATGATTGTTATTCTTT
                                                                 1224
TATGAATAAAATGTATGTAGAAACAAGCAAATTTACTGAAACAAGCAGAATTAAAAGACAACTGTAACAGT
                                                                 1296
CTATATCACTATACCCTTTTAGTTTTATAATTAGCATATATTTTGTTGTGATTATTTTTTTTGTTGGTGTGA
                1316
ATAAATCTTGTAACGAATGT ... (polyA tail)
```

contains a number of remarkable features, namely: a hydrophobic leader sequence; a potential N-linked glycosylation site (Lennarz, 1983); a series of ten consecutive aspartate residues; a potential Ca^{2+}-binding site; and a GlyArgGlyAspSer (GRGDS) potential cell adhesion site. The RGD sequence is present in fibronectin, vitronectin, and a number of extracellular matrix proteins, and is part of the recognition site for the integrin family of receptors (Ruoslahti and Pierschbacher, 1986, 1987). The potential Ca^{2+}-binding site conforms with the consensus sequence (Szebenyi and Moffat, 1986) for an E-F hand except for the -z position:

	+x	+y	+z	-y	-x	-z	
consensus:	D -	D N -	D N S	G -	n	* - -	E

n = non-polar

* = E,Q,D,N,S,T

2ar: **D Q D N N G** K **G S** H **E** S

The 2ar polypeptide is highly acidic, and the acidic and basic residues are loosely clustered. There is a cluster of acidic amino acids from residues 60 to 120, surrounding the run of aspartates; this region contains 50.9% acidic amino acids versus 22.8% for the entire polypeptide. Many of the basic residues are present in a cluster near the C-terminus; residues 249-285 contain 37.8% basic residues versus 13.3% for the entire polypeptide.

3.2.2 Similarity Between 2ar and Osteopontin

The sequence of the 2ar cDNA is similar to the sequence reported for a rat osteopontin cDNA isolated from ROS 17/2.8 osteosarcoma cells (Oldberg et al., 1986). Osteopontin is a 41.5 kDa (rat) glycosylated phosphoprotein known also as 44K bone phosphoprotein and bone sialoprotein I (Prince et al., 1987; Fisher et al., 1987). It is secreted from osteo-

sarcoma cells in culture (Oldberg et al., 1986). The mouse 2ar and rat osteopontin sequences are 87% identical at the nucleotide level and 84% identical at the amino acid level (Fig. 3.3). There are 23 amino acids (amino acids 233-255 corresponding to nucleotides 776-844 in Oldberg et al., 1986) in rat osteopontin that are not present in mouse 2ar. The last 18 of these 23 amino acids form the first of two imperfect repeats in osteopontin. The site of this discontinuity likely falls between amino acids 232 and 233 (Fig. 3.2, nucleotides 696 and 697) in 2ar. The presence of an AG/G sequence at this location may indicate that the 2ar clone lacks an exon relative to the osteopontin clone.

The amino acid compositions of the predicted proteins are compared in Table 3.1. Both polypeptides are extremely rich in serine, aspartate and glutamate residues, which together account for 40.1% of mouse 2ar and 41.3% of rat osteopontin residues.

The overall structure, as well as several of the interesting features, is conserved between mouse 2ar and rat osteopontin (Fig. 3.3C). The hydropathy profiles (Kyte and Doolittle, 1982) reveal the highly charged character of the proteins [GRAVY (average hydropathy) = -10.11 and -10.53 for 2ar and osteopontin, respectively, compared with a mean value of -4 for sequenced soluble proteins]. The hydrophobic leader sequence, the potential N-linked glycosylation site, the series of 9-10 aspartate residues, the potential Ca^{2+}-binding site, the GRGDS potential cell adhesion site, and a seven-fold repeat of SerXaaXaa are present in both proteins at identical positions. The predicted site of cleavage of the leader sequence of 2ar (Fig. 3.2, between amino acids 16 and 17) was deduced by homology with the amino terminal sequence of mature rat osteopontin (Prince et al., 1987). The predicted secondary structure

Figure 3.3

Comparison of mouse 2ar and rat osteopontin primary and predicted secondary structures.

A. Dot matrix comparison of mouse 2ar (1385 nt) and rat osteopontin (1473 nt) nucleotide sequences. Brackets indicate the coding region.

B. Dot matrix comparison of mouse 2ar (294 aa) and rat osteopontin (317 aa) deduced amino acid sequences.

C. Hydropathicity and predicted secondary structure of mouse 2ar (lower) and rat osteopontin (upper). The hydropathy profiles were generated using the method of Kyte and Doolittle (1982) with an interval of 9 amino acids. The scale at left refers to the 2ar plot. The secondary structures were predicted using the algorithms of Garnier et al. (1978) with decision constants chosen for proteins with over 50% secondary structure. H, E, T, and C indicate regions of α-helix, extended chain (ß-sheet), reverse turn, and random coil structures, respectively, that were common to both proteins (with the exception of the bracketed designations). Three PEST regions rich in proline, glutamic acid, serine, and threonine are indicated by open boxes (Rogers et al., 1986). Unmarked arrows indicate regions predicted to be most flexible (Karplus and Schulz, 1985). The computer analyses were performed by D.T. Denhardt using the PC Gene software from IntelliGenetics Inc.

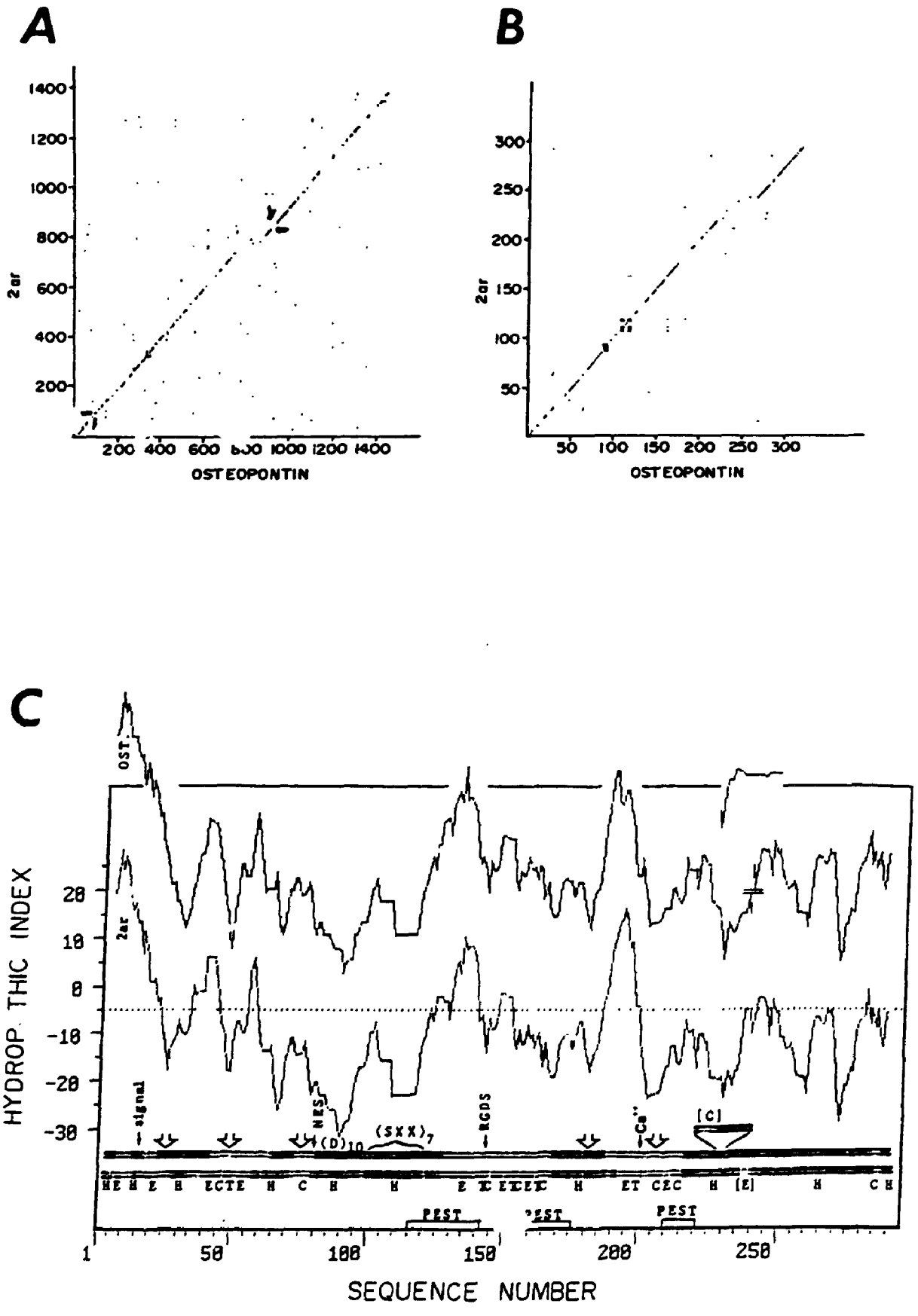

A
1400
1200
1000
800
600
400
200
2ar
200 400 600 800 1000 1200 1400
OSTEOPONTIN
B
300
250
200
150
100
50
2ar
50 100 150 200 250 300
OSTEOPONTIN
C
OST
2ar
HYDROPATHIC INDEX
20
10
0
-10
-20
-30
signal
NES
(D)10
(SXX)7
RGDS
Ca
[C]
PEST
PEST
PEST
1
50
100
150
200
250
SEQUENCE NUMBER

TABLE 3.1

AMINO ACID COMPOSITIONS OF MOUSE 2AR AND RAT OSTEOPONTIN

Amino Acid	2ar (%)	Osteopontin (%)[1]
alanine	14 (4.8)	23 (7.3)
arginine	7 (2.4)	10 (3.2)
asparagine	8 (2.7)	7 (2.2)
aspartate	41 (13.9)	42 (13.2)
cysteine	2 (0.7)	3 (0.9)
glutamate	26 (8.8)	33 (10.4)
glutamine	14 (4.8)	15 (4.7)
glycine	10 (3.4)	7 (2.2)
histidine	15 (5.1)	15 (4.7)
isoleucine	7 (2.4)	7 (2.2)
leucine	23 (7.8)	21 (6.6)
lysine	17 (5.8)	18 (5.7)
methionine	4 (1.4)	3 (0.9)
phenylalanine	7 (2.4)	7 (2.2)
proline	15 (5.1)	15 (4.7)
serine	51 (17.3)	56 (17.7)
threonine	12 (4.1)	12 (3.8)
tryptophan	1 (0.3)	1 (0.3)
tyrosine	4 (1.4)	5 (1.6)
valine	16 (5.4)	17 (5.4)
(total) .	294	317

[1]From Oldberg et al. (1986).

(Garnier et al., 1978) of the GRGDS sequence is a turn/coil between two regions of ß-pleated sheet, suggesting that this site is at the surface of the molecule. However, the potential Ca^{2+}-binding site is not predicted as part of an E-F hand since it is not flanked by α-helices. These observations were also noted by Prince (1988) based on five different secondary structure predictions of osteopontin. Both mouse 2ar and rat osteopontin contain three regions of sequence rich in proline, glutamate, serine and threonine. These "PEST" sequences are associated with a short half-life for intracellular proteins (Rogers et al., 1986), and may also signify the presence of regions inhibitory to protein phosphatases (Rechsteiner, 1987). The tripeptide SerXaaGlu occurs 26 times in osteopontin and 20 times in 2ar, 19 of which are at the identical position as in osteopontin. It was suggested (Oldberg et al., 1986) that this tripeptide may be a signal sequence for O-linked glycosylation.

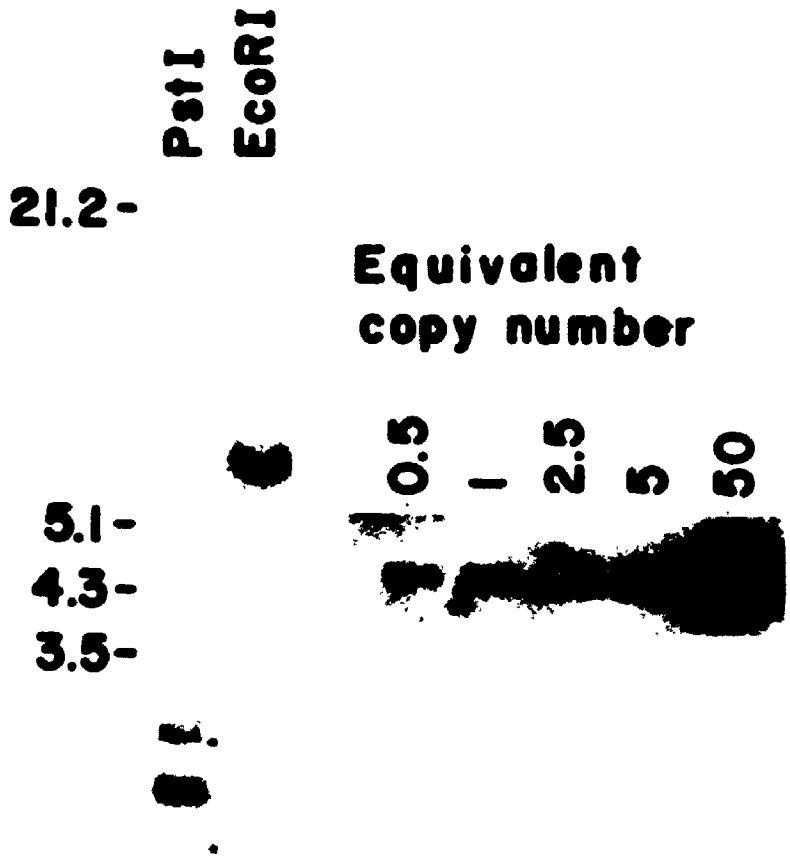
Human osteopontin has been purified and partially characterized (Fisher et al., 1987). In the limited region of the N-terminal 16 amino acids, there is 56% identity between the human and mouse proteins. Analysis of a human osteopontin cDNA also indicated that the GRGDS sequence was completely preserved (Fisher et al., 1989).

3.2.3 2ar is a Single Copy Gene

Southern blot analysis of mouse embryo fibroblast DNA probed with the original 1.4-kb 2ar cDNA revealed one EcoRI and two PstI restriction fragments containing 2ar-hybridizable sequence (Fig. 3.4). A comparison of the intensity of the hybridization signal to the adjacent standards on the same blot suggests that 2ar is a single copy gene. The calculations were based on the size of the 2ar cDNA relative to the size of the mouse

Figure 3.4

Southern blot estimation of copy number of 2ar gene in mouse genome. Mouse embryo fibroblast DNA was restricted with PstI or EcoRI and electrophoresed (5 μg/lane) adjacent to appropriate amounts of linearized plasmid carrying the original 1.4-kb 2ar cDNA. These standards contained amounts of DNA representative of 0.5-50 copies/haploid genome (1.2×10^{-6} to 1.2×10^{-4} μg); calculations were based on the size of the 2ar cDNA relative to the size of the mouse haploid genome. The resultant blot was hybridized with ^{32}P-labeled plasmid carrying the original 1.4-kb 2ar cDNA.


PstI
EcoRI
21.2-
Equivalent
copy number
0.5
—
2.5
5
50
5.1-
4.3-
3.5-

haploid genome. These results indicate that 2ar and osteopontin are not related members of a multi-gene family, but that 2ar is the murine homolog of rat (and human) osteopontin.

3.2.4 Production of ß-Galactosidase-2ar Fusion Proteins and Antisera

In order to study the expression of the 2ar protein, the cDNA was used to produce cro-ß-galactosidase-2ar fusion proteins, which were then used to raise antisera. Plasmids for expression of the fusion proteins were constructed by cloning two non-overlapping segments of the 2ar cDNA into the pEX2 expression vector (Stanley and Luzio, 1984), as described in Fig. 3.5A. This system was chosen because it allows inducible expression from the λP_R promoter in a cIts strain, which can be important if the mammalian gene product is deleterious to the bacteria. The ß-galactosidase segment allows for easy isolation of the fusion protein and may help increase the stability of the mammalian gene product (Stanley and Luzio, 1984; Denhardt and Colasanti, 1988). The two fusion genes contain ≈400 bp inserts, which is the size reported to give optimum expression; the two fusion proteins are also completely independent.

These plasmids were transfected into E. coli, and the fusion proteins were purified from induced cell lysates (Fig. 3.5B). The 2ar N-proximal and C-terminal fusion proteins contain an additional 132 or 80 amino acids, respectively, from the predicted 2ar protein. The electrophoretic mobility indicated apparent molecular weights of 120,000 (cro-ß-galactosidase), 157,000 (2ar N-proximal derivative), and 141,000 (2ar C-terminal derivative; Fig. 3.5C). These three fusion proteins were isolated from SDS-polyacrylamide gels, injected into rabbits, and the immune sera collected.

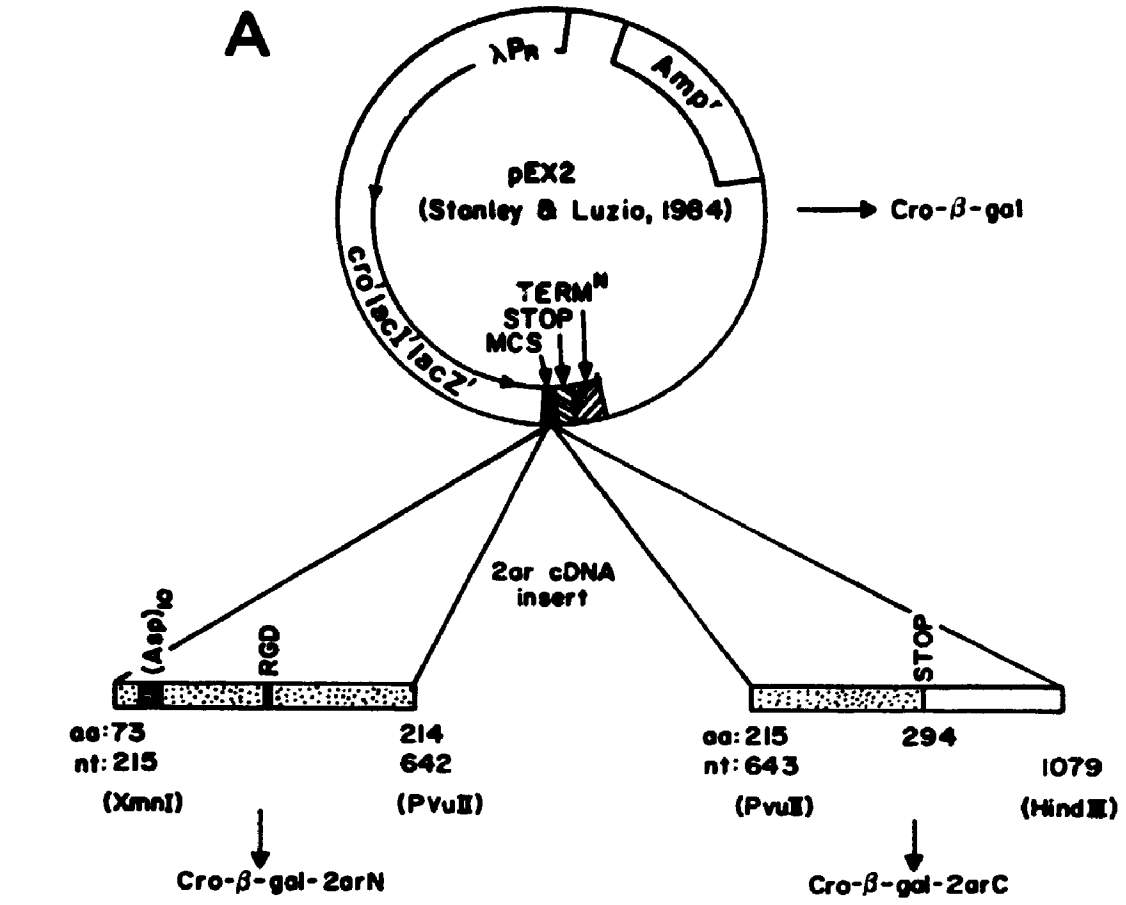
Figure 3.5

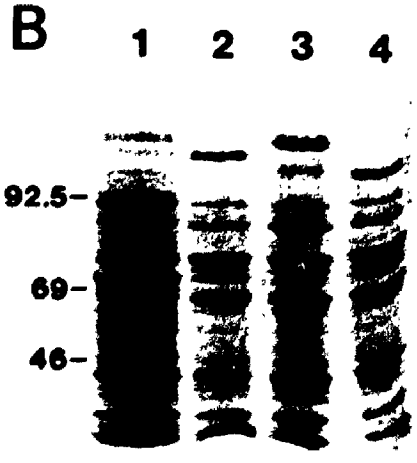
Production of cro-ß-galactosidase-2ar fusion proteins.

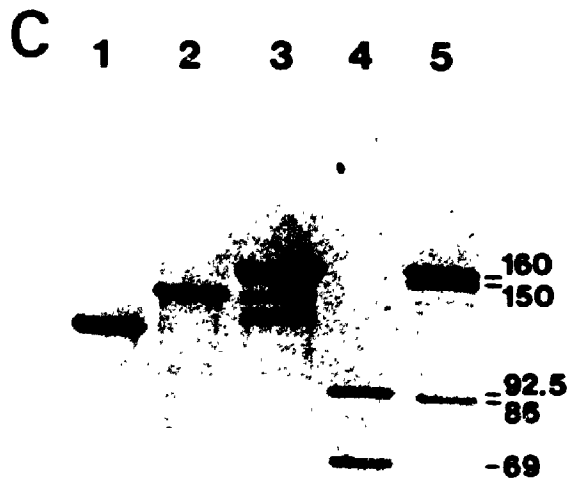
A. Construction of plasmids for expression of cro-ß-galactosidase (Cro-ß-gal)-2ar fusion proteins. Two fragments of the 2ar cDNA were cloned into the multiple cloning site (MCS) of the pEX2 expression vector, so that transcription from the λP_R promoter would direct production of a fusion protein with 2ar sequence at the carboxyl terminus. Following the insertion site, there are stop codons in all three reading frames (STOP) and transcription termination signals ($TERM^N$).

B. Induction of fusion protein synthesis in E. coli NF1 carrying plasmid expression constructs. Total cell proteins were isolated from bacteria grown at the non-permissive temperature (30°C) or permissive temperature (42°C) for expression from the λP_R promoter of the plasmid: lane 1, 30°C, pEX2; lane 2, 42°C, pEX2-2ar C-terminal derivative; lane 3, 42°C, pEX2-2ar N-proximal derivative; lane 4, 42°C, pEX2. The SDS-polyacrylamide gel (7.5%) was stained with Coomassie Blue.

C. Purified cro-ß-galactosidase-2ar fusion proteins. Proteins were purified as described and analyzed on a reducing SDS-polyacrylamide gel (7.5%, 30:0.4 acrylamide:bisacrylamide): lane 1, cro-ß-galactosidase; lane 2, cro-ß-galactosidase-2arC; lane 3, cro-ß-galactosidase-2arN; lanes 4 and 5, molecular mass markers. The gel was stained with Coomassie Blue.


A
λPR
Amp'
pEX2
(Stanley & Luzio, 1984)
Cro-β-gal
cro'lacI'lacZ'
TERM^N
STOP
MCS
2ar cDNA
insert
(Asp)10
RGD
STOP
aa:73
nt:215
(XmnI)
214
642
(PVuII)
aa:215
nt:643
(PvuII)
294
1079
(HindIII)
Cro-β-gal-2arN
Cro-β-gal-2arC


B
1
2
3
4
92.5-
69-
46-


C
1
2
3
4
5
160
150
92.5
86
69

3.2.5 Immunoprecipitation of 2ar from In Vitro Translation Lysates

The anti-cro-ß-galactosidase-2ar(N+C) antiserum was able to specifically immunoprecipitate in vitro translation products of hybrid-selected 2ar mRNA derived from TPA-treated JB6 mouse epidermal cells (Fig. 3.6A). The anti-cro-ß-galactosidase-2arN and -2arC sera were also effective independently. This result demonstrates that the open reading frame indicated in Fig. 3.2 is used to encode a protein. Immunoprecipitation of in vitro translation products of hybrid-selected 2ar mRNA from mouse kidney yielded similar results. In both cases, three discrete polypeptide species with electrophoretic mobilities corresponding to M_r 56,000, 60,000 and 67,000 were immunoprecipitated.

3.2.6 Immunoprecipitation of 2ar Secreted by JB6 Cells

The anti-cro-ß-galactosidase-2arN and -2arC sera were able to specifically precipitate a protein secreted by TPA-treated JB6 mouse epidermal cells labeled with [^{35}S]methionine (Fig. 3.6B). Thus, antisera raised against the 2ar polypeptide backbone as part of fusion proteins are able to recognize the glycosylated, phosphorylated protein secreted by mammalian cells. This protein was the major phosphoprotein secreted by [^{32}P]orthophosphate-labeled TPA-treated JB6 cells, and was also recognized by an antiserum raised against osteopontin purified from rat bone (a gift of C.W. Prince and W.T. Butler; Fig. 3.6C). The electrophoretic mobility indicated microheterogeneous species of apparent M_r 57,000-65,000; these were often separable into two diffuse bands centered on apparent M_r 60,000 and 63,000 (see also Fig. 4.1B). No specific immunoprecipitation was observed from cellular proteins or proteins attached to the tissue culture

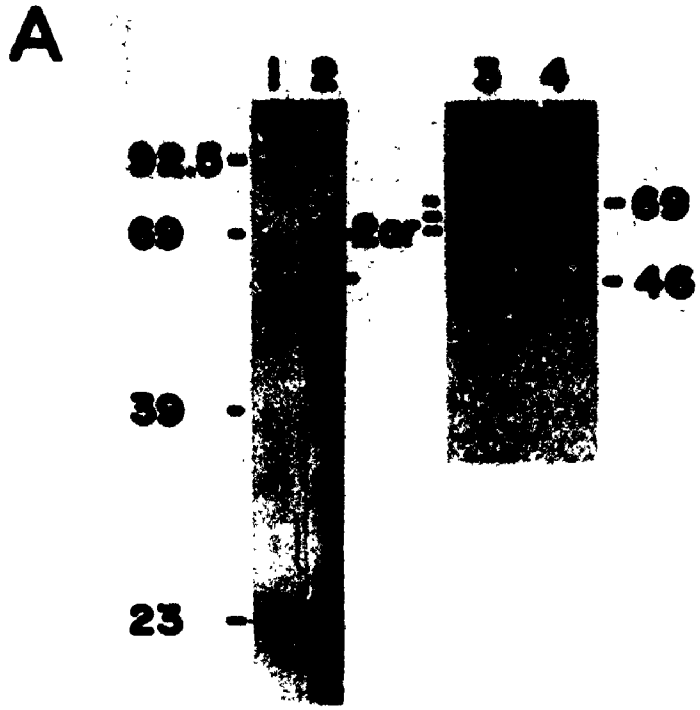
Figure 3.6

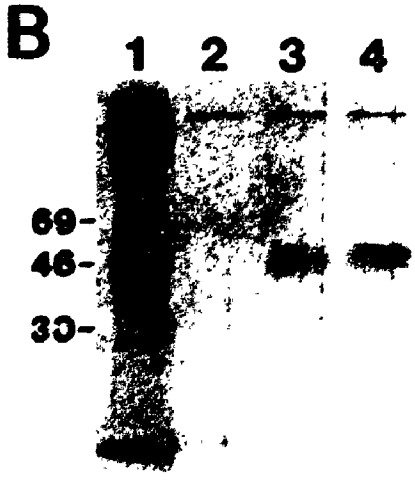
Immunoprecipitation of 2ar protein: (A) produced by in vitro translation of hybrid-selected mRNA; and (B,C) secreted by JB6 epidermal cells.

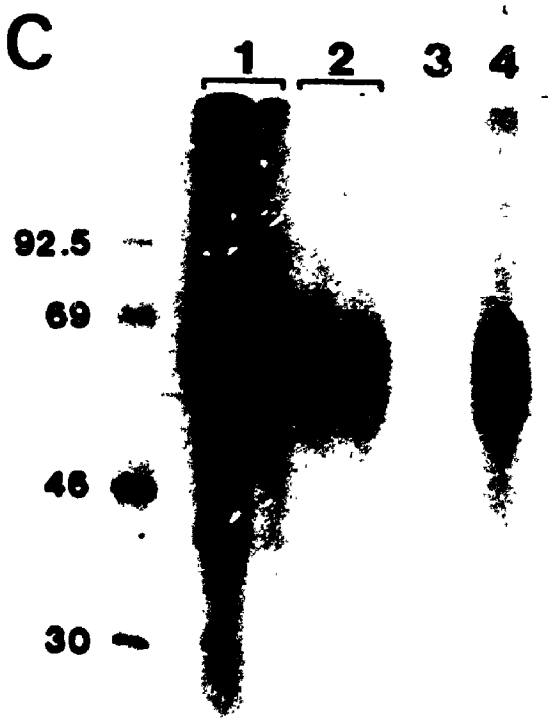
A. Immunoprecipitation of in vitro translation products of hybrid-selected 2ar mRNA. Shown are fluorograms of SDS-polyacrylamide gels (12%) of the proteins produced by in vitro translation of hybrid-selected mRNA (the 11-cm gel on the left) followed by immunoprecipitation (the 6-cm mini-gel on the right). The hybrid selection was programmed with control plasmid (lane 1) or plasmid carrying the original 2ar cDNA (lanes 2, 3, and 4). The translation products shown in lane 2 were immunoprecipitated with anti-cro-ß-galactosidase-2ar(N+C) antiserum (lane 3) or anti-cro-ß-galactosidase control serum (lane 4). The gel on the left and the hybrid-selected mRNA were provided by D.T. Denhardt.

B. Immunoprecipitation of [^{35}S]methionine-labeled 2ar secreted by TPA-treated JB6 epidermal cells. JB6 cells were treated with TPA for 8 h and labeled for the last 2 h of treatment with [^{35}S]methionine. Conditioned medium was electrophoresed directly (lane 1) or after immunoprecipitation of equal volumes with the following antisera: lane 2, anti-cro-ß-galactosidase; lane 3, anti-cro-ß-galactosidase-2arN; lane 4, anti-cro-ß-galactosidase-2arC. The SDS-polyacrylamide mini-gel (10%) was subjected to fluorography.

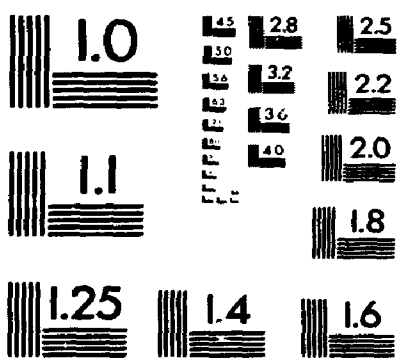
C. Immunoprecipitation of [^{32}P]orthophosphate-labeled 2ar secreted by TPA-treated JB6 epidermal cells. JB6 cells were treated with TPA for 9 h and labeled for the last 3 h of treatment with [^{32}P]orthophosphate. Conditioned medium was electrophoresed directly (lane 1) or after immunoprecipitation of equal volumes with the following antisera: lane 2, anti-cro-ß-galactosidase-2arC; lane 3, anti-cro-ß-galactosidase; lane 4, anti-osteopontin. The SDS-polyacrylamide gel (10%) was autoradiographed.


A
1 2
3 4
92.5
69
39
23
69
46


B
1 2 3 4
69
46
30


C
1 2 3 4
92.5
69
46
30

2



dishes of [^{35}S]methionine-labeled TPA-treated JB6 cells.

Both the best batch of antiserum against 2ar and the osteopontin antiserum consistently precipitated only ≈1/5th of the total ^{32}P-labeled major phosphoprotein. This phenomenon was observed even when the secreted proteins were first precipitated with trichloroacetic acid and then redissolved, when only phosphate-buffered saline was used in the immunoprecipitation and washes, and when the volume of immune serum used was increased by 10-fold. However, re-precipitation of the supernatant from an initial immunoprecipitation again resulted in precipitation of ≈1/5th of the remaining ^{32}P-labeled major phosphoprotein. These results suggest that only ≈1/5th of the 2ar protein in the conditioned medium is "available" for immunoprecipitation.

3.2.7 Characterization and Affinity Purification of 2ar Antisera

The different batches of 2ar fusion protein antisera are identified by number (17 and 6 for 2arC; 5 and 1 for 2arN) and either 1st batch, 2nd batch or CP (final cardiac puncture). The relative abilities of equal amounts of each batch to immunoprecipitate 2ar protein secreted by JB6 cells are as follows: 6(CP) > 1(2nd) ≥ 6(1st) ≥ 5(1st) ≈ 6(2nd) ≥ 17(1st) ≥ 1(1st) ≥ 17(2nd) ≥ 1(CP) > 5(CP) ≈ 5(2nd). Batch 6(CP) was used for all of the studies described in Chapter 4.

With the help of H. Gilman, portions of the anti-cro-ß-galactosidase-2arN and -2arC sera were subjected to a purification scheme to specifically isolate the anti-2ar antibodies. This consisted of isolation of IgG with Protein A-Sepharose, removal of anti-cro-ß-galactosidase antibodies by passage through a cro-ß-galactosidase-Sepharose column, and

Figure 3.7

Affinity purification of 2ar antibodies.

A. Summary of affinity purification scheme and properties of the 2ar antibodies. The relative abilities of the 2ar antisera and affinity purified antibodies to recognize the fusion proteins by Western blot analysis (part B), and to immunoprecipitate ^{32}P-labeled 2ar secreted by TPA-treated JB6 epidermal cells (part C) are compared. The CP sera were used for antibody purification, since these sera were available in the greatest quantity.

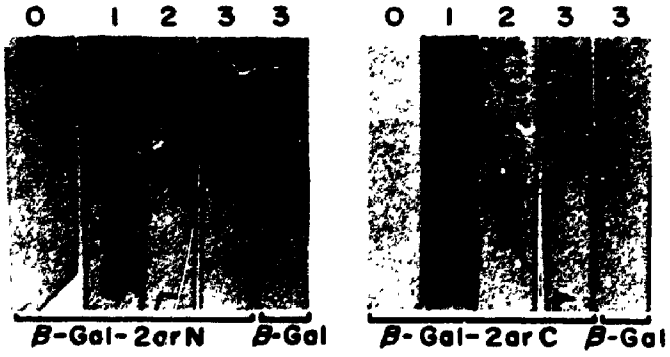
B. Western blot analysis of fusion protein recognition by the anti-2arN antibodies (left panel) and anti-2arC antibodies (right panel). Fusion proteins were isolated as described, electrophoresed on an SDS-polyacrylamide gel (7.5%), and transferred to nitrocellulose. Designations below the lanes indicate the fusion protein in each lane, while numbers on top indicate the antibody probe, according to the legend in part A. Binding of primary antibody was detected by the peroxidase-conjugated secondary antibody method.

C. Immunoprecipitation of metabolically labeled secreted 2ar with the affinity purified anti-2arC antibodies. JB6 cells were treated with TPA for 9 h and labeled for the last 3 h of treatment with [^{32}P]orthophosphate. Conditioned medium was electrophoresed directly (lane 1) or after immunoprecipitation of equal volumes (lanes 3-7, as indicated in part A). Molecular mass markers were run in lane 2. An autoradiogram of the SDS-polyacrylamide gel (10%) is shown.

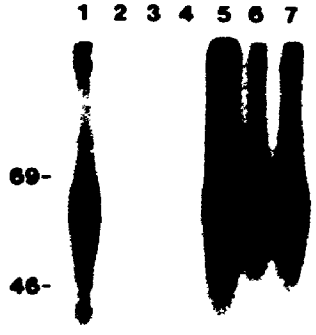
A

ANTIGEN	ANTIBODY	FUSION PROTEIN RECOGNITION		FIG. 3.7B	RECOGNITION OF 2AR SECRETED BY JB6 CELLS	FIG. 3.7C
		β-gal	β-gal-2arN+C			
β-gal	immune serum	+++++	+++++	-	-	3
		β-gal	β-gal-2arN			
β-gal-2arN	pre-immune serum	-	-	0	-	-
	immune serum	+++++	+++++	1	++	-
	↓					
	β-gal-Sepharose unbound	+/-	+++	2	+/-	-
	↓					
	β-gal-2arN-Sepharose bound	-	++	3	+	-
		β-gal	β-gal-2arC			
β-gal-2arC	pre-immune serum	-	-	0	-	4
	immune serum	+++++	+++++	1	++++++	5
	↓					
	β-gal-Sepharose unbound	+/-	++	2	+++	6
	↓					
	β-gal-2arC-Sepharose bound	-	++	3	++++	7

B



C



binding to and elution from a cro-ß-galactosidase-2arN(or C)-Sepharose column. The affinity purified 2ar antibodies were unable to recognize cro-ß-galactosidase but were able to recognize the 2ar fusion proteins and metabolically labeled 2ar secreted by JB6 cells (Fig. 3.7).

A number of attempts were made to immunolocalize 2ar protein in mouse kidney cryostat sections, using various fixatives and either fluorescein isothiocyanate-conjugated secondary antibody or the peroxidase anti-peroxidase method. The crude 2ar sera as well as the anti-cro-ß-galactosidase control sera gave a signal in the brush border region of proximal tubules. No specific reproducible signal was obtained with the affinity purified sera.

3.2.8 Localization of 2ar mRNA in Kidney by *In Situ* Hybridization

Northern blot and slot blot analysis using aliquots of plasmid DNA as standards indicated that the level of 2ar mRNA in kidney is in the range of 0.01-0.1% of the total messenger RNA, approximately the same as in TPA-treated JB6 cells and in exponentially growing primary mouse embryo fibroblasts. A *Hin*dIII fragment of the 2ar cDNA (nt 89-1079 in Fig. 3.2) was subcloned into the pGEM4 vector in both orientations so that transcription from the SP6 promoter would produce sense (control) or antisense 2ar mRNA. These RNA probes were transcribed *in vitro* in the presence of an ^{35}S-labeled precursor and used for *in situ* hybridization of mouse kidney cryostat sections. This experiment was done in collaboration with D.T. Denhardt and M. McLeod.

The antisense 2ar probe gave signals over the background seen with the control probe (Fig. 3.8). The signals corresponded to scattered tubular profiles distributed at highest concentration in the inner

Figure 3.8

In situ hybridization of 2ar mRNA in adult mouse kidney. Kidney sections were hybridized with ^{35}S-labeled control sense 2ar probe (A) or antisense 2ar probe (B, C) and processed as described. Tissues were not stained. These pictures of whole kidney sections (X10) were obtained by placing the slides on an enlarger and producing a negative image directly onto photographic paper. This experiment was done in collaboration with M. McLeod and D.T. Denhardt.

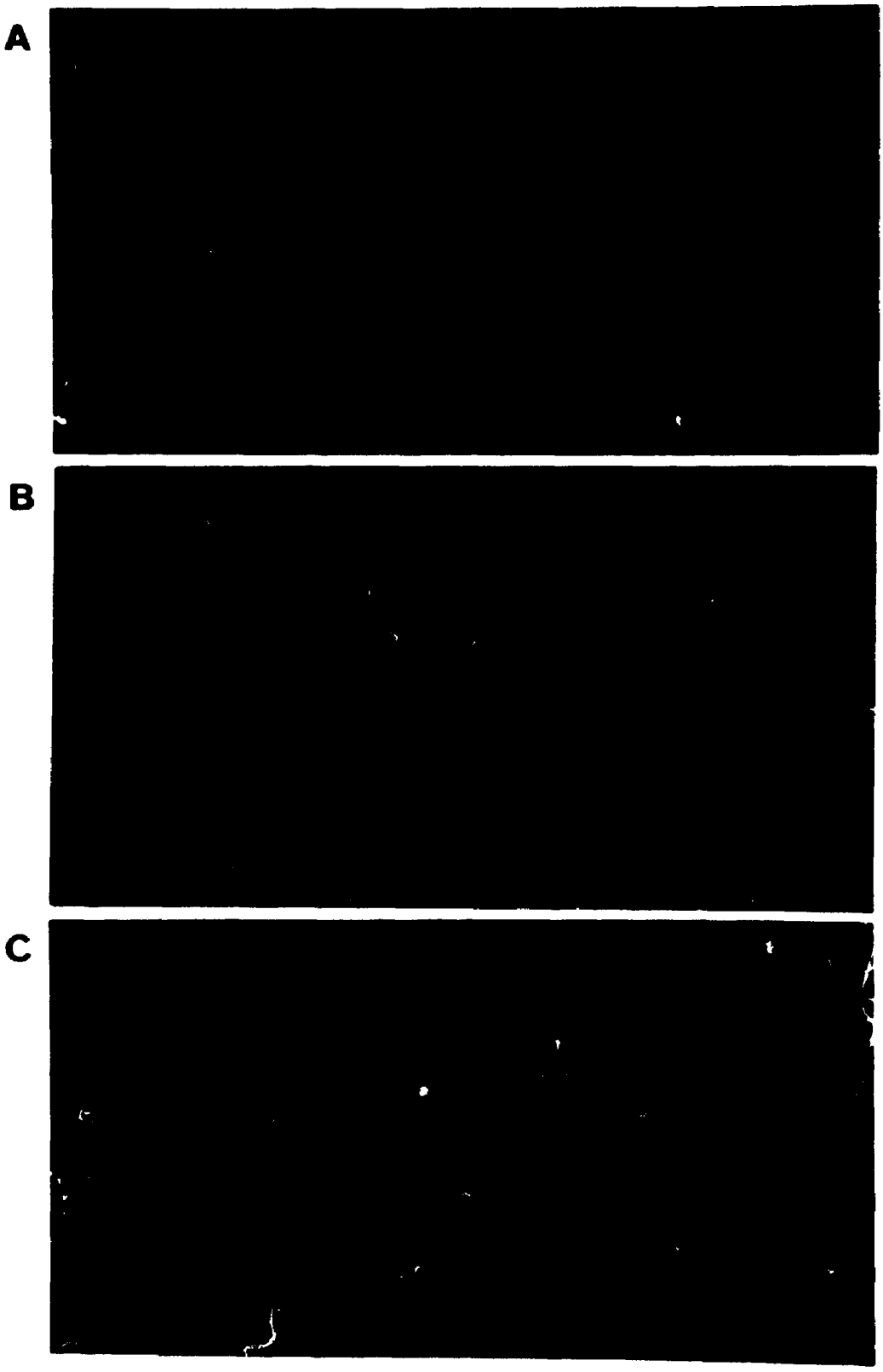
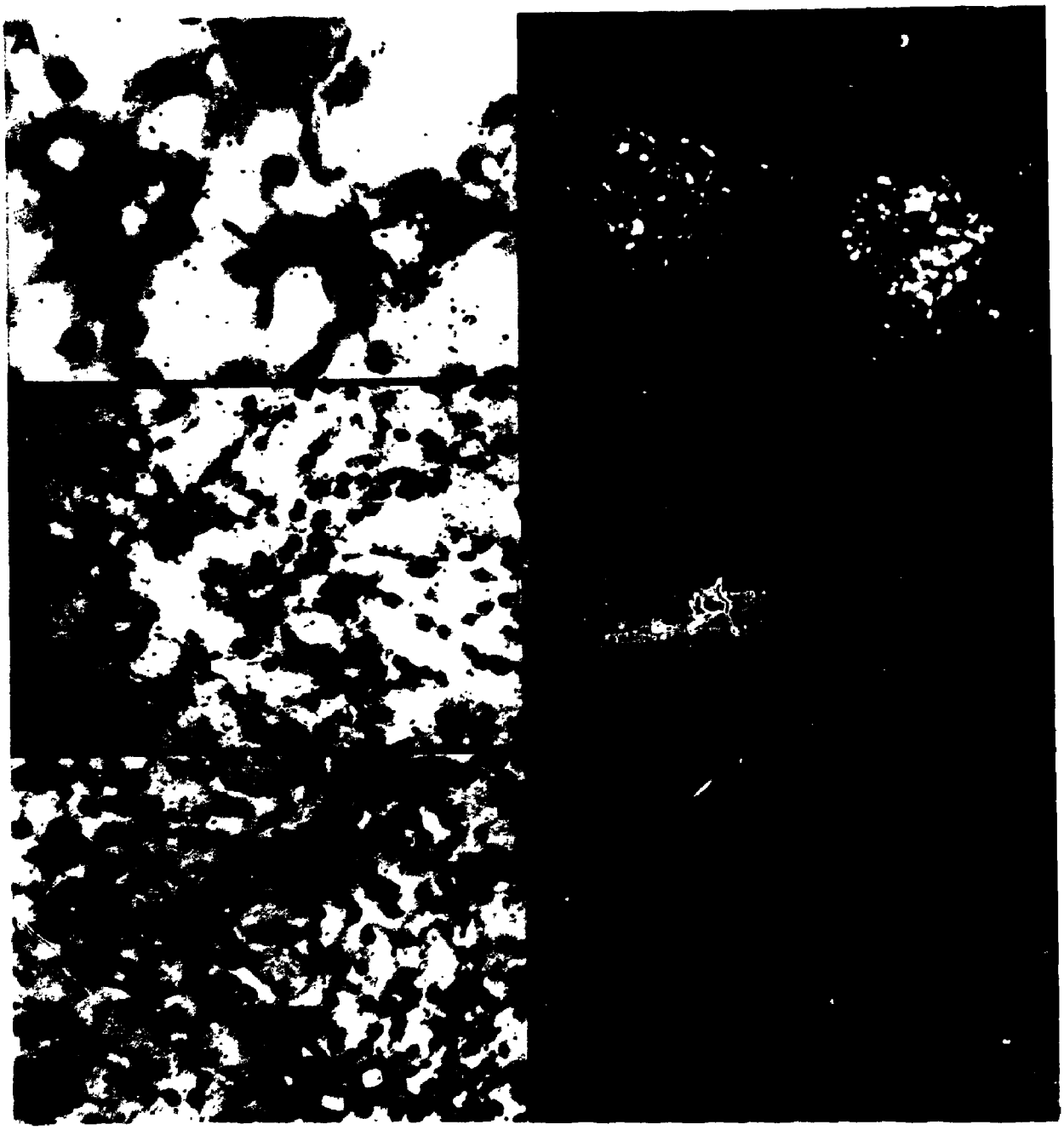

A
B
C

Figure 3.9

In situ hybridization of 2ar mRNA in the medulla of adult mouse kidney. Kidney sections were hybridized with ^{35}S-labeled antisense 2ar probe (A, B, C, D) or control sense 2ar probe (E, F) and processed as described. Sections were then stained with haematoxylin and eosin. Photographs of regions of the medulla were taken with a Zeiss Photomicroscope III under white light (A, C, E) or polarized light (B, D, F). (Magnifications: A, B, 1500X; C, D, E, F, 600X). This experiment was done in collaboration with M. McLeod and D.T. Denhardt.



medulla, and at a lower concentration in the outer medulla and inner cortex. No reproducible signal was seen in the area of the outer cortex, and all glomeruli were negative. The tubules which expressed 2ar mRNA were generally thin and of small diameter compared with the surrounding non-expressing tubules, and nuclei often appeared to be "flattened" against the sides (Fig. 3.9). Although the tissue morphology and staining did not allow for an unambiguous determination of tubule and cell types, the observations are consistent with expression of 2ar mRNA by the cells forming the thin segment of Henle's loop. Blood capillaries (vasa recta) are also thin and of small diameter, and form long bundles of parallel vessels (retia mirabilia) in the medulla (Bloom and Fawcett, 1975). The 2ar-expressing tubules did not seem to be clustered in bundles.

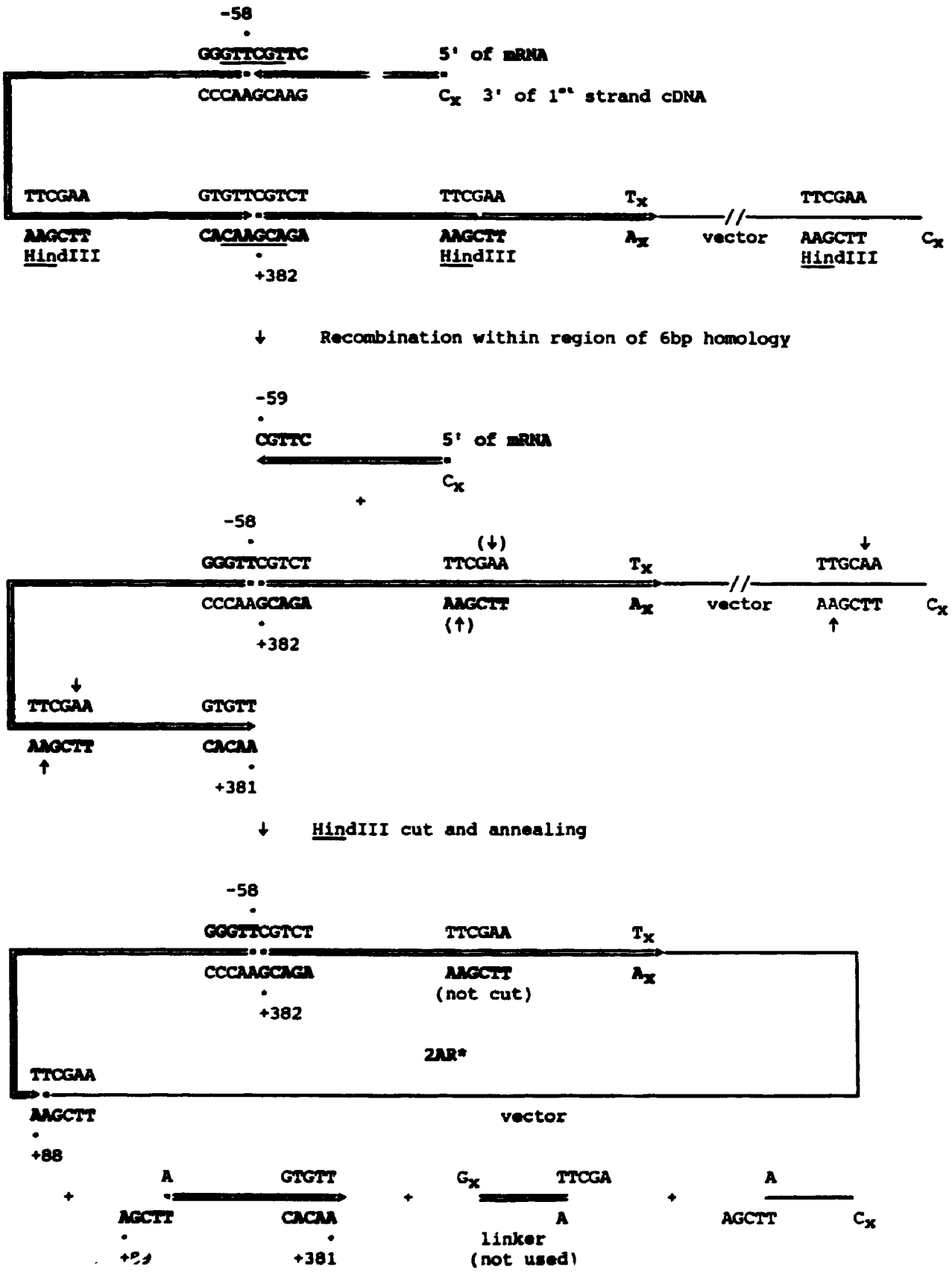
3.3 DISCUSSION

3.3.1 Cloning Sidenotes

The difficulties encountered in obtaining a full-length 2ar cDNA can be partially understood. Since the rat osteopontin cDNA is overall 87% identical to 2ar, and the similarity is maintained in the 3' non-coding region as well as in the coding region, it was expected that an oligonucleotide probe against the 5' non-coding region of osteopontin (position 1-25) would also be very similar to the homologous sequence in 2ar. However, based on the sequence of the primer-extended 2ar cDNA and of the first exon (Chapter 5), it is now evident that only 13 of these particular 25 bp are identical between 2ar and the osteopontin clone (even though an average of 23/25 are identical for the rest of the 5' non-coding region). Thus, it is now not surprising that the osteopontin 25-mer did not hybridize to 2ar mRNA or to a 2ar genomic clone.

Figure 3.10

Proposed mechanism of formation of the aberrant 2ar* cDNA. Recombination in a 6 bp region of internal homology in the 2ar mRNA-cDNA hybrid, followed by restriction at one of the internal HindIII sites, and ligation to the HindIII end of the vector could have produced the 2ar* clone. This low frequency event could have been selected by screening with both 5' and 3' 2ar probes (since restriction at the internal HindIII site appears to have prevented cloning of normal full-length cDNAs).


-58
GGGTTCGTTC
5' of mRNA
CCCAAGCAAG
C_x 3' of 1st strand cDNA
TTCGAA
GTGTTCGTCT
TTCGAA
T_x
TTCGAA
AAGCTT
HindIII
CACAAGCAGA
+382
AAGCTT
HindIII
A_x
vector
AAGCTT
HindIII
C_x
Recombination within region of 6bp homology
-59
CGTTC
5' of mRNA
C_x
-58
GGGTTCGTCT
TTCGAA
T_x
TTGCAA
CCCAAGCAGA
AAGCTT
A_x
vector
AAGCTT
C_x
+382
TTCGAA
GTGTT
AAGCTT
CACAA
+381
HindIII cut and annealing
-58
GGGTTCGTCT
TTCGAA
T_x
CCCAAGCAGA
AAGCTT
A_x
(not cut)
+382
2AR*
TTCGAA
AAGCTT
vector
+88
A
GTGTT
G_x
TTCGA
A
AGCTT
CACAA
A
AGCTT
C_x
+381
linker
(not used)

The apparent absence of a full-length 2ar cDNA in the original JB6 Cl22 library (Smith and Denhardt, 1987) can also be rationalized. The library was cut with HindIII at the mRNA-cDNA hybrid stage (Kowalski et al., 1985). The internal HindIII site at +88 of the 2ar mRNA-cDNA must have been susceptible to cleavage, since this is the 5' end of the longest 2ar cDNA obtained from this library (and the same clone was independently isolated several times). The other internal HindIII site at +1075 of 2ar must have been resistant to cleavage in at least some clones, suggesting that conformational effects may determine susceptibility or resistance to cleavage. The result of cleavage of 2ar clones at the +88 HindIII site, and selection for clones containing sequence 5' to this using the upstream exon probe was the isolation of rare, rearranged clones, such as 2ar*. A possible mechanism of formation of the 2ar* clone, by recombination within a 6 bp region of homology, is depicted in Fig. 3.10. Such a recombination could not have occurred in the mRNA itself, and thus the clone is not a copy of an aberrant mRNA, but simply a cloning artefact. Limited analysis of the other two clones isolated in a similar manner indicated that they also likely arose by internal recombinations resulting in even larger internal deletions.

3.3.2 Relationship to Osteopontin and Other Proteins

The discovery that 2ar, a TPA-inducible gene in cultured epidermal cells, codes for murine osteopontin came as a surprise. Osteopontin (also known as bone sialoprotein I and 44K bone phosphoprotein; reviewed by Butler, 1989) was initially described as a bone-specific protein. The sialic acid-rich bone proteins were first identified by Herring and Kent (1963), although the intact sialoproteins I and II were not isolated until

22 years later, from rat and bovine bone (Franzén and Heinegård, 1985a,b). Osteopontin (sialoprotein I) has been isolated independently from developing bovine bone (Fisher and Termine, 1985), rat bone (Prince et al., 1987), and human bone (Fisher et al., 1987).

In <u>in vitro</u> experiments, osteopontin functions in cell adhesion (Oldberg et al., 1986; Somerman et al., 1987), binds to the hydroxyapatite bone matrix (Oldberg et al., 1986), and has an apparent binding constant for Ca^{2+} in the mM range at pH 7.4 (Farach et al., 1988). Mature osteopontin from rat bone has a predicted M_r of 41,285, of which 33,268 is due to the 301 aa peptide backbone (Butler, 1989). The M_r determined by sedimentation equilibrium analysis is 44,000 (Prince et al., 1987). The protein contains about 12 phosphoserines and 1 phosphothreonine, and slightly over 30 monosaccharides (including 10 sialic acid residues) present in 1 N-linked and 5-6 O-linked oligosaccharides (Prince et al., 1987). The isoelectric point was determined as 4.9-5.1, compared with a predicted value of 4.0 (Butler, 1989). Osteopontin tends to form aggregates in non-denaturing solutions (Butler, 1989).

Isolation of murine osteopontin (protein) has not been reported. The high degree of conservation demonstrated here between the mouse and rat cDNA sequences and predicted secondary structures suggests that the mouse protein would also be very similar to the rat protein in function. Prince (1988) predicted a similar secondary structure for osteopontin, except for the highly acidic region which may be lacking secondary structure. The very limited protein analysis presented here indicated that mouse osteopontin is highly phosphorylated and likely glycosylated. The single N-linked glycosylation site is conserved, as are most of the

SerXaaGlu tripeptides (postulated by Oldberg et al., 1986 as a recognition sequence for O-linked glycosylation).

Sialoprotein II (also known simply as bone sialoprotein, BSP) is distinct from osteopontin (Fisher et al., 1987). The two proteins and cDNAs share no extended sequence homology, but do have several features in common (Oldberg et al., 1988a). Features of rat sialoprotein II which are reminiscent of osteopontin include: size (320 aa with a polypeptide core of 33.6 kDa); a 16-aa signal peptide; three potential N-linked glycosylation sites; an RGD potential cell adhesion site (near the C-terminus); and a high content of acidic amino acids (25%), including a series of 10 glutamates in the middle of the molecule (Oldberg et al., 1988a). The mature, glycosylated protein is 57 kDa and contains 5% sialic acid (Franzén and Heinegård, 1985a,b). Sialoprotein II is encoded by two mRNAs, 1600 and 2000 nt, which originate by use of alternative 3' polyadenylation sites. The mRNAs were found in bone, but not in kidney or several other tissues, and not in ROS17/2.8 osteosarcoma cells (Oldberg et al., 1988a). Rat sialoprotein II binds to the vitronectin receptor on ROS17/2.8 cells (Oldberg et al., 1988b), and human sialoprotein II promotes RGD-mediated adhesion to gingivial fibroblasts, but to a lesser extent than osteopontin (Somerman et al., 1988). Thus, it seems likely that sialoprotein II may share some functional similarity with osteopontin.

There is another rat bone phosphoprotein with reported structural similarity to osteopontin, designated bone acidic glycoprotein-75 (BAG-75; Gorski and Shimizu, 1988). This 75,000 kDa protein contains 29% acidic amino acids, 7.9% phosphate, and 7% (w/w) sialic acid, and also has a high affinity for hydroxyapatite. The amino acid composition and N-terminal

sequence are distinct from both osteopontin and sialoprotein II, although there is 33% homology of the N-terminal sequence with osteopontin. There is also a series of at least six (Asp/Glu) residues near the N-terminus.

The extent of structural and sequence similarity of osteopontin or sialoprotein II with BAG-75 is unclear. The results presented here indicate that murine osteopontin is encoded by a single gene, distinct from those that may encode the murine homolog of sialoprotein II or BAG-75. The differences in amino acid composition and N-terminal sequence also suggests that these three proteins are encoded by three separate genes. Immunological cross-reactivity among osteopontin, sialoprotein II, and BAG-75 (Franzén and Heinegård, 1985b; Gorski and Shimizu, 1988) may be due to shared epitopes, or due to cross-contamination of immunizing preparations (which were all bone-derived). The anti-2ar sera described here, which were raised against portions of the murine polypeptide backbone free of any contaminating mammalian bone proteins, may be useful in determining whether there are shared epitopes. It is interesting that the same 5 of the first 15 amino acids (1, 2, 3, 12, and 13) that are conserved between mature rat osteopontin and BAG-75 are also conserved in 2ar, suggesting a functional significance.

There was a recent observation of two forms of bone osteopontin, a 44 kDa form similar to that isolated previously from bone, and a 67 kDa form (T. Kubota, J.L. Wrana, R. Ber, Q. Zhang, J.E. Aubin, W.T. Butler, and J. Sodek, personal communication). Higher levels of the less phosphorylated 67 kDa phosphoprotein were found in bone extracts, and were secreted by primary rat calvarial cells, ROS17/2.8 osteosarcoma cells, and the calvarial osteoblastic RCA 11 cell line. The evidence that the 44 kDa and 67 kDa proteins are two forms of osteopontin was based largely on

recognition of these two proteins by affinity purified antibodies against rat osteopontin. However, these same affinity purified antibodies (from Mark et al., 1987b) were shown by Gorski and Shimizu (1988) to recognize sialoprotein II and BAG-75 as well as osteopontin on Western blots. The level of metabolically labeled secreted 67 kDa protein did also correlate with the level of osteopontin (2ar-hybridizable) mRNA. Immunoprecipitation with the anti-2ar sera, if it were found to specifically recognize osteopontin and not BAG-75 or sialoprotein II, might also be used to support the identification of the 44 kDa and 67 kDa proteins as two forms of osteopontin.

In this work, several murine polypeptide species were immunoprecipitated by anti-2ar sera, both in the case of metabolically labeled secreted proteins and in vitro translation products of hybrid-selected mRNA. The evidence seems convincing that the 2ar cDNA corresponds to a single gene and likely a single mRNA. The original 2ar cDNA was independently isolated several times, hybridizes to a single band on a Northern blot, and appears to be encoded by a single gene. Analysis of the first few exons of that gene (Chapter 5) revealed no evidence of alternate splicing. The region from nt 89-642 of the cDNA was completely protected from S1 nuclease by hybridization to mRNA. In this experiment, there was a minor species corresponding to protection of only nt ≈222-642. It is possible that this minor species is due to an alternately spliced mRNA; there is an intron located somewhere between nt 212 and 220 (Chapter 5). However, there could also be more trivial explanations, such as protection of an mRNA degradation intermediate or breathing of the cDNA-mRNA hybrid in this region, which is relatively A-T rich. Although the possibility of an

alternately spliced mRNA cannot be ruled out, the simplest hypothesis is that there is only a single 2ar mRNA.

The three distinct immunoprecipitated in vitro translation products could be derived from a single polypeptide by differential phosphorylation or by degradation of the highest M_r species. This experiment was performed in the absence of microsomal membranes, so it is expected that signal peptide cleavage and glycosylation would not have occurred (Anderson and Blobel, 1983). Heterogeneity in the 2ar protein secreted by JB6 cells is almost certainly due at least in part to differences in glycosylation; such microheterogeneity leading to the appearance of diffuse bands is often a result of different glycoforms (Rademacher et al., 1988). Differences in phosphorylation could also contribute to heterogeneity. There do appear to be differences in the post-translational modifications to osteopontin among different cell lines or tissues (see Chapter 4). Yet another possible explanation of the several immunoprecipitated species would be the presence of epitopes on other proteins, such as BAG-75 or sialoprotein II, that cross-react with the anti-2ar sera.

As will be documented in Chapter 4, 2ar/osteopontin is identical or closely related to a major tumor-associated phosphoprotein (Chackalaparampil et al., 1985; Senger et al., 1988). Aspects of this work relevant to protein structure and possible physiological role will be discussed here. The tumor-associated phosphoprotein displays microheterogeneity in the mouse cell lines, and the apparent M_r depends on the species of origin and the cell type (Senger et al., 1983). Chackalaparampil et al. (1985) reported two forms of the protein that differ in M_r (pp69 and pp62), and whose relative levels are related to the

ability of the cells to sustain anchorage-independent growth. Both of these forms are recognized by anti-2ar sera (Chapter 4). However, using the same cell lines, I have been unable to reproducibly detect pp62 (it was detected only in a mycoplasma-infected line; Chapter 4). Senger and colleagues have reported only one major form of the protein (with micro-heterogeneity) in all of the cell lines they have studied. Again, the data are conflicting with regard to the possibility of more than one major form of the protein, and this question is still not resolved. The production of two major forms may depend on cell type and culture conditions. Only a single major species of the same apparent M_r was detected in both normal human plasma and plasma of patients with disseminated carcinomas (Senger et al., 1988).

In many of these studies, including the immunoprecipitaions shown here, the apparent M_r of the protein was greater than the M_r calculated on the basis of the protein composition. This is likely due to anomalous migration on SDS-polyacrylamide gels, which has been demonstrated for mature rat osteopontin. The predicted M_r is 41,285 (Butler, submitted), while the apparent M_r is 45,000 on a uniform 15% SDS-polyacrylamide gel, and 75,000 on a 5-15% gradient polyacrylamide gel (Prince et al., 1987). The apparent M_r of the 2ar <u>in vitro</u> translation products on a 12% polyacrylamide gel, 56,000-67,000, is much greater than the M_r of 32,350 predicted from the amino acid composition. This suggests that the anomalous migration is largely not due to glycosylation, but to the polypeptide backbone, and possibly phosphorylation. The apparent M_r of the two cro-ß-galactosidase-2ar fusion proteins on a 7.5% polyacrylamide gel was also much greater than predicted (157 kDa versus a predicted ≈131.5 kDa for cro-ß-galactosidase-2arN; and 141 kDa versus a predicted

≈126 kDa for cro-ß-galactosidase-2arC). This supports the idea that the anomalously slow migration is due to the unusual amino acid composition of the polypeptide, perhaps through decreased binding of SDS compared with the "average" protein. SDS-polyacrylamide gel electrophoresis led to an overestimate of the M_r of several other proteins with a high content of acidic amino acids and clusters similar to the Asp_{10}, namely: aspartactin (54 kDa versus predicted 45 kDa; Clegg et al., 1988); ubiquinol-cytochrome c reductase subunit VI (26 kDa versus predicted 17.4 kDa; Loon et al., 1984); and the hinge protein of beef complex III of cytochrome c and c_1 (15 kDa versus predicted 9.2 kDa; Wakabayashi et al.,1982).

The consistent precipitation of only $1/5^{th}$ of the total metabolically labeled 2ar protein secreted by JB6 cells suggests that there are two conformations, only one of which is "available" or recognized by the antisera. After one immunoprecipitation, the equilibrium is re-established, releasing another $1/5^{th}$ of the remaining protein. This phenomenon may be related to the tendency of osteopontin to form aggregates in non-denaturing solvents (Butler, submitted).

3.3.3 Sequence Features

A. $(Aspartate)_{10}$

As alluded to above, there are several other proteins with a sequence similar to the run of 9-10 aspartates in 2ar/osteopontin. One is sialoprotein II, as mentioned above. Another, which may also have a functional similarity to 2ar, is aspartactin, a chicken extracellular matrix-associated skeletal muscle laminin-binding protein (Clegg et al., 1988). This 406-aa polypeptide (45 kDa) contains 28% acidic residues, including a series of 33 aspartates at the C-terminus. The secreted,

glycosylated protein binds to cells as a peripheral membrane protein, is localized to basal lamina surrounding individual muscle fibers, and is thought to modulate cell adhesion to the extracellular matrix via laminin. A 4.4-kDa (43 aa) soybean seed polypeptide contains at its C-terminus the sequence GlyArgGly$(Asp)_9$ (Odani et al., 1987), which combines the RGD sequence with a series of aspartates. Yeast ubiquinol-cytochrome c reductase 17-kDa subunit VI (147 aa) contains 40% acidic residues, including an internal $(Asp\ or\ Glu)_{25}$ (Van Loon et al., 1984). The homologous 9-kDa "hinge" protein of beef complex III (78 aa), which is thought to mediate the interaction between cytochrome c and c_1, contains 30% acidic residues, including Glu_8 (Wakabayashi et al., 1982). Finally, several Drosophila homeotic gene products (in the Notch, Antennapedia, and Ultrabithorax loci) contain a sometimes imperfect Gln_{31}; this sequence is encoded by the repetitive DNA element, opa (Regulski et al., 1985; Wharton et al., 1985a,b). Murine Hox 1.1 encodes a 229-aa polypeptide with Glu_{15} at the C-terminus (Kessel et al., 1987).

In all of these proteins, the function of the series of aspartates, glutamates, or glutamines is not known. Suggested functions include: interaction with basic residues on other proteins; binding to free Ca^{2+} and cooperative binding to Ca^{2+} on the surface of hydroxyapatite; and Ca^{2+}-independent activation of a Ca^{2+}/calmodulin-dependent cyclic nucleotide phosphodiesterase. Synthetic poly(L-Asp) does exhibit these activities (Gorbunoff, 1984; Tanaka et al., 1985). Other possible functions of an acidic amino acid cluster could be to render the polypeptide susceptible to proteolysis, to act as a proton sink, or to mediate pH-dependent changes in secondary structure (Van Loon et al., 1984; Wharton et al., 1985a,b).

It has been suggested that the Asp_9 in osteopontin may mediate its tight binding to hydroxyapatite, the most abundant component of bone calcified matrix (Oldberg et al., 1986). Osteopontin (the tumor-associated phosphoprotein) was isolated from plasma by virtue of its tight binding to a barium citrate precipitate (Senger et al., 1988). The Ba^{2+} moieties of this matrix may mimic the immobilized Ca^{2+} in hydroxyapatite. This suggests that osteopontin may bind to any Ca^{2+}, or perhaps even Mg^{2+}, matrix with the appropriate distribution of positive charges. The aspartate stretch may also contribute to free Ca^{2+}-binding, although this may be mediated instead or in addition by the potential E-F hand at amino acid 185-196 of osteopontin (Prince, 1988; and above).

B. RGD Sequence

The RGD sequence present in 2ar is a potential recognition site for the integrin family of cell surface receptors. Integrins and RGD-mediated interactions have been reviewed (Hynes, 1987; Ruoslahti and Pierschbacher, 1986, 1987; Rouslahti, 1988) and are briefly described below. RGD mediates cell binding of several proteins present in blood and/or in the extracellular matrix, including fibronectin, vitronectin, collagen, thrombospondin, fibrinogen, and von Willebrand factor. The role of the RGD sequence was demonstrated by the ability of RGD-containing peptides to competitively block cell binding.

Integrins are a family of receptors related by structure and sequence, and include the RGD-recognizing receptors, adhesive leukocyte surface proteins (LFA-1, Mac-1, and p150,95), very late antigens of activation on T-cells, and Drosophila position-specific antigens. Integrins are composed of one α (140-160 kDa) and one β (90-150 kDa) subunit. The α subunit has a Ca^{2+} and Mg^{2+} binding site and is often

composed of two polypeptides joined by a disulfate bridge, the ß subunit contains a cysteine-rich repeat, and both subunits have a transmembrane domain and a short cytoplasmic domain that binds to the cytoskeleton via talin. Some integrins appear to be specific for a single ligand, such as the human fibronectin receptor ($\alpha_F\beta_1$). In contrast, the integrin glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$) on the surface of platelets binds fibronectin, vitronectin, fibrinogen, von Willebrand factor, and possibly thrombospondin and collagen. Known functions of RGD-mediated integrin-ligand interactions include effects on extracellular matrix formation, cell adhesion, cytoskeletal organization and cell morphology, cell migration, cell differentiation, and platelet adhesion and aggregation. These events are essential for such physiological processes as embryogenesis and development, thrombosis and wound healing, phagocytosis, and likely metastasis of tumor cells. Other effects of integrin-ligand interactions, which may or may not be mediated via RGD, include adhesion of leukocytes, monocytes and neutrophils, complement binding, and T lymphocyte help and cytotoxicity.

The RGD site in rat osteopontin is functional in mediating adhesion to ROS17/2.8 osteosarcoma cells (Oldberg et al., 1986). Rat osteopontin, at 1-20 µg/ml (the same concentration range as fibronectin), stimulated attachment and spreading of human gingival fibroblasts, several other fibroblast or bone cell clones, and one epithelial-like cell line, but not A431 epidermal carcinoma cells (Somerman et al., 1987; Butler, 1989). Human osteopontin also stimulated RGD-dependent attachment of gingival fibroblasts, and this effect persisted for at least 8 days, whereas fibronectin-mediated attachment declined by 3 hours (Somerman et al.,

1987, 1988). Unlike fibronectin, osteopontin did not promote chemotactic migration of two cell types tested (Butler, 1989).

Inhibition experiments with variant RGD-containing peptides and with a vitronectin receptor antiserum (which is not completely specific) suggested that osteopontin may bind to the vitronectin receptor on ROS17/2.8 cells (Oldberg et al., 1988b). The existence of more than one type of osteopontin receptor is supported by cell type variability in trypsin sensitivity of the receptor (Butler, 1989). Cell surface localization of a phosphorylated form of osteopontin (pp69) indicated that normal rat kidney cells also have receptors (M. Nemir and B.B. Mukherjee, personal communication). It is not known whether JB6 epidermal cells have receptors for 2ar.

Osteopontin apparently does not bind to collagen (Somerman et al., 1987; Butler, 1989). A non-phosphorylated form of osteopontin (np69) may bind to fibronectin (M. Nemir and B.B. Mukherjee, personal communication). Osteopontin also binds heparin; predicted binding sites rich in basic, aromatic and hydrophobic residues are at amino acids 134-143 and 283-289 of mature osteopontin (Prince, 1988). These sites are completely conserved in 2ar, at amino acids 150-159 (YGLRSKSRSF) and 276-282 (RYLKFRI) in Fig. 3.2. Thus, osteopontin may bind to extracellular matrix via fibronectin and/or heparin.

3.3.4 Localization and Speculations on Physiological Role

As shown by the <u>in situ</u> hybridization studies presented here, the localization of 2ar mRNA in adult mouse kidney is clearly very different than in newborn or embryonic kidney, as determined by Nomura et al. (1988) with an identical 2ar probe. In the newborn kidney, 2ar mRNA was

expressed mainly in the outer cortex, in proximal and distal tubules and loops of Henle, and was not expressed in the medulla (Nomura et al., 1988). In our studies in adult kidney, 2ar mRNA was absent from the outer cortex, and was expressed mainly in the inner medulla, and to a lesser extent in the outer medulla and inner cortex. Reinholt et al. (1988) have also reported synthesis of rat osteopontin mRNA in the medulla of the kidney.

The localization of 2ar mRNA is consistent with expression by cells forming the thin segments of Henle's loop, or, less likely, blood capillaries. Thin segments of Henle's loop are lined by squamous epithelia, in which the nuclei appear to bulge into the lumen, and most of the wall is made of thin segments (1-3 μm) of interdigitated cell processes (Bloom and Fawcett, 1975). The cells rest on a fairly thick basal lamina, and there are short microvilli on the luminal surface. One of the major functions of the loop of Henle is the production of hypertonic urine, by means of a sodium pump to create an osmotic gradient, a descending limb which is permeable to water, and an ascending limb which is impermeable to water (Bloom and Fawcett, 1975).

The attempted immunolocalization of 2ar protein in mouse kidney was unsuccessful. There are many possible reasons for this, but the most likely is the quality of the antibodies, since the same antisera or affinity purified antibodies did not yield specific signals in two other systems, using a variety of methods (J. Aubin, and B. Hogan, personal communications). Immunolocalization of rat osteopontin in bone, dentin, kidney and nervous tissues has been reported (Mark et al., 1987a,b, 1988a,b). However, the affinity purified antibodies used also reacted with sialoprotein II and BAG-75 on a Western blot (Gorski and Shimizu,

1988). In kidney, the protein was found in apical endocytotic vacuoles and lysosomes of proximal tubules, a pattern typical of uptake and degradation (Mark et al., 1988b). It was surprising that this group did not detect synthesis of osteopontin protein (i.e. localization to the Golgi region) anywhere in kidney, which produces such large amounts of the mRNA. It is possible that antibodies against the processed bone protein did not recognize the protein as processed in the kidney. Production of tissue-specific glycoforms of other proteins has been reported (Rademacher et al., 1988). Using antibodies against a rat osteopontin fusion protein rather than against the mature bone protein, Reinholt et al. (1988) detected osteopontin in epithelial cells in the papilla (i.e. inner medulla) region of kidney. Synthesis of the protein in this region would be consistent with the localization of 2ar mRNA we have found. The protein was concentrated mainly at the basal surface, to a lesser extent at the luminal surface, and intracellularly at the mitochondria (Reinholt et al., 1988).

The function of 2ar/osteopontin in kidney is not likely to relate to its ability to bind to calcified matrices, but may involve binding of free Ca^{2+}, and/or attachment of the epithelia to the basal lamina or to each other. There was a marked change in localization of expression of 2ar mRNA within the kidney, from scattered unidentified tubules at 16.5 days post-coitum, to proximal and distal tubules and loops of Henle in the cortex in the newborn, to scattered tubules (likely thin segments of Henle's loop) in the medulla in the adult (Nomura et al., 1988; and here). It is possible that attachment mediated by 2ar/osteopontin and its receptor(s) is necessary for proper kidney development, in a manner analogous to the requirement for Drosophila position-specific antigen

integrins for attachment of mesoderm to ectoderm and later attachment of muscles to epidermis and gut (Leptin et al., 1989).

Expression of osteopontin/2ar in developing bone has been extensively studied (Mark et al., 1987a,b, 1988a; Yoon et al., 1987; Evered and Harnett, 1988; Nomura et al., 1988; Reinholt et al., 1988). Briefly, osteopontin is synthesized by preosteoblasts, osteoblasts, and osteocytes beginning at 15-17 days of gestation, and deposited into the extracellular matrix prior to mineralization. Osteopontin was detected in both endochondral and membranous bone from every location, in the mineralization front of newly formed bone, and sometimes in the osteoid and at the cell/osteoid surface. The protein was detected in the Golgi apparatus of osteoblasts, indicating secretion. Evidence of secretion by odontoblasts into predentin, and by chondrocytes into areas of cartilage-to-bone transition was also found. High level expression of 2ar/osteopontin in bone is consistent with a role in cell adhesion to the extracellular matrix, and possibly in mineral metabolism. It appears to act as an attachment factor for osteoblasts to the osteoid matrix, as suggested by the name osteopontin (bridge), and then becomes trapped in the matrix upon mineralization. It has been suggested that osteopontin may play a direct role in mineralization, but this idea is controversial (Evered and Harnett, 1988).

The localization of 2ar mRNA to certain regions of the inner ear, as described in the introduction to this chapter, is supported by immunostaining of similar structures for rat osteopontin (Mark et al., 1988b). Expression in the sensory epithelia of the maculae, which is overlain by the otoconia (a matrix containing calcium carbonate), and in the endolymphatic sacs, which secrete immature otoconial material, is

consistent with a role in binding cells to calcified matrix, and possibly matrix formation. 2ar/osteopontin mRNA and protein were also detected in the acoustic and vestibular ganglia, and cross-reacting protein in neurons of the trigeminal ganglion (Swanson et al., 1989; Mark et al., 1988b).

In addition to bone, kidney, inner ear, brain, decidua, placenta, and uterus, 2ar/osteopontin mRNA has been found in ovary and at low levels in lung (D.T. Denhardt, personal communication; Yoon et al., 1987). High levels of 2ar mRNA were also found in ventral but not dorsal skin of pregnant mice, and in both ventral and dorsal skin of lactating mice (D.T. Denhardt, personal communication). The protein (tumor-associated phosphoprotein) is present in human milk at ≈3-10 μg/ml (Senger et al., 1989). The biological significance of 2ar/osteopontin expression in these tissues is not obvious. This protein may have another function independent of binding to cells and calcified matrix.

The pattern of expression described above strongly suggests that 2ar/osteopontin is under hormonal regulation. It seems likely that it may be induced in the appropriate cell type by estrogens, progesterone, and/or prolactin. Although a large number of samples were not analyzed, 2ar mRNA appeared to be expressed at a higher level in the kidneys of female versus male mice. This could be due to a generalized effect of estrogens. An analogous induction of ornithine decarboxylase mRNA in kidney by androgens has been reported (Hickok et al., 1986). Regulation of expression of osteopontin by other agents will be discussed in Chapters 4 and 5.

A final site of localization reported for 2ar (tumor-associated phosphoprotein) is in blood (Senger et al., 1988). This was first suggested by Mark et al. (1987b), based on osteopontin immunolocalization. The protein is secreted by macrophages, is found at low levels in normal

serum, and is cleaved by thrombin during blood coagulation (Senger et al., 1988). These results suggest that 2ar may play a role in hemostasis or thrombosis. The binding of osteopontin to heparin (an anticoagulant) could also be significant in the regulation of thrombosis. Cleavage by thrombin could potentially activate or inactivate cell adhesion, Ca^{2+} binding, or another function of osteopontin. There are three possible sites of cleavage consistent with the known specificity of thrombin (Mann and Lundblad, 1987), with the production of a cleavage product about half the size of the original protein (Senger et al., 1988), and with the residues conserved between mouse and rat osteopontin. These possible cleavage sites are between residues 144 and 145 (Arg/Gly), 153 and 154 (Arg/Ser), or 157 and 158 (Arg/Ser) in Fig. 2. Amino-terminal analyses by Senger et al. (1989) indicated that the second of these sites is used, i.e. between residues 153 and 154. This cleavage site is in one of the potential heparin-binding domains, and would split the molecule between the RGD site and the potential E-F Ca^{2+} binding loop.

High levels of 2ar (tumor-associated phosphoprotein) were found in 4 of 4 patients with gram-negative sepsis, leading Senger et al. (1988) to suggest that the protein may normally be involved in a response to bacterial infection. This is consistent with the possibility of allelism between 2ar and Ric (resistance to the bacterium which causes scrub typhus), as suggested in the introduction. If this is the case, it again suggests a function other than binding cells to mineral matrices.

CHAPTER 4

THE ROLE OF 2AR IN CARCINOGENESIS

4.1 INTRODUCTION

The initial interest in 2ar stemmed from the marked induction of this mRNA by the tumor promoter TPA in JB6 epidermal cells, suggesting that enhanced expression of 2ar may be relevant to carcinogenesis (Smith and Denhardt, 1987). Induction was transient in subconfluent cells, and sustained in confluent cells. At the same time, it was found that, while the message was induced by TPA in all JB6 subclones, the level of message induction did not correlate with the ability of the clones to form colonies in soft agar in the presence of TPA. This characteristic has been referred to as promotability (Colburn et al., 1979), although the irreversible acquisition of anchorage independent growth in response to TPA might more aptly be considered part of progression, i.e. malignant conversion. In any case, the lack of correlation of 2ar expression with this attribute of in vitro transformation in these cell lines still leaves open the question of its relevance to carcinogenesis. It is expected that any effect of 2ar expression on carcinogenesis would be in cooperation with other gene products, some of which may be defective in the "non-promotable" JB6 clones.

2ar mRNA was induced in JB6 cells by platelet derived growth factor (PDGF) and epidermal growth factor (EGF), but not by benzoyl peroxide, a tumor promoter which does not activate PKC (Smith and Denhardt, 1989). Induction in this system was inhibited by some (retinoic acid, dexamethasone), but not all (indomethacin, difluoromethyl ornithine) inhibitors of tumor promotion. Induction did correlate with activation of PKC and with the mitogenic response (Smith and Denhardt, 1989). 2ar mRNA was also

induced by treatment of quiescent mouse fibroblasts with serum or purified PDGF, EGF, basic fibroblast growth factor (bFGF), or embryonal carcinoma-derived growth factor (ECDGF) (Smith and Denhardt, 1987; Nomura et al., 1988). Cancer is essentially a disease of uncontrolled growth, and as mentioned earlier, several of the oncogenes encode aberrant growth factors or growth factor receptors. Thus, induction of 2ar mRNA by growth factors is again suggestive of a contribution of this gene product to some aspect of cancerous growth.

If 2ar does play a role in carcinogenesis, what is that role likely to be? Based on its function as a cell adhesion protein, it seems most likely that 2ar would be involved in invasion and/or metastasis, later stages in the progression of a tumor. Synthetic peptides containing the RGD sequence can inhibit both tumor cell invasion through the human amniotic membrane in vitro (Gehlsen et al., 1988) and experimental lung metastasis of murine melanoma cells (Humphries et al., 1986). The specificity of the inhibitory peptides in the in vitro assay suggested a role for fibronectin and type I collagen interactions, but not vitronectin (Gehlsen et al., 1988).

Changes in fibronectin and/or its receptors have been studied as a possible basis for the changes in adhesive properties of tumor cells, i.e. invasion and metastasis in vivo, and foci formation, anchorage-independent growth and invasion in vitro. Malignant cells lack an organized cytoskeleton and do not form a proper extracellular matrix (ECM) (Ruoslahti and Pierschbacher, 1987). This may be due to defects in specific ECM components and/or in cellular receptors. Some transformed cells secrete lesser amounts of a more highly phosphorylated form of fibronectin (Ali and Hunter, 1981), but other transformed cells secrete normal amounts of

fibronectin, and it is apparently functional (Ruoslahti, 1988). Addition of excess fibronectin will still mediate attachment of these cells. A reduction in the $\alpha_5\beta_1$ fibronectin receptor, and in two other integrins, $\alpha_A\beta_1$ and $\alpha_B\beta_1$, may contribute to the reduced adhesion of the transformed cells (Plantefaber and Hynes, 1989). Phosphorylation of a tyrosine in the cytoplasmic domain of integrin RGD-receptors may also regulate adhesion (Ruoslahti and Pierschbacher, 1987). Since many malignant cells are free of a proper ECM, they may use their adhesion receptors to facilitate migration through tissues. In addition to promoting cell attachment and spreading, fibronectin and vitronectin also modulate the growth rate and differentiation of various cell types, and promote both directed and random migration of tumor cells (Ali and Hynes, 1978; Basara et al., 1985).

Thus, integrin-cell adhesion protein interactions have been implicated in the spread of tumor cells. Unlike fibronectin, 2ar/osteopontin does not seem to be a component of the extracellular matrix surrounding normal cells, except in bone, and perhaps kidney and a very limited number of other tissues. Inappropriate expression of this protein (e.g. due to induction by tumor promoters) could interfere with cell-ECM interactions and enhance the release of tumor cells from a primary site, or promote the migration of tumor cells and/or adhesion at a secondary site.

The experiments presented below were aimed initially at determining whether enhanced expression of 2ar correlated with transformation *in vitro* and/or carcinogenesis *in vivo*. This included the demonstration that induction of 2ar by the tumor promoter TPA occurred not only in cultured cells, but also in mouse epidermis *in vivo*. Some of the results also help to differentiate between a correlation with initiation and promotion, or

progression and metastasis. Construction of 2ar mammalian expression vectors are described here as part of a final experiment still in progress. They are being used to up-regulate or down-regulate 2ar specifically to attempt to demonstrate more than a correlation, i.e. to determine whether 2ar expression contributes to the metastatic ability of certain cell lines.

4.2 RESULTS

4.2.1 Induction of 2ar by the Tumor Promoter TPA

Smith and Denhardt (1987) showed that 2ar mRNA was induced by 10 ng/ml TPA in JB6 epidermal cells. The dose-response in subconfluent JB6 cells was determined, for a range of 1-40 ng/ml TPA and a treatment of 6 h (Fig. 4.1A). It was found that maximal induction (≈52-fold by densitometry) was achieved even at 1 ng/ml TPA. In contrast, ODC mRNA increased with increasing TPA concentration from 0.06 ng/ml (0.1 nM) to a maximum at 600 ng/ml (1000 nM) in primary mouse epidermal cells (Verma et al., 1986).

TPA-induction of 2ar protein in JB6 cells was observed, using the anti-cro-ß-galactosidase-2ar sera for immunoprecipitation of secreted 2ar protein metabolically labeled with either [^{35}S]methionine or [^{32}P]ortho-phosphate (Fig. 4.1B). A longer exposure of these gels indicated that the untreated cells secreted much lower but detectable levels of labeled 2ar. 2ar protein was detectable by Western blot analysis of conditioned media proteins from TPA-treated cells, but not from untreated cells (Fig. 4.1C). In each case, the TPA treatment was for 24 h. Thus, the induction of mouse 2ar/osteopontin by TPA occurs not only at the mRNA level, but also at the protein level, at least <u>in vitro</u>. These methods did not provide

Figure 4.1

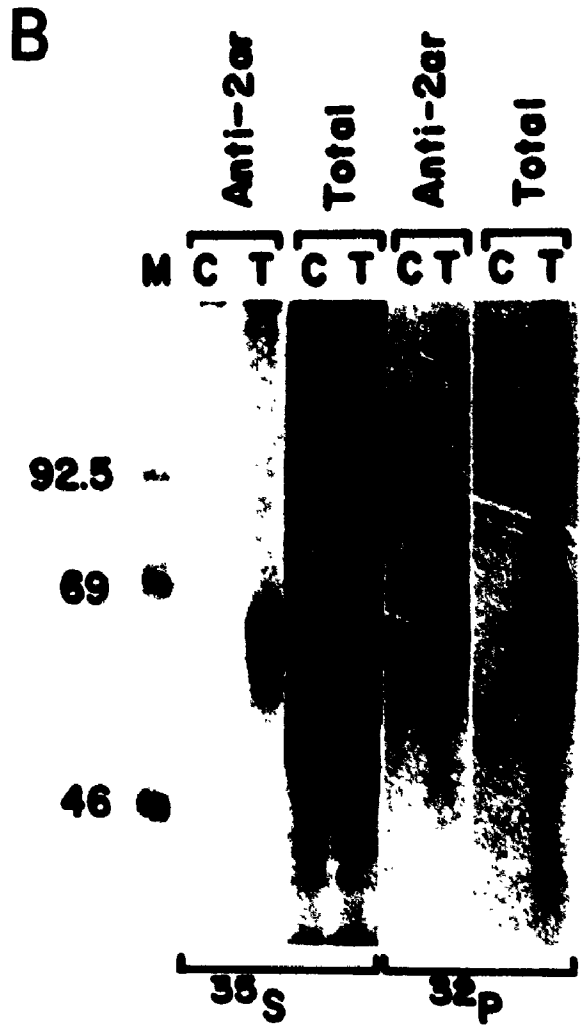
Effect of the tumor promoter TPA on 2ar expression in JB6 epidermal cells.

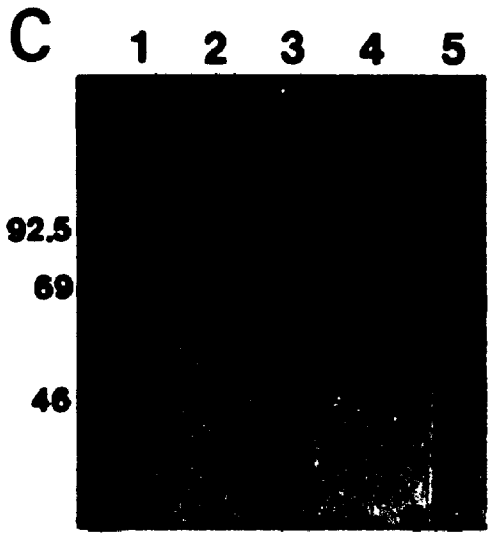
A. Dose-response of TPA-induction of 2ar mRNA in JB6 epidermal cells. JB6 cells were treated for 6 h with TPA in DMSO. Numbers above the lanes indicate increasing concentrations of TPA: 1, no TPA; 2, 1 ng/ml TPA; 3, 4 ng/ml TPA; 4, 10 ng/ml TPA; 5, 40 ng/ml TPA. Cytoplasmic RNA was electrophoresed (10 μg/lane in the left panel; 3 μg/lane in the right panel), blotted, and probed with ^{32}P-labeled 2ar cDNA.

B. Immunoprecipitation of metabolically labeled 2ar protein secreted by control or TPA-treated JB6 epidermal cells. Cells were treated with DMSO alone (C lanes) or 10 ng/ml TPA in DMSO (T lanes) for 72 h and labeled for the last 3 h of treatment with either [^{35}S]methionine or [^{32}P]ortho-phosphate, as indicated. Secreted proteins were electrophoresed on reducing SDS-polyacrylamide gels (12%) directly (Total) or after immunoprecipitation of volumes normalized to ^{35}S-incorporation with cro-ß-galactosidase-2arC antiserum (Anti-2ar). Gels were fluorographed (^{35}S) or autoradiographed (^{32}P). A composite of two autoradiograms is shown.

C. Western blot analysis of 2ar protein secreted by control or TPA-treated JB6 epidermal cells. Cells were treated for 24 h with DMSO alone (lanes 2, 4) or 10 ng/ml TPA in DMSO (lanes 1, 3, 5) in media containing low serum (0.2%). Conditioned media were electrophoresed on an SDS-polyacrylamide mini-gel (10%) (5 μl/lane in lanes 1 and 2; 50 μl/lane in lanes 3-5). Proteins were transferred to nitrocellulose and stained with amido black (lanes 1 and 5) or probed with anti-cro-ß-galactosidase-2arC sera (lanes 2-4). Control anti-cro-ß-galactosidase sera and pre-immune sera gave no signal (not shown). Primary antibody binding was detected by immunogold staining and silver enhancement.


A
1 2 3 4 5 1 2 3 4 5
4.3
3.5
2.0
1.9
1.5
1.4
0.9


B
Anti-2ar
Total
Anti-2ar
Total
M C T C T C T C T
92.5
69
46
35S
32P


C
1 2 3 4 5
92.5
69
46

a quantitative determination of the level of induction of 2ar protein, but it is clearly a marked induction, as in the case of the mRNA.

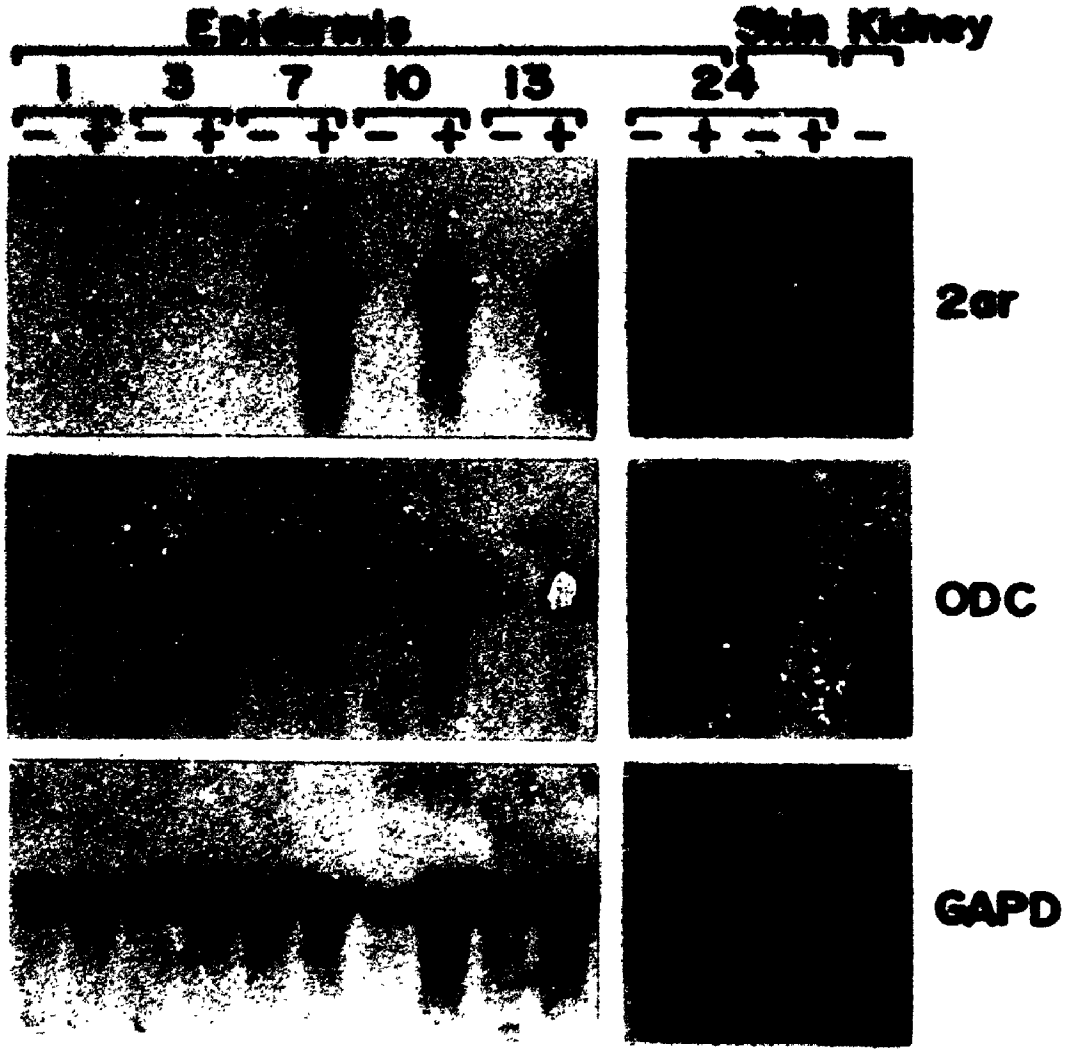
TPA treatment (24 h) of exponentially growing Swiss 3T3 fibroblasts also resulted in a marked induction of 2ar protein, as determined by immunoprecipitation of secreted proteins metabolically labeled with [^{32}P]orthophosphate (not shown). Although not quantitated, the extent of tumor promoter-induction in the mouse 3T3 fibroblasts appeared to be about the same as in the JB6 epidermal cells.

A limited study was done to determine the effect of TPA on the extent of phosphorylation of 2ar. The last 1/2 h of a 5 or 15.5 h metabolic labelling of subconfluent JB6 cells with [^{32}P]orthophosphate was performed in the presence or absence of 10 ng/ml TPA. Under these conditions, TPA had no effect on the amount of ^{32}P-radioactivity incorporated into secreted immunoprecipitable 2ar (data not shown). Incorporation of ^{32}P was greater after 15.5 h than after 5 h of labelling, indicating that the [^{32}P]orthophosphate had not been exhausted after 5 h. Thus, TPA did not immediately stimulate phosphorylation of 2ar under these conditions.

The <u>in vitro</u> studies were followed by an <u>in vivo</u> assay for tumor promoter induction of 2ar mRNA. The 2ar mRNA, present at very low levels in untreated epidermis, was induced by a single application of TPA to the back of a CD-1 mouse (Fig. 4.2). Induction was transient, peaking at 7-13 h and returning to basal levels by 24 h after application of the tumor promoter. Ornithine decarboxylase mRNA was also transiently induced, with maximal induction ≈3 h after TPA treatment (Gilmour et al., 1987; and Fig. 4.2). Glyceraldehyde phosphate dehydrogenase mRNA served as a control for the quality and amount of mRNA on the Northern blots.

Figure 4.2

Northern blot analysis of the level of 2ar mRNA in control or TPA-treated mouse epidermis. Mouse skin (5 cm^2) was painted with acetone alone (- lanes) or acetone containing 5 μg of TPA (+ lanes) for the times indicated, in hours, prior to killing. Total RNA was isolated from the tissues as described and electrophoresed (10 μg/lane). The resulting blot was probed successively with ^{32}P-labeled 2ar cDNA (987-bp HindIII fragment) and plasmids carrying cDNA corresponding to ornithine decarboxylase (ODC) and glyceraldehyde-3-phosphate dehydrogenase (GAPD). Mobilities of the messages relative to λ HindIII-EcoRI markers indicated sizes of approximately 1.6 kb (2ar), 2.5-2.7 kb (ODC), and 1.6 kb (GAPD).


Epidermis
Skin Kidney
1
3
7
10
13
24
2cr
ODC
GAPD

4.2.2 Relationship of 2ar to a Major Secreted Phosphoprotein

It was demonstrated in Chapter 3 that 2ar is the major phosphoprotein secreted by TPA-treated JB6 cells. Osteopontin is also the major phosphoprotein secreted by ROS17/2.8 osteosarcoma cells (Prince and Butler, 1987). These observations led us to pursue the idea that 2ar/osteopontin might be identical or closely related to the major phosphoprotein secreted by many rodent cell lines, known as a transformation-related phosphoprotein (Senger et al., 1980), or pp69 and the related protein pp62 (Chackalamparampil et al., 1985). Chackalaparampil et al. (1985) reported that pp69 is secreted by normal rat kidney cells (NRK-49F; a fibroblastic subclone) or retinoic acid-treated RR1022 cells (a Rous sarcoma virus-transformed rat cell line), and pp62 by RR1022 cells or TPA-treated NRK-49F cells.

NRK-49F cells, untreated or TPA-treated, and RR1022 cells, untreated or retinoic acid-treated, were metabolically labeled with [^{35}S]methionine or [^{32}P]orthophosphate. The secreted proteins were electrophoresed directly, or after immunoprecipitation with anti-cro-ß-galactosidase-2ar sera or control sera (Fig. 4.3). The major secreted phosphoprotein from all cell lines was specifically immunoprecipitated by the anti-2ar sera. However, in contrast to the expected 69 kDa and 62 kDa proteins, only one species (corresponding to pp69; see below) was secreted by all of the rat cell lines. While the apparent molecular weight of the protein secreted by NRK-49F cells was not changed by TPA, the tumor promoter did cause a slight but specific induction in the amount of 2ar-related protein produced.

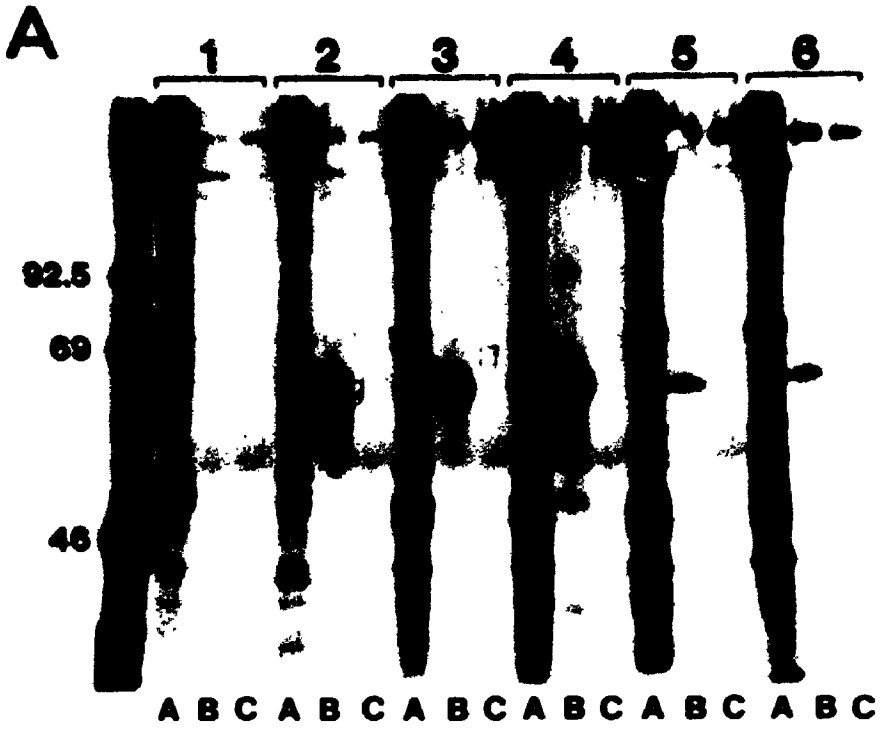
The apparent relationship of 2ar with pp69 and pp62 was further explored in collaboration with M. Nemir and B.B. Mukherjee. Conditioned

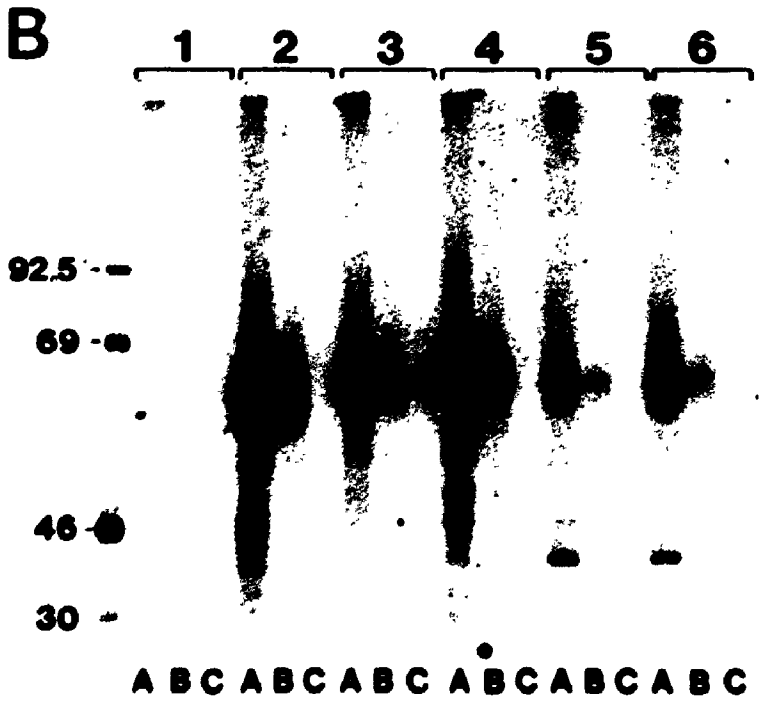
Figure 4.3

Immunoprecipitation of 2ar-related protein secreted by normal rat kidney (NRK-49F) and RR1022 cells.

A. Immunoprecipitation of [^{35}S]methionine-labeled proteins. Numbers above the lanes indicate the cell lines and treatments: 1, mouse JB6 epidermal cells treated with DMSO; 2, JB6 cells treated with 10 ng/ml TPA in DMSO; 3, rat NRK-49F cells treated with DMSO; 4, NRK-49F cells treated with 10 ng/ml TPA in DMSO; 5, rat RR1022 fibroblasts treated with DMSO; 6, RR1022 cells treated with 10^{-5} M retinoic acid in DMSO. All treatments were for 72 h, and cells were labeled with [^{35}S]methionine for the last 3 h of treatment. Secreted proteins were electrophoresed directly (A) or after immunoprecipitation of equal volumes with anti-cro-ß-galactosidase-2arC sera (B) or control anti-cro-ß-galactosidase sera (C). A fluorogram of the SDS-polyacrylamide gel (10%) is shown.

B. Immunoprecipitation of [^{32}P]orthophosphate-labeled proteins. The methods and symbols are the same as in part A, except that the cells were labeled with [^{32}P]orthophosphate instead of [^{35}S]methionine.


A
1 2 3 4 5 6
92.5
69
46
A B C A B C A B C A B C A B C A B C


B
1 2 3 4 5 6
92.5
69
46
30
A B C A B C A B C A B C A B C A B C

media containing [^{32}P]orthophosphate-labeled proteins from late passage rat NRK-49F cells, KNRK cells, and mouse KA31 cells were immunoprecipitated with antiserum raised against gel-purified pp69 (Laverdure et al., 1987) or the antisera against the 2ar fusion proteins. As shown in Fig. 4.4A, the anti-pp69 and anti-2ar sera recognized the same species secreted by every one of these cell lines. A second precipitation of the supernatant from the first immunoprecipitation with both antisera ruled out the possibility of two unrelated phosphoproteins with the same electrophoretic mobility (not shown).

The anti-pp69 and anti-2ar sera recognized the secreted phosphoprotein from rat cells with approximately equal efficiency. However, while the anti-2ar sera was specific (Fig. 4.3), the anti-pp69 sera also recognized several other [^{35}S]methionine-labeled secreted proteins (not shown). The anti-pp69 (rat) antiserum was also less efficient at precipitating the protein secreted from mouse KA31 cells (Fig. 4.4A) or JB6 epidermal cells (Fig. 4.4B). Furthermore, the anti-pp69 serum did not recognize the cro-ß-galactosidase-2ar fusion proteins by Western blot analysis (Fig. 4.4C). [In contrast, an antiserum against rat bone osteopontin (a gift of C.W. Prince and W.T. Butler) did specifically recognize the 2ar fusion proteins.] These data suggest that the anti-pp69 serum mainly recognizes epitopes containing post-translational modifications on pp69, such as glycosyl and phosphoryl groups, and that these modifications are different on the homologous proteins secreted by mouse cells (and presumably absent from the cro-ß-galactosidase-2ar fusion proteins isolated from E. coli).

For reasons we do not understand, only under certain culture conditions (compare Fig. 4.3 and 4.4A) NRK cells and derivatives

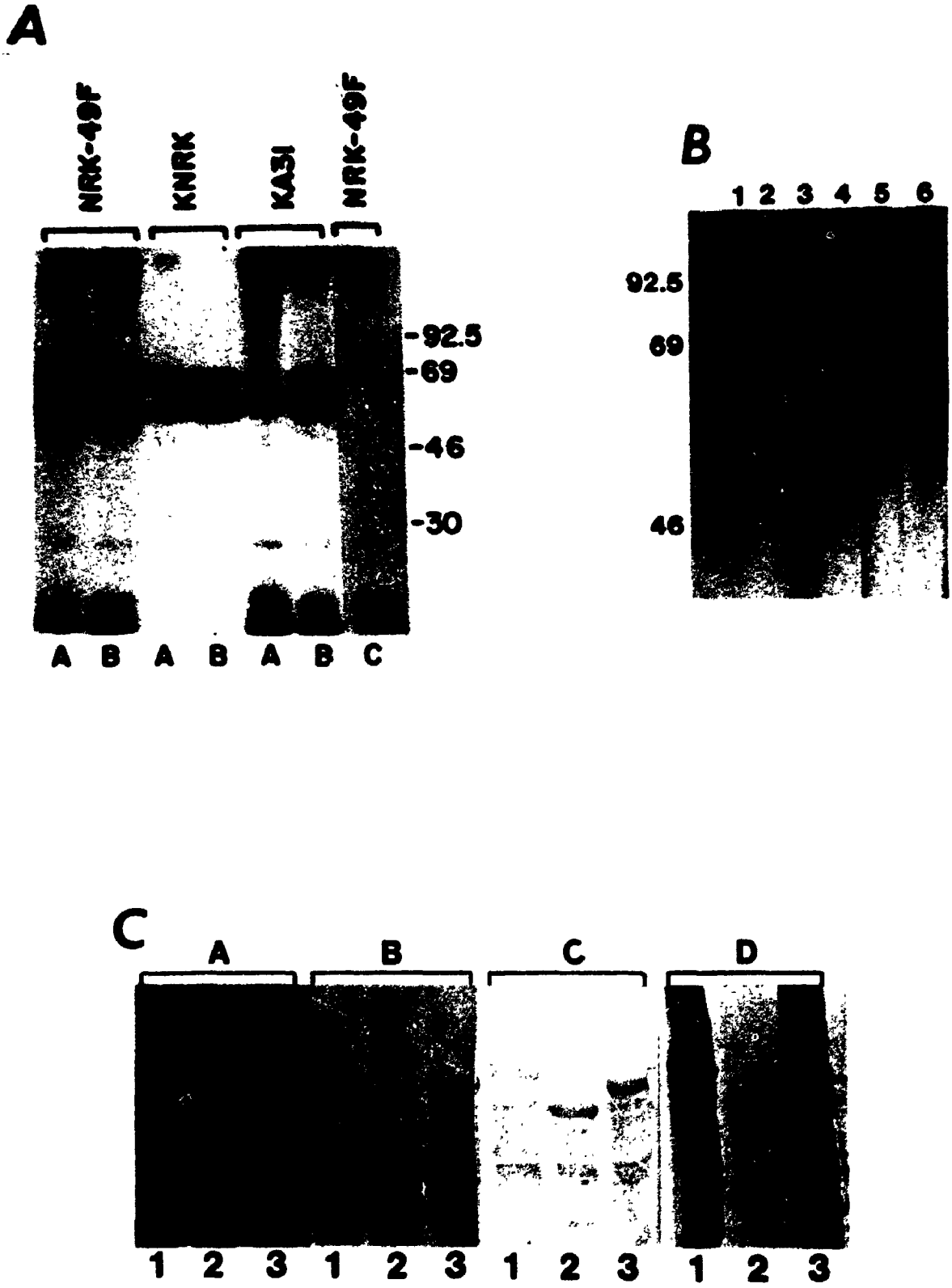
Figure 4.4

Immunological relationship between 2ar and pp69.

A. Immunprecipitation of ^{32}P-labeled proteins secreted by rat or mouse fibroblasts with anti-2ar or anti-pp69 sera. The indicated cells lines were labeled with [^{32}P]orthophosphate and media containing equal amounts of acid-precipitable radioactivity were immunoprecipitated with the following antisera: A, anti-pp69; B, anti-cro-ß-galactosidase-2arC; C, normal rabbit serum. This experiment was done in collaboration with M. Nemir and B.B. Mukherjee.

B. Immunoprecipitation of ^{32}P-labeled proteins secreted by JB6 epidermal cells with anti-2ar or anti-pp69 sera. JB6 cells were treated for 24 h with DMSO (lanes 1-4) or TPA in DMSO (lanes 5, 6) and labeled with [^{32}P]orthophosphate for the last 4 h of treatment. Secreted proteins were electrophoresed directly (lane 3) or after immunoprecipitation with the following antisera: anti-osteopontin (lane 1); anti-pp69 (lanes 2, 5); or anti-cro-ß-galactosidase-2arC (lanes 4, 6). The SDS-polyacrylamide gel (10%) was autoradiographed.

C. Western blot analysis of fusion protein recognition by anti-cro-ß-galactosidase-2arC (A), anti-pp69 (B), pre-immune serum (C), or anti-osteopontin (D). The fusion proteins were isolated as described and electrophoresed on an SDS-polyacrylamide gel (7.5%): lanes 1, cro-ß-galactosidase-2arN; lanes 2, cro-ß-galactosidase; lanes 3, cro-ß-galactosidase-2arC. The blot was developed by the peroxidase-conjugated secondary antibody method.


A
NRK-49F
KNRK
KA31
NRK-49F
-92.5
-69
-46
-30
A B A B A B C
B
1 2 3 4 5 6
92.5
69
46
C
A
B
C
D
1 2 3 1 2 3 1 2 3 1 2 3

apparently secreted a second major phosphoprotein, pp62, which is related to pp69 both immunologically (as shown here) and by peptide mapping (Chackalamparampil et al., 1985; Laverdure et al., 1987). The secretion of pp62 is reported to be correlated with the anchorage-independent growth of transformed or partially-transformed cells (Chackalaparampil et al., 1985). However, in this laboratory, pp62 has not been detected by metabolic labelling of several transformed cell lines (Fig. 4.3 and 4.5), except in one instance, by [^{32}P]orthophosphate-labelling of mycoplasma-infected KNRK cells (not shown). The single species detected in all other cases co-electrophoresed with the higher M_r of the two species, i.e. pp69.

4.2.3 Effect of Transformation on Expression of 2ar

The level of expression of 2ar was determined in several pairs of transformed and parental untransformed cells (Fig. 4.5). PAP2 cells, derived from pooled clones of NIH3T3 cells transformed with the human bladder cancer (T24) H-ras oncogene (Hill et al., 1988), secreted more 2ar than the parental NIH3T3 cells. Densitometric analysis indicated a greater than 10-fold difference in the level of labeled immunoprecipitated protein. In response to serum stimulation of quiescent cells, a DMBA-transformed derivative of mouse Balb/c3T3 fibroblasts (obtained from C. Parfett) also secreted much higher levels of metabolically labeled 2ar than the parental cells. The expression of 2ar protein by exponentially growing Balb/c and DMEA-transformed cells was not studied, but it is expected that there would be a difference, since the level of 2ar mRNA is greater in the exponentially growing DMBA-transformed cell line (C. Parfett, personal communication).

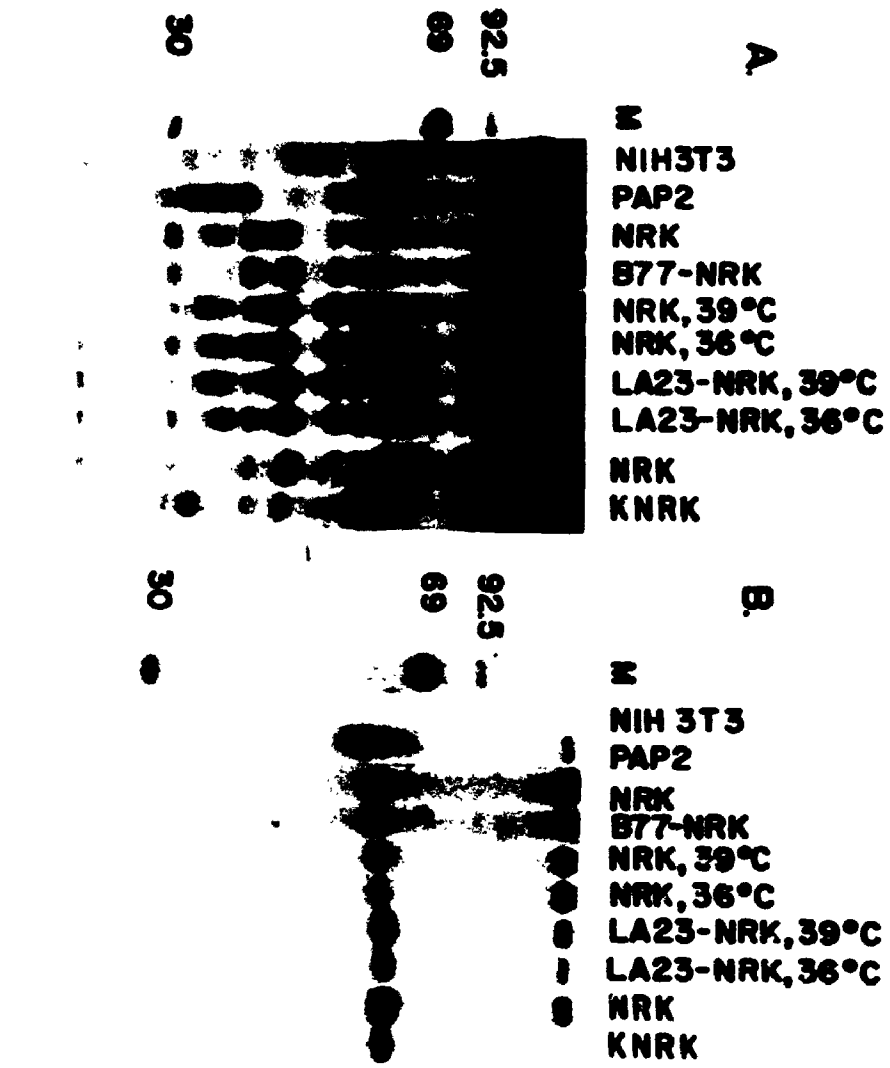
Figure 4.5

Immunoprecipitation of 2ar protein secreted by transformed and parental untransformed mouse and rat cells.

A. Total protein secreted by transformed derivatives of NIH3T3 and NRK cells. The indicated cell lines were metabolically labeled with [^{35}S]methionine for 3 h. Portions of conditioned media containing equal amounts of acid-precipitable radioactivity were electrophoresed on SDS-polyacrylamide gels (12%) and fluorographed. Some of these ^{35}S-labeled protein samples were obtained from A.F. Chambers.

B. Immunoprecipitated 2ar protein secreted by transformed derivatives of NIH3T3 and NRK cells. Conditioned media as in part A were immunoprecipitated with cro-ß-galactosidase-2arC antiserum prior to electrophoresis and fluorography. The protein immunoprecipitated from NRK cells and derivatives corresponds to pp69 (the higher M_r species in Fig. 4.4A); this was determined by electrophoresis of both samples on a single gel. The mobility of 2ar/osteopontin is known to depend on electrophoretic conditions (Prince et al., 1987).

C. Immunoprecipitated 2ar protein secreted by serum-stimulated Balb/c 3T3 fibroblasts and a DMBA-transformed derivative. Cells were made quiescent by growth in media containing 1% serum for 6 days, and then stimulated with 10% serum for 0, 6, 12, 18, or 24 h (left to right in the figure). Proteins were labeled with [^{35}S]methionine for the last 3 h prior to harvest. Media containing equal amounts of acid-precipitable radioactivity were immunoprecipitated with anti-cro-ß-galactosidase-2arC serum and electrophoresed on an SDS-polyacrylamide gel (10%). A fluorogram of the gel is shown.


A.
30
69
92.5
M
NIH3T3
PAP2
NRK
B77-NRK
NRK, 39°C
NRK, 36°C
LA23-NRK, 39°C
LA23-NRK, 36°C
NRK
KNRK
B.
30
69
92.5
M
NIH 3T3
PAP2
NRK
B77-NRK
NRK, 39°C
NRK, 36°C
LA23-NRK, 39°C
LA23-NRK, 36°C
NRK
KNRK
C
46
69
92.5
0→24
0→24
BALB/c 3T3
DMBA 50

In contrast to the mouse fibroblast cells, NRK cells transformed with viral K-ras (KNRK) or viral src (B77-NRK) showed no obvious change in the level of expression of 2ar. This result was confirmed with LA23-NRK, a cell line that expresses a ts src oncogene and thus is transformed at 36°C but not at 39°C (Chen et al., 1977). Expression of 2ar by these cells did not show a corresponding temperature sensitive phenotype.

To confirm these results and to determine at what level the induction of 2ar occurred, cytoplasmic RNA was isolated from subconfluent cells and subjected to Northern blot analysis (Fig. 4.6A). Densitometry of the hybridization signal obtained with the 2ar cDNA probe normalized to the actin signal showed no difference in 2ar mRNA steady state level due to transformation of NRK cells with viral src. This was the case both for B77-NRK versus NRK, and for LA23-NRK at 36°C versus 39°C, in agreement with the protein data. In the T24 H-ras transformed PAP2 cells, there was a ≈3.5-fold increase in H-ras mRNA and a ≈3-fold increase in 2ar mRNA over the untransformed parental cells.

Two human cell lines, the non-tumorigenic human osteosarcoma (HOS) cells and a tumorigenic derivative, MNNG-HOS, were also assayed for expression of a 2ar-related mRNA and protein. Northern blot analysis of total cytoplasmic RNA probed with ^{32}P-labeled 2ar cDNA resulted only in background hybridization to ribosomal RNA. Similarly, the anti-2ar sera were unable to immunoprecipitate any secreted [^{32}P]orthophosphate-labeled protein (data not shown).

4.2.4 Correlation of 2ar Expression with Metastatic Potential

The PAP2 cells are not only tumorigenic, but also highly metastatic in an assay for experimental metastasis in the chick embryo (Hill et al.,

Figure 4.6

Northern blot analysis of 2ar mRNA levels in untransformed and transformed cell lines differing in metastatic ability.

A. Northern blot analysis of 2ar mRNA levels in normal rat kidney (NRK) cells and transformed derivatives. Cytoplasmic RNA was electrophoresed (10 µg/lane), blotted, and probed successively with ^{32}P-labeled 2ar and ß-actin cDNAs. In the blot hybridized to the actin probe, the lower M_r species in the four right lanes is a residual signal from the 2ar probe.

B. Northern blot analysis of 2ar mRNA levels in non-metastatic mouse cell lines (NIH3T3, LTA) and metastatic derivatives (PAP2, 6bL4b). Cytoplasmic RNA (obtained from A.F. Chambers) was electrophoresed (10 µg/lane), blotted, and probed successively with ^{32}P-labeled 2ar, v-H-<u>ras</u>, and ß-actin.

C. Northern blot analysis of 2ar mRNA levels in mouse 10T1/2 fibroblasts and T24 <u>ras</u>-transfected derivatives. A blot containing cytoplasmic RNA (20 µg/lane) was obtained from A.H. Greenberg. The blot was probed successively with ^{32}P-labeled 2ar, ß-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNAs. Numbers at the bottom indicate the relative levels of <u>ras</u> mRNA, which correlated with metastatic ability (from Egan et al., 1987).

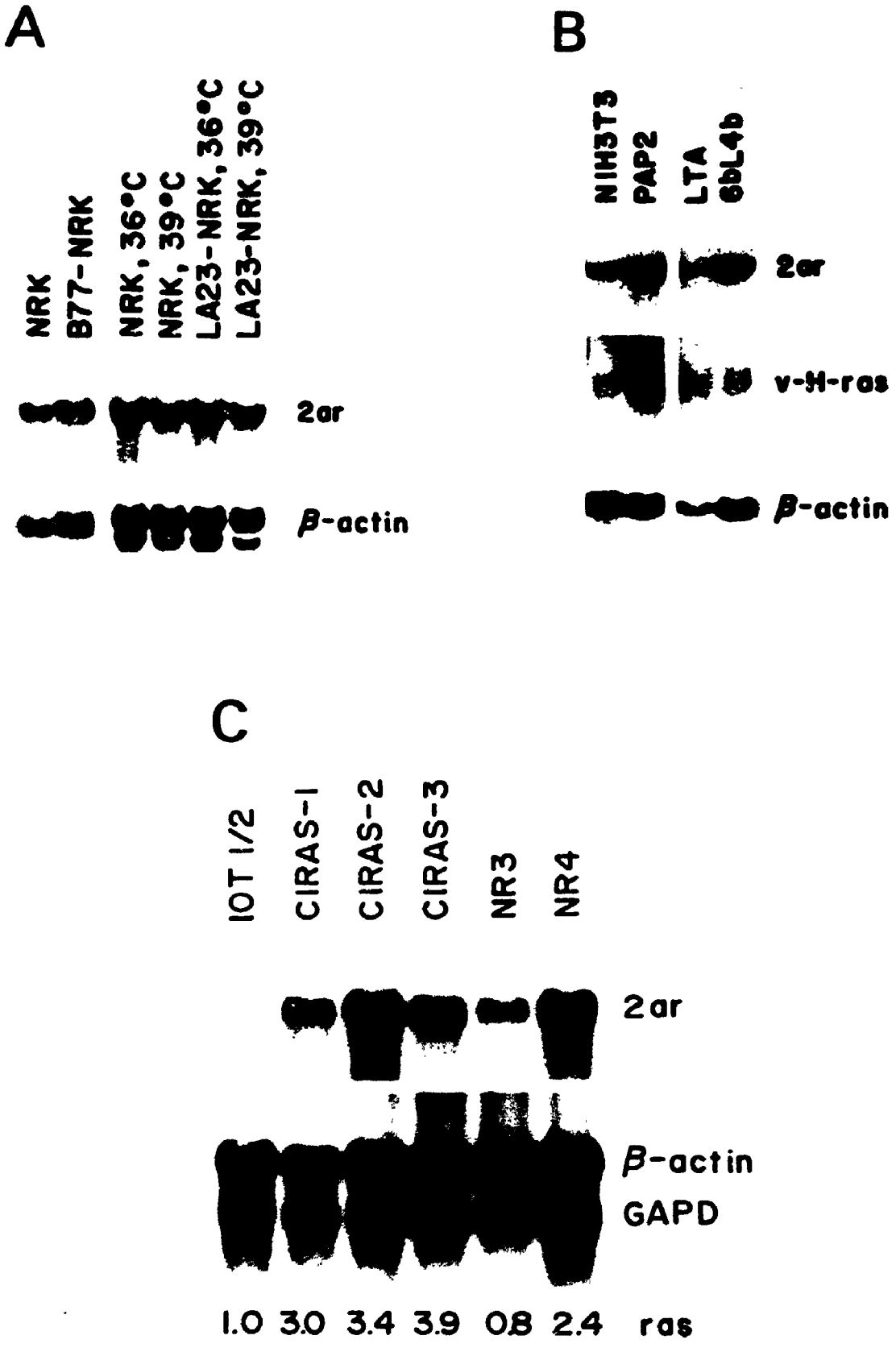

A
NRK
B77-NRK
NRK, 36°C
NRK, 39°C
LA23-NRK, 36°C
LA23-NRK, 39°C
2ar
β-actin
B
NIH3T3
PAP2
LTA
6bL4b
2ar
v-H-ras
β-actin
C
IOT 1/2
CIRAS-1
CIRAS-2
CIRAS-3
NR3
NR4
2ar
β-actin
GAPD
1.0 3.0 3.4 3.9 0.8 2.4 ras

TABLE 4.1

COMPARISON OF 2AR EXPRESSION WITH METASTATIC POTENTIAL

A Cell line	Tumorigenicity	Experimental metastasis[1] (cells recovered per liver)	ras mRNA[2] (relative amount)	2ar mRNA[2] (relative amount)
NIH3T3	-	$< 10^3$	(1)	(1)
PAP2	+	10^4	3.4	2.9
LTA	+	$< 10^3$	(1)	(1)
GbL4b	+	10^5	0.6	6.0

B Cell line	Tumorigenicity[3] (frequency)	Experimental[3] metastases (frequency)	 (No. ± SE)	Spontaneous[4] metastases (frequency)	ras mRNA[3] (relative amount)	2ar mRNA[2] (relative amount)
10T1/2	0/12	0/12	0	0/10	(1)	(1)
C1RAS-1	13/13	20/27	14 ± 5	7/13	3.0	9.4
C1RAS-2	11/11	8/8	118 ± 6	13/14	3.4	17.1
C1RAS-3	11/11	14/14	121 ± 20	15/15	3.9	11.6
NR3	6/8	1/13	0.1 ± 0.1	0/10	0.8	2.5
NR4	10/10	12/19	2 ± 0.5	4/15	2.4	14.6

[1]From Chambers and Ling (1984) and Hill et al. (1988),by intravenous injection of 1-5 X 10^5 cells into chick embryos and recovery of ouabain^r cells from liver.

[2]The relative amounts of 2ar (or ras mRNA) were estimated by densitometry of Northern blots shown in Fig. 4.6; the 2ar (or ras) signal was normalized to the actin signal.

[3]From Egan et al. (1987), by injection of 3 X 10^5 cells into syngeneic C3H/HeN mice, and analysis of lung metastases and ras mRNA levels.

[4]From Denhardt et al. (1987), by subcutaneous injection of 3 X 10^5 cells into syngeneic C3H/HeN mice; only lung metastases were found.

1988; Table 4.1). The above results, and the ability of 2ar/osteopontin to mediate cell adhesion, led us to determine the level of expression of 2ar mRNA in several tumorigenic cell lines which vary in metastatic ability. 6bL4b is a cell line derived by transfection of mouse LTA cells (fibroblast-like L-cells) with genomic DNA from the highly metastatic B16F1 murine melanoma cells (Chambers and Ling, 1984). While LTA cells are tumorigenic but non-metastatic, 6bL4b cells are both tumorigenic and metastatic (in the chick embryo experimental metastasis assay). Northern blot analysis indicated that the steady-state level of 2ar mRNA was 6-fold higher in 6bL4b cells than in LTA cells (normalized to actin; Fig. 4.6B; Table 4.1).

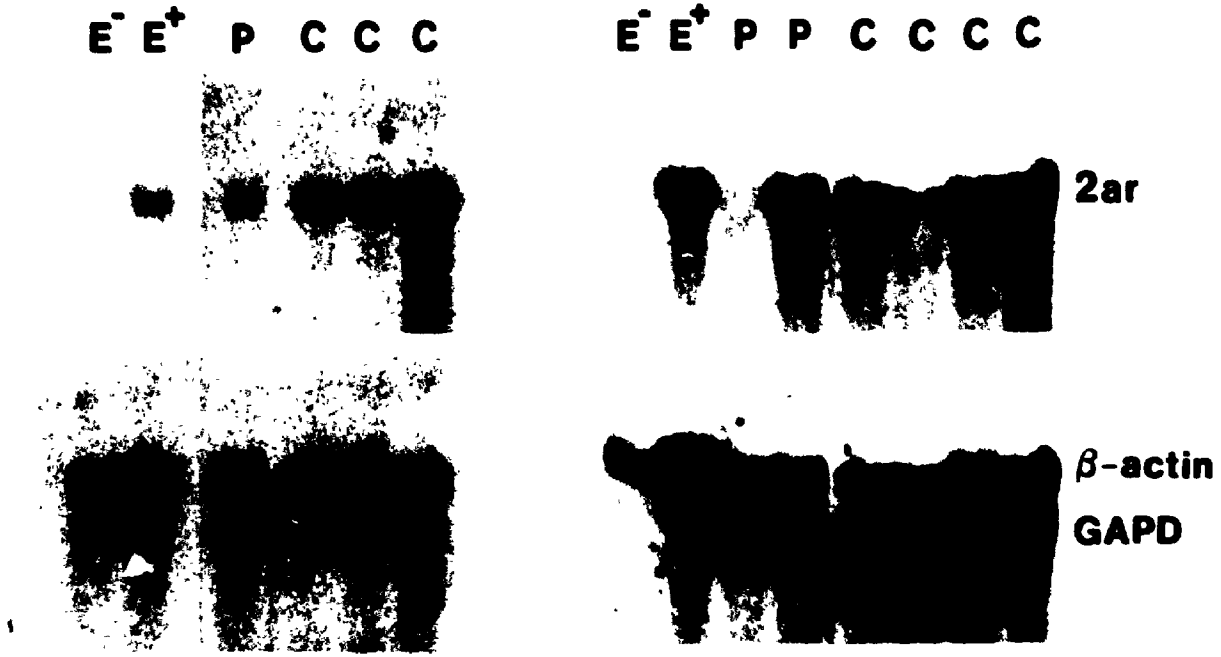
Another set of mouse fibroblast lines varying in metastatic potential were constructed by Egan et al. (1987) by transfection of 10T1/2 cells (non-tumorigenic and non-metastatic) with the T24 H-ras oncogene. Selecting for the G418 resistance marker on the transfected plasmid, two classes of cell lines were isolated: C1RAS-1, -2, and -3 from transformed foci, and NR3 and NR4 from flat, contact-inhibited colonies. The expression of ras mRNA was found to correlate with anchorage-independent growth and in vivo metastatic potential (Denhardt et al., 1987; Egan et al., 1987). We report here that the expression of 2ar mRNA also correlates with ras expression, anchorage-independent growth and metastatic ability of these cell lines (Fig. 4.6C and Table 4.1).

4.2.5 Enhanced Expression of 2ar in Epidermal Carcinomas

The possible link between 2ar expression and carcinogenesis in vivo was studied by a determination of the level of 2ar mRNA in mouse epidermal papillomas and carcinomas, in collaboration with G.T. Bowden (Fig. 4.7).

Figure 4.7

Northern blot analysis of 2ar mRNA levels in mouse epidermal papillomas and carcinomas. Total RNA from epidermal papillomas (P) and squamous cell carcinomas (C) was obtained from G.T. Bowden. The papillomas and carcinomas were induced in Sencar mice by initiation with either 20 μg of DMBA or 100 μg of Cisplatin followed by promotion with twice weekly applications of 2.5-5 μg of TPA. RNA from control epidermis (E^-) or epidermis treated with 5 μg of TPA for 13 h (E^+) was isolated as described. RNA was electrophoresed (10 μg/lane in the left panel; 30 μg/lane in the right panel), blotted, and probed successively with ^{32}P-labeled 2ar, ß-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNAs.


E- E+ P C C C
E- E+ P P C C C C
2ar
β-actin
GAPD

Papillomas and squamous cell carcinomas were induced in Sencar mice by initiation with DMBA or Cisplatin, followed by TPA promotion. Carcinomas were distinguished from papillomas by histological signs of invasive growth. In two of three papillomas, and seven of seven carcinomas, 2ar mRNA was constitutively expressed. The level of constitutive expression was approximately the same as or greater than the maximal induction obtained transiently after a single TPA treatment.

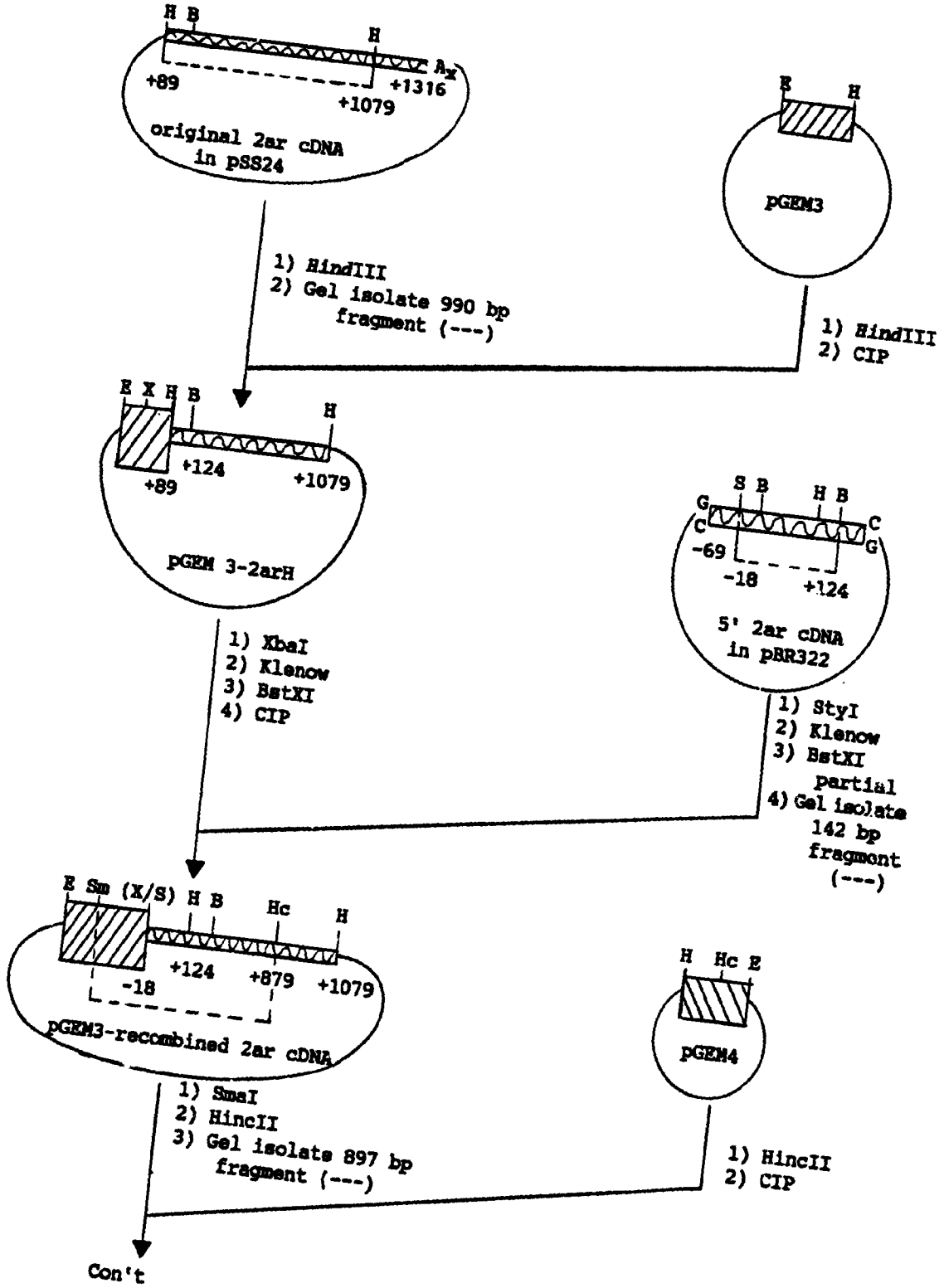
4.2.6 Construction of Vectors for Up- and Down-Modulation of 2ar

A final experiment described here is the construction of expression vectors which would express sense or antisense 2ar mRNA in mammalian cells. The cloning strategy is described in Fig. 4.8. By various manipulations of the original cDNA, the 5' cDNA, and the pGEM cloning vectors, a "complete 2ar cDNA" (-18 to +1079 with respect to translation) flanked by BamHI sites was obtained. This cDNA contains the complete coding region, 18 bp of 5' untranslated sequence, and 197 bp of 3' untranslated sequence.

The cDNA was cloned in both orientations into the mammalian expression vector pNMT (Khokha and Denhardt, 1987). This vector contains the metallothionein promoter to direct expression of the sense or antisense RNA, and transcription termination and polyadenylation signals from the human growth hormone gene. Ideally, transcription from the metallothionein promoter will be inducible by zinc or cadmiun, but in practice this has not always been the case (Khokha et al., 1989). The pNMT vector has been used successfully for constitutive expression of antisense RNA corresponding to tissue inhibitor of metalloproteinase, and consequent down-modulation of the protein (Khokha et al., 1989).

Figure 4.8

Cloning strategy for reconstitution of a 2ar cDNA containing the complete coding region and insertion into a vector for sense and antisense expression in mammalian cells. Wavy lines indicate 2ar cDNA, hatched boxes indicate multiple cloning sites (from the pGEM vectors), and single lines indicate vector DNA. Numbers refer to the position in the 2ar cDNA relative to the translation start site. Klenow was used to render DNA ends blunt, and calf intestinal phosphatase (CIP) was used to dephosphorylate vectors, as indicated. The pNMT vector contains the metallothionein promoter (MT prom.) and human growth hormone gene polyadenylation signal (hGH polyA) flanking the cloning site, and the selectable neomycin resistance gene (Neo^r). Restriction enzyme sites are indicated as follows: B, BstXI; Bm, BamHI; E, EcoRI; H, HindIII; Hc, HincII; P, PstI; S, StyI; Sm, SmaI; Sp, SphI; X, XbaI.


H B
H
A_x
+89
+1316
+1079
original 2ar cDNA
in pSS24
1) HindIII
2) Gel isolate 990 bp
fragment (---)
E H
pGEM3
1) HindIII
2) CIP
E X H B
H
+124
+89
+1079
pGEM 3-2arH
1) XbaI
2) Klenow
3) BstXI
4) CIP
S B H B
G
C
C
G
-69
-18
+124
5' 2ar cDNA
in pBR322
1) StyI
2) Klenow
3) BstXI
partial
4) Gel isolate
142 bp
fragment
(---)
E Sm (X/S) H B
Hc
H
+124
+879
+1079
-18
pGEM3-recombined 2ar cDNA
1) SmaI
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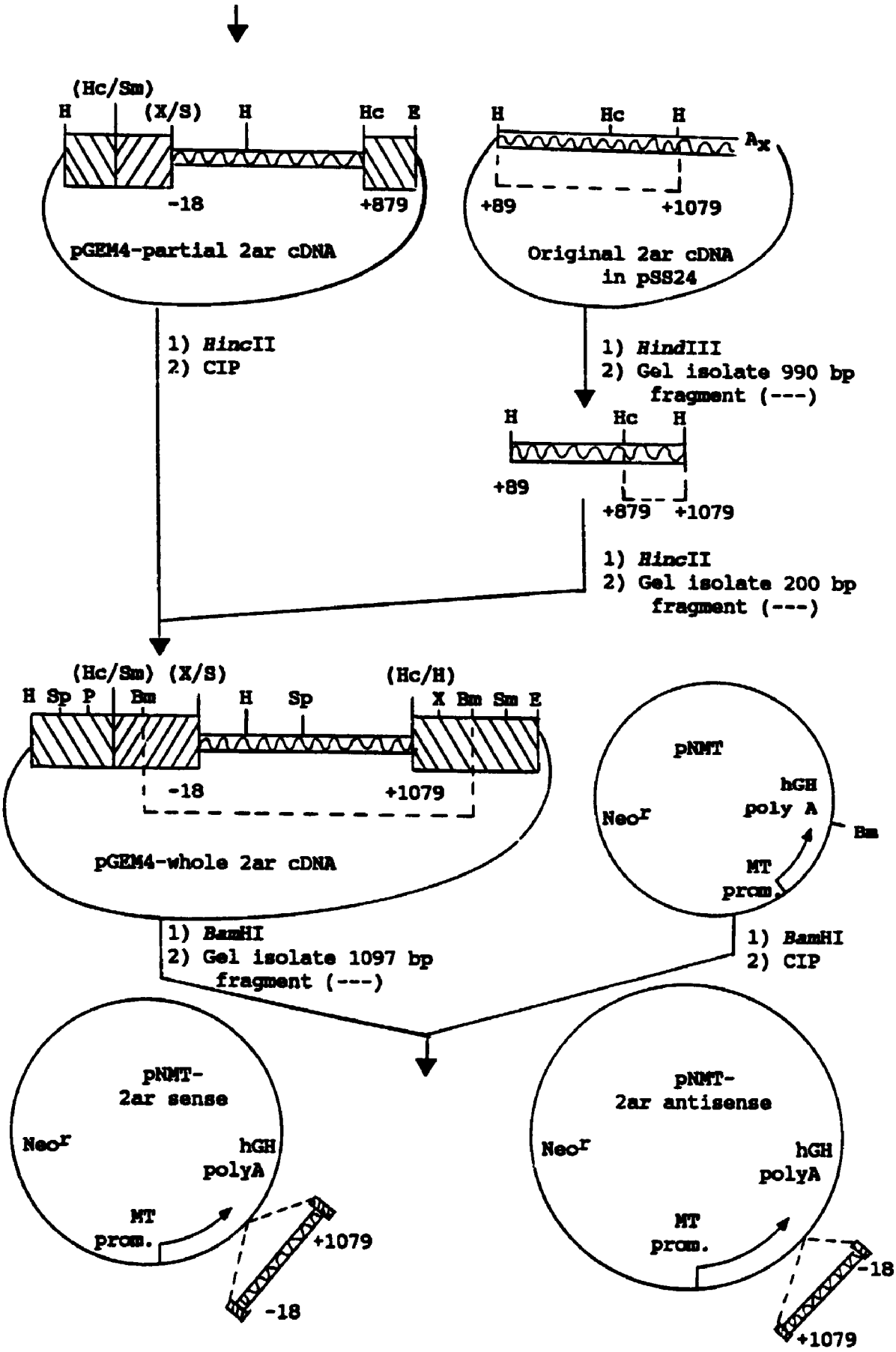
3) Gel isolate 897 bp
fragment (---)
H Hc E
pGEM4
1) HincII
2) CIP
Con't


(Hc/Sm)
H
(X/S)
H
Hc
E
-18
+879
pGEM4-partial 2ar cDNA
H
Hc
H
A_x
+89
+1079
Original 2ar cDNA
in pSS24
1) HincII
2) CIP
1) HindIII
2) Gel isolate 990 bp
fragment (---)
H
Hc
H
+89
+879
+1079
1) HincII
2) Gel isolate 200 bp
fragment (---)
(Hc/Sm)
(X/S)
(Hc/H)
H Sp P Bm
H
Sp
X Bm Sm E
-18
+1079
pGEM4-whole 2ar cDNA
pNMT
hGH
poly A
Neo^r
Bm
MT
prom.
1) BamHI
2) Gel isolate 1097 bp
fragment (---)
1) BamHI
2) CIP
pNMT-
2ar sense
Neo^r
hGH
polyA
MT
prom.
+1079
-18
pNMT-
2ar antisense
Neo^r
hGH
polyA
MT
prom.
-18
+1079

These 2ar expression vectors have been described here because they were constructed to further study the function of 2ar in carcinogenesis. Using these vectors, along with the antisera for screening 2ar protein levels, 2ar could be specifically up-regulated (e.g. in non-metastatic cells, such as LTA cells) or down-regulated (e.g. in metastatic cells, such as the PAP2 cell line). The resultant stably transfected cell lines up- or down-modulated for 2ar could then be tested for any change in metastatic potential. Thus, the expression vectors described here could be used to determine more directly whether 2ar enhances and/or is necessary for metastasis of certain cell lines.

4.3 DISCUSSION

4.3.1 Induction of 2ar by the Tumor Promoter TPA

2ar mRNA is transiently induced by TPA in mouse epidermis, as was suggested by its induction in cultured epidermal cells (Smith and Denhardt, 1987). Secretion of 2ar protein was also markedly induced by TPA in JB6 epidermal cells and 3T3 fibroblasts, and a very slight induction was observed in NRK-49F cells. It is not known whether TPA induction of 2ar also occurs in fibroblasts in vivo. In situ hybridization in TPA-treated skin or other organs would likely answer this question.

As mentioned in the introduction, while many genes are induced by TPA in various cultured cells, induction in vivo has been demonstrated for relatively few, and many of those were found to be constitutively expressed in epidermal carcinomas. Constitutive expression in epidermal carcinomas has now also been demonstrated for 2ar. This result suggests that it is the stem cells induced to proliferate by TPA which express high levels of 2ar, rather than the cells induced to terminally differentiate.

Constitutive expression of 2ar in the papillomas and carcinomas, compared with transient induction after a single TPA treatment of normal epidermis, indicate that the carcinogen treatment and/or multiple TPA treatments led to a change in the cellular factors which regulate expression of 2ar. The induction of 2ar *in vitro* by transformation with activated H-*ras* or DMBA suggests that carcinogen treatment is likely required for constitutive induction of 2ar. Tumor promotion to expand the initated cell population is also likely required. The constitutive versus transient induction might also be related to the biphasic induction of 2ar mRNA in JB6 cells, where TPA treatment led to a transient induction in subconfluent cells and a stable induction in confluent cells (Smith and Denhardt, 1987). As the tumor cells proliferate, cell-cell interactions may be necessary for constitutive expression of 2ar.

4.3.2 2ar Induction by H-*ras* and Chemical Transformation

As stated above, induction of 2ar by transformation of 3T3 cells with activated H-*ras* or DMBA indicates that 2ar can be induced not only by tumor promoters, but also by agents which stimulate initiation and progression. This observation stresses the fact that initiation, promotion and progression are operationally defined stages which often overlap. It seems likely that 2ar/osteopontin may be one of a number of mediators of the carcinogenic effects of DMBA and activated *ras*. The apparent redundancy in the effects of tumor promoters, carcinogens such as DMBA, and oncogenes such as H-*ras* likely reflects common targets. For example, the *ras* gene was frequently found mutated after DMBA initiation (Quintanilla et al., 1988). The *ras* gene product and TPA may both act along a pathway which involves PKC activation (see Fig. 1.1); *ras*

transformation led to elevated levels of DG and decreased phorbol ester sensitivity (Wolfman and Macara, 1987). While TPA treatment leads to a transient activation of this pathway, transformation by activated H-ras or DMBA may lead to a permanent induction or other perturbation of this pathway.

Enhanced expression of 2ar resulted from T24-H-ras transformation of both 3T3 and 10T1/2 fibroblasts, and the level of expression of 2ar mRNA correlated roughly with that of ras mRNA (Table 4.1). Modulation of the expression of other genes by transfection of cultured cells with activated ras oncogenes has been reported. In the same 10T1/2 derivatives studied here, enhanced expression of the cysteine proteinase cathepsin L also correlated with the enhanced ras expression (Denhardt et al., 1987). In other cell lines, ras transfection has led to increased expression of plasminogen activator, a type IV collagenase activity, a 66 kDa protein with hemolytic activity, and the mRNAs encoding metallothionein and the glucose transporter (Thorgeirsson et al., 1985; Schmidt and Hamer, 1986; Wieman et al., 1986; Flier et al., 1987; Garbisa et al., 1987; Sistonen et al., 1987). Microinjection of transforming ras protein induced c-fos expression (Stacey et al., 1987). Reduced expression of Ly-6.2 and Thy-1 antigens and the α_1(III) and α_2(I) collagen mRNAs resulted from ras transfection of 3T3 fibroblasts (Liau et al., 1985; Ivanyi et al., 1986). Mouse mammary epithelial cells transfected with EJ-H-ras exhibited decreased collagen synthesis and deposition in response to growth on collagen (Warburton et al., 1986).

Ras transfection also led to changes in N-linked carbohydrate modification of proteins, including increased sialylation and ß-1-6-linked branching of gp130, a major cell surface glycoprotein (Dennis et al.,

1987; Bolscher et al., 1988). The high degree of glycosylation of 2ar makes it a prime candidate for transformation-induced changes in activity mediated by changes in glycosylation. We have not detected any change in electrophoretic mobility of the protein due to transformation (with ras or src) or TPA treatment. Mukherjee and colleagues (Chackalaparampil et al., 1985; and Fig. 4.4A) have found a decrease in apparent M_r from 69,000 to 62,000 associated with transformation or partial transformation, e.g. effected by TPA. However, this phenomenon might be due to some characteristic of the culture conditions.

In contrast to the induction of 2ar by transformation of mouse 3T3 fibroblasts, transformation of NRK cells with viral K-ras or viral src did not significantly affect the level of expression of 2ar mRNA or protein. This is likely due to the high basal level of expression of 2ar in NRK cells, which in turn is likely due to the kidney derivation of these cells and the abundance of 2ar in normal kidney. The results are consistent with the idea that a high level of 2ar in a cell type in which it is not normally expressed may actively contribute to carcinogenesis.

4.3.3 Relationship of 2ar to Other Secreted Phosphoproteins

An immunological relationship has been demonstrated here between 2ar/osteopontin and pp69, a major phosphoprotein secreted by normal rat kidney cells. 2ar is encoded by a unique single-copy gene (Chapter 3), and only one hybridizing mRNA species has been detected in mouse and rat cells. The simplest interpretation of these results is that pp69 and osteopontin are encoded by a single rat gene and mRNA, and 2ar by the mouse homolog. In accordance with this interpretation, it is most likely

that the various forms of this protein differ only in species differences and post-translational modifications.

M. Nemir and B.B. Mukherjee (personal communication) found that induction of the transformed phenotype by vanadyl treatment of NRK cells increased the secretion of the 69 kDa protein, but decreased its level of phosphorylation. They also reported that only the non-phosphorylated form was associated with fibronectin, while only the phosphorylated form was associated with the NRK cell surface. Protein phosphorylation and dephosphorylation is a well-known mechanism of regulating cellular functions, including the response to growth factors and transforming agents (reviewed by Krebs, 1986). It is unusual for an extracellular protein to be phosphorylated. Fibronectin is also phosphorylated, and the extent of phosphorylation is increased in transformed NRK cells (Ali and Hunter, 1981).

The relationship of 2ar to pp69 and its induction by transformation of 3T3 fibroblasts led us to believe that it might be the same as a "transformation-associated phosphoprotein" studied by Senger and colleagues (Senger et al., 1979, 1980, 1983; Senger and Peruzzi, 1985). Like 2ar and pp69, this was the major phosphoprotein secreted by all rodent cell lines studied. The conclusion that the transformation-associated phosphoprotein is identical or closely related to 2ar/osteopontin is supported by sequence identity of the N-terminal nine amino acids of this protein (Senger et al., 1988) with the N-terminal sequence of mature rat osteopontin (Oldberg et al., 1986; Prince et al., 1987). The transformation-associated phosphoprotein was secreted in larger amounts by MNNG-HOS cells compared with HOS cells, although in these and other human cell lines it was not the major secreted phosphoprotein, but

was only detectable by immunoprecipitation (Senger and Perruzzi, 1985). The inability of the anti-2ar sera or 2ar cDNA to detect a homologue in the MNNG-HOS cells may be due to the limited sequence conservation between the human and rat (and therefore mouse) osteopontin cDNA sequences (Fisher et al., 1989). It is now clear that 2ar is related to osteopontin (44 kDa bone phosphoprotein; sialoprotein I), pp69, and the transformation-associated phosphoprotein. These relationships are summarized in Table 4.2. These various names appear to refer to a single protein, although the form may depend on the cell of origin and possibly other factors.

Elevated secretion of this phosphoprotein by transformation of mammalian fibroblasts and epithelial cells with a wide variety of agents was often correlated with tumorigenicity (Senger et al., 1979, 1980, 1983; Senger and Peruzzi, 1985). The cells studied were from several species (hamster, guinea pig, mouse, rat, or human), and were derived from tumors or by transformation *in vitro* with chemical carcinogens, polyoma virus, or retroviruses (including those carrying *src* or *ras*). High level expression of this protein in cells derived from both carcinomas and sarcomas suggests that the induction of 2ar in fibroblasts as well as epidermal cells may be relevant to the *in vivo* situation. Secretion of the protein by mouse macrophages was also enhanced by viral transformation (Senger et al., 1988). The apparent molecular weight of the protein was 58-69 kDa, depending on the species, and displayed microheterogeneity in the mouse cell lines (as reported here for 2ar in PAP2 cells). The presence of elevated levels of this protein in the serum of human patients with disseminated carcinomas (10 of 14 cases), but not in two cases of localized nonmetastatic tumors (Senger et al., 1988) suggests that this protein may play a role in the later stages of cancer progression.

TABLE 4.2

PROTEINS WHICH ARE IDENTICAL OR CLOSELY RELATED TO 2AR

PROTEIN	PROPERTIES	RELATIONSHIP TO 2AR
OSTEOPONTIN[1] (44 kDa bone phosphoprotein[2]; bone sialoprotein I[3])	41.5 kDa (rat bone)[2]; binds cells via RGD[1]; binds hydroxyapatite bone matrix[1]	87% identity of rat osteopontin and mouse 2ar cDNA sequences[6]; immunological relationship[6]
pp69[4,5]	the major phosphoprotein secreted by many rodent cell lines[4,5]; a related form (pp62) secreted by some transformed lines[4]	immunological relationship[6]
TRANSFORMATION-ASSOCIATED PHOSPHOPROTEIN[7,8]	enhanced secretion by many transformed cell lines correlates with tumorigenicity[7]; high level in sera of patients with disseminated carcinomas[8]; cleaved by thrombin during blood coagulation[8]	sequence of the first nine amino acids identical with that of mature rat osteopontin[1,6,8]; like 2ar, osteopontin, & pp69, it is the major phosphoprotein secreted by all rodent cell lines studied

[1]Oldberg et al. (1986).
[2]Butler (1989).
[3]Fisher et al. (1987).
[4]Chackalaparampil et al. (1985).
[5]Laverdure et al. (1987).
[6]This work.
[7]Senger et al. (1979).
[8]Senger et al. (1988).

4.3.4 Correlation of 2ar Expression with Metastatis

We have found enhanced expression of 2ar in all metastatic cell lines analyzed, whether derived by transfection of the T24-H-ras oncogene or transfection of genomic DNA from metastatic melanoma cells. The level of 2ar mRNA in the ras-transformed 10T1/2 cells correlated roughly with the ability of the cells to induce both experimental and spontaneous metastases, although the level of 2ar mRNA was still quite high in the moderately metastatic NR4 cells. The consistently elevated level of 2ar mRNA in the epidermal carcinomas also indicates an association of 2ar expression with tumor progression in vivo. Eight human cell lines or fresh explants which produced lethal, progressively growing, invasive tumors in nude mice secreted higher levels of 2ar (transformation-associated phosphoprotein) than six non-tumorigenic controls (Senger and Perruzzi, 1985). The metastatic ability of these cells was not reported. As mentioned above, the circulating levels of this protein in cancer patients supports a role for 2ar in progression and metastatic spread.

2ar expression was also elevated (to a lesser extent) above control levels in the papilloma and in the NR3 cells, which are tumorigenic but poorly metastatic. A contribution of 2ar to tumor growth cannot be ruled out, although the evidence is much stronger for a contribution to metastasis. Expression of 2ar may be an early event in the progression of some tumors, which still lack other characteristics necessary for efficient metastasis. It is also possible that one gene product can affect both primary tumor formation and metastasis, as appears to be the case for tissue inhibitor of metalloproteinases (Khokha et al., 1989).

It is not clear from these studies which step(s) of metastasis are associated with high expression of 2ar. Where determined, the experi-

mental and spontaneous metastatic potentials were in agreement. As suggested in the introduction, there are various means by which 2ar could affect escape from the primary tumor, migration to a secondary site, and/or adhesion at the secondary site. 2ar produced by tumor cells may act as an autocrine adhesion factor. Much of the other work on the contribution of gene products to metastasis has been focussed on proteases, protease inhibitors, and cell surface proteins, and so no parallel can be drawn with 2ar. It is hoped that use of the vectors for expression of sense and antisense RNA will determine more directly whether 2ar stimulates and/or is necessary for metastasis of certain cells, and perhaps how this is achieved.

CHAPTER 5

REGULATION OF EXPRESSION OF 2AR

5.1 INTRODUCTION

The 2ar gene product is regulated in an interesting manner. As described in Chapter 3, expression is limited to a specific set of tissues, most notably bone, kidney, inner ear, ovary, decidual metrial gland, and circulating blood. 2ar expression by specific cells within these tissues changes during development; for example, 2ar mRNA was localized mainly to the cortex of the newborn mouse (Nomura et al., 1988) and the medulla of the adult mouse (here). The induction of 2ar mRNA in the uterus and skin of pregnant and lactating mice (D.T. Denhardt, personal communication), and the presence of the protein in human milk (Senger et al., 1989), indicates that 2ar expression is apparently under hormonal control in vivo.

In cultured ROS17/2.8 osteoblast-like osteosarcoma cells, 2ar (osteopontin) mRNA expression was stimulated by 1,25-dihydroxyvitamin D_3 (10-fold) and TGFß (3-fold), and inhibited by dexamethasone and parathyroid hormone (3-fold) (Prince and Butler, 1987; Yoon et al., 1987; Noda et al., 1989; Noda and Rodan, 1989). Similar effects of 1,25-dihydroxyvitamin D_3, TGFß, and parathyroid hormone on osteopontin protein secretion were demonstrated. These effects further associate osteopontin expression with the osteoblastic phenotype, and may reflect in vivo responses to regulation of bone metabolism. Inhibition by parathyroid hormone still occurred when the basal level of expression was modified by treatment with 1,25-dihydroxyvitamin D_3, TGFß, or dexamethasone (Noda and Rodan, 1989). However, while TGFß still enhanced osteopontin expression in dexamethasone-treated cells, it had no effect on the osteopontin mRNA level in

1,25-dihydroxyvitamin D_3-treated cells, indicating complex multihormonal regulation (Noda et al., 1988).

Modulation of osteopontin expression by TGFß and parathyroid hormone occurred largely at the transcriptional level, as shown by nuclear run-on assays (Noda et al., 1988; Noda and Rodan, 1989). cAMP mediation of the response to parathyroid hormone was suggested by the ability of parathyroid hormone to stimulate adenylate cyclase in these cells, and the ability of a cAMP-analog and other cAMP-elevating agents to inhibit osteopontin expression (Noda and Rodan, 1989). Blockage of the response to 1,25-dihydroxyvitamin D_3 with Actinomycin D also suggested that this effect occurs largely at the transcriptional level (Prince and Butler, 1987).

Expression of 2ar by normal rat kidney (NRK) cells is also modulated by growth factors. 2ar (pp69) protein secretion by NRK cells was induced by EGF and TPA and inhibited by TGFß (Laverdure et al., 1987; and here). In an independent study (Binas and Grosse, 1987), [^{32}P]orthophosphate incorporation into the major phosphoprotein secreted by NRK cells (presumably 2ar) was stimulated by EGF and TPA, inhibited by TGFß, and unaffected by PDGF, FGF, insulin, and nerve growth factor. It is interesting that the effect of TGFß on 2ar expression was dependent on the cell type, i.e. induction in osteoblastic cells as opposed to inhibition in normal rat kidney cells. This may be due to tissue-specific differences in components of regulatory pathways.

2ar mRNA was induced in quiescent mouse 3T3 or 10T1/2 fibroblasts by TPA, serum, PDGF, EGF, bFGF, and ECDGF, but not by somatomedin C, progesterone or dexamethasone (Smith and Denhardt, 1987; Nomura et al., 1988; and here). The induction of 2ar by growth factors likely reflects

the association of 2ar expression with carcinogenesis, as discussed in Chapter 4. 2ar was induced by the tumor promoter TPA in mouse epidermal cells both *in vitro* and *in vivo*. It was also induced by transforming agents such as the T24 H-*ras* oncogene, and its induction correlated roughly with the metastatic ability of *ras*-transfected fibroblasts. Expression of 2ar was elevated *in vivo* in mouse epidermal squamous cell carcinomas and the serum of human patients with disseminated carcinomas (Senger et al., 1988).

TPA-induction of 2ar mRNA in JB6 epidermal cells was inhibited by cycloheximide, indicating a requirement for protein synthesis (Smith and Denhardt, 1987). Thus, 2ar induction is not a primary response to TPA treatment. Induction is at least partially at the transcriptional level, as indicated by run-on transcription assays (Smith and Denhardt, 1987). As described in the introduction, a major cellular receptor for TPA is protein kinase C (PKC). The tumor promoters phorbol dibutyrate, teleocidin, and aplysiatoxin, and the stage II promoter mezerein, which are all activators of PKC, also induced 2ar in JB6 cells (Smith and Denhardt, 1989). PDGF and EGF, both of which indirectly activate PKC, induced 2ar mRNA in JB6 cells. TPA-induction was inhibited by H-7 [1-(5-isoquinoline sulfonyl)-2-methylpiperazine-2HCl], a specific inhibitor of PKC (Smith and Denhardt, 1989). These results suggest that TPA-induction of 2ar is mediated by activation of PKC. Benzoyl peroxide, a tumor promoter which does not activate PKC but likely acts through the generation of reactive oxygen species, did not induce 2ar (Smith and Denhardt, 1989).

The effects of inhibitors of tumor promotion on TPA-induction of 2ar in JB6 cells was varied (Smith and Denhardt, 1989). Retinoic acid and the anti-inflammatory steroids dexamethasone and fluocinoline acetonide

inhibited 2ar induction. Indomethacin, a cyclooxygenase inhibitor, and difluoromethyl ornithine, an inhibitor of ODC, did not inhibit 2ar induction. Although 2ar induction in this in vitro system did not correlate well with the known effects of these agents on tumor promtion in vivo, a complete correlation was observed between 2ar induction and the mitogenic response (Smith and Denhardt, 1989).

A peculiar feature of the TPA induction of 2ar in JB6 cells is the effect of cell density (Smith and Denhardt, 1987). In subconfluent cells, 2ar mRNA was transiently induced, peaking 6 h after addition of the tumor promoter. However, in confluent cells, 2ar mRNA was again induced by TPA, but the induction was sustained rather than transient, and the time of induction appeared to depend on the cell density rather than the duration of exposure to TPA. Induction occurred at the point at which the control cells reached plateau density, but the TPA-stimulated cells underwent a final division. Inhibition of TPA-stimulated 2ar induction by difluoromethyl ornithine (an inhibitor of ODC) in confluent but not in subconfluent cells suggested that induction under these two conditions occurs by different mechanisms (Smith and Denhardt, 1989).

Thus, 2ar would be an interesting model gene with which to study mechanisms of tissue-specific expression, regulation by hormones, growth factors, and tumor promoters, and cell density-dependent expression. In order to make such studies possible, a 2ar genomic clone was isolated from a lambda mouse library and partially characterized. The promoter region was characterized by sequence analysis, S1 mapping of the transcriptional start site, and transient transfection assays for expression of a fused marker gene. Both the transient transfection assays and mRNA stability assays were used to study the mechanisms of induction of 2ar by TPA.

5.2 RESULTS

5.2.1 Isolation and Characterization of a 2ar Genomic Clone

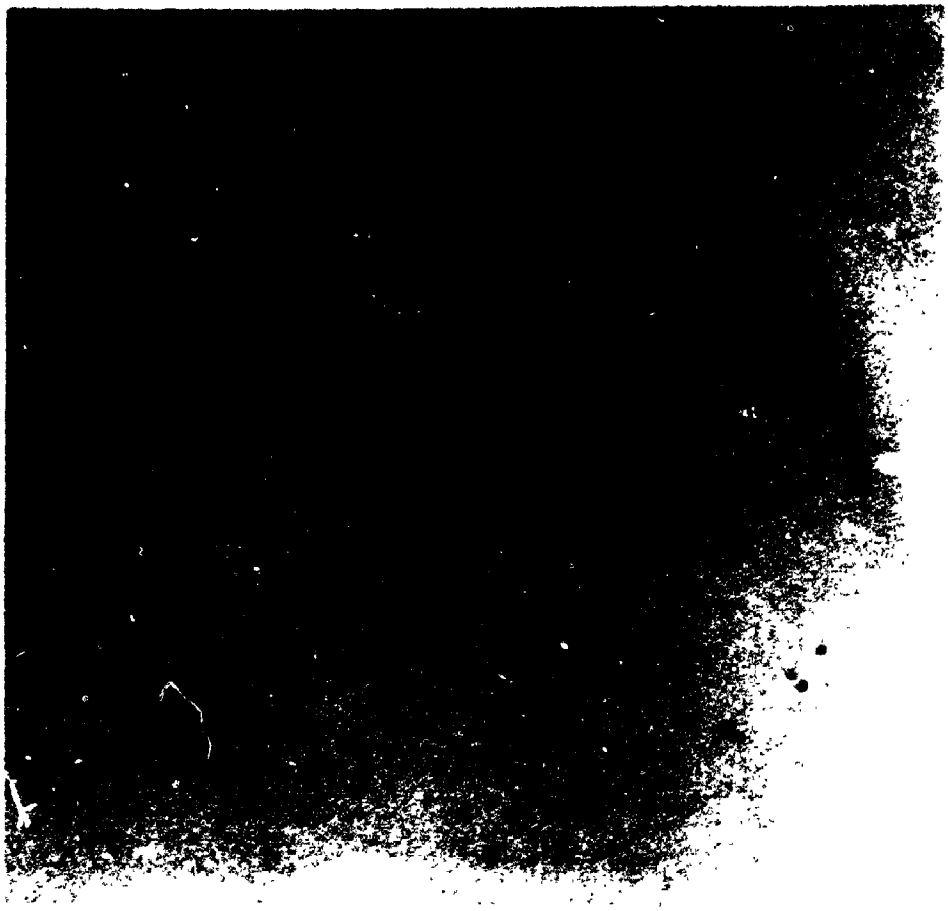
The 9090 mouse genomic library, made from Balb/c embryo DNA partially restricted with HaeIII and cloned with linkers into the EcoRI site of the lambda phage Charon4A (J. Sideman, obtained from V. Morris), was screened for a 2ar genomic clone. The probe was a mixture of ^{32}P-labelled HindIII-PvuII fragments of the original 2ar cDNA corresponding to nts 89-642, 643-1079, and 1080-1316 in Fig. 3.2 (plus the polyA tail plus a 3' linker). Of 750,000 plaques screened, one clone was obtained which gave positive signals on a second screen; it was subsequently purified (Fig. 5.1).

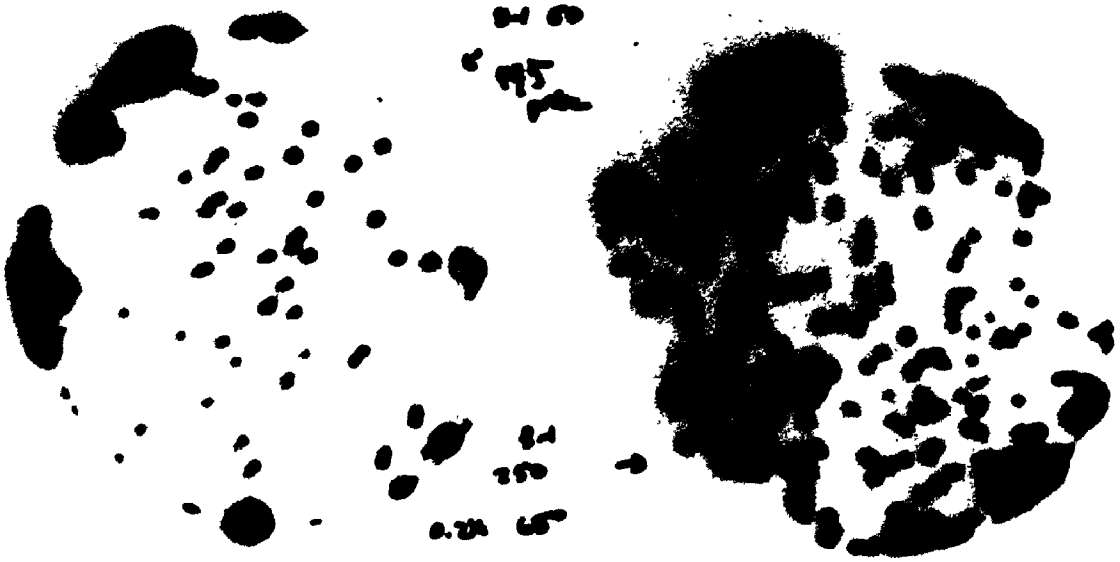
An interesting phenomenon was observed during the screening. Under low stringency hybridization conditions (washing in 0.2X SSC at 42°C, as described in the methods section), approximately 1% of all plaques gave a positive signal. The signals were lost during higher stringency washes (in 0.2X SSC at 65°C). The *same* plaques also gave a positive signal when probed with a cDNA corresponding to tissue inhibitor of metalloproteinase, which is not related to 2ar. The only sequences in common between the two probes were the polyA tails and a 3' linker sequence containing 24 bp from the enhancer region of SV40 (the PvuII-KpnI fragment at position 208-230; see Kowalski et al., 1985 for pSS24, the cDNA vector). The high frequency signals were not seen with shorter cDNA probes lacking both the polyA tails and SV40 enhance. sequences. These results indicate that there are either long stretches of polyA-polyT or sequences highly homologous to the SV40 enhancer at high frequency in the mouse genome.

The 2ar genomic clone ("λ 9090 2ar") was analyzed by Southern blot analyses of restriction enzyme digests probed with various segments of the

Figure 5.1

Isolation of the mouse λ 9090 2ar genomic clone. Phage were grown on a lawn of E. coli LE392 on agar plates, transferred to nitrocellulose, and probed with ^{32}P-labeled 2ar cDNA insert. The autoradiograms shown are of the second screen (top, ≈20,000 phage) and of the final screen (bottom, ≈50 phage (left) or ≈250 phage (right)). All of the phage gave a positive signal on the final screen.





2ar cDNA or of the genomic clone itself. Some of the restriction fragments from the genomic clone were subcloned into pBR322 or the pGEM vectors and subjected to further analysis. The cumulative results led to the determination of a structural and restriction map of part of the 2ar gene (Fig. 5.2). The λ 9090 2ar clone does not contain the 3' end of the gene, but does contain at least the first six exons and ≈16 kb of 5' flanking DNA.

A sample Southern blot analysis is shown in Fig. 5.3. The 5' cDNA probe used in Fig. 5.3B (nt -69 to +131 of the cDNA) contains sequence corresponding to exons 1, 2, 3, and 4, and so hybridized to multiple restriction fragments for most enzymes tested. The PvuII genomic fragments which hybridized this cDNA probe were 2.0 kb (containing exons 1 and 2), 0.8 kb (containing exon 4), 0.5 kb (containing exon 3), and products of partial digestion which gave a very weak signal. The same blot was stripped and reprobed with a shorter cDNA probe (nt -69 to -15) containing only exon 1 (Fig. 5.3C); only the 2.0 kb PvuII fragment (containing exons 1 and 2) hybridized to this probe. The size of the major species which hybridized to this probe in all cases indicated the size of the restriction fragment cor ining exon 1. The presence of more weakly hybridizing species is likely due to partial restrictions and contamination of the gel-eluted probe with sequences corresponding to exon 2.

Exons 1, 3, and 4 have been sequenced (after subcloning appropriate fragments into M13 phages), whereas exons 2, 5, and 6 have only been roughly mapped. [The reason for sequencing exons 3 and 4 was to aid in isolation of a full-length cDNA (Chapter 3)]. The exon sizes and exon/intron boundary sequences are shown in Table 5.1. There are a

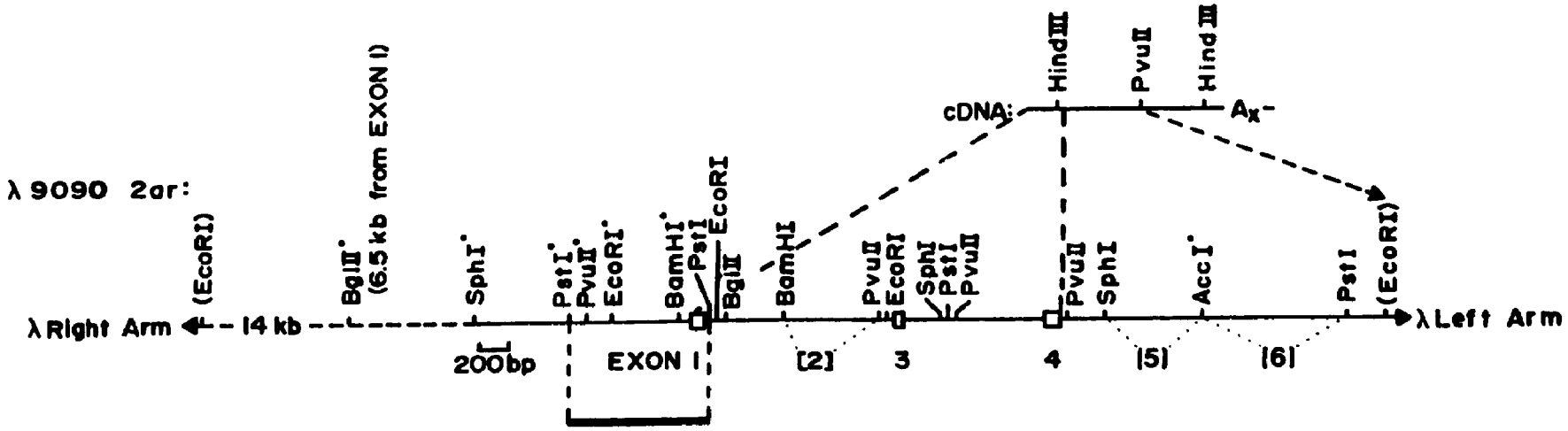
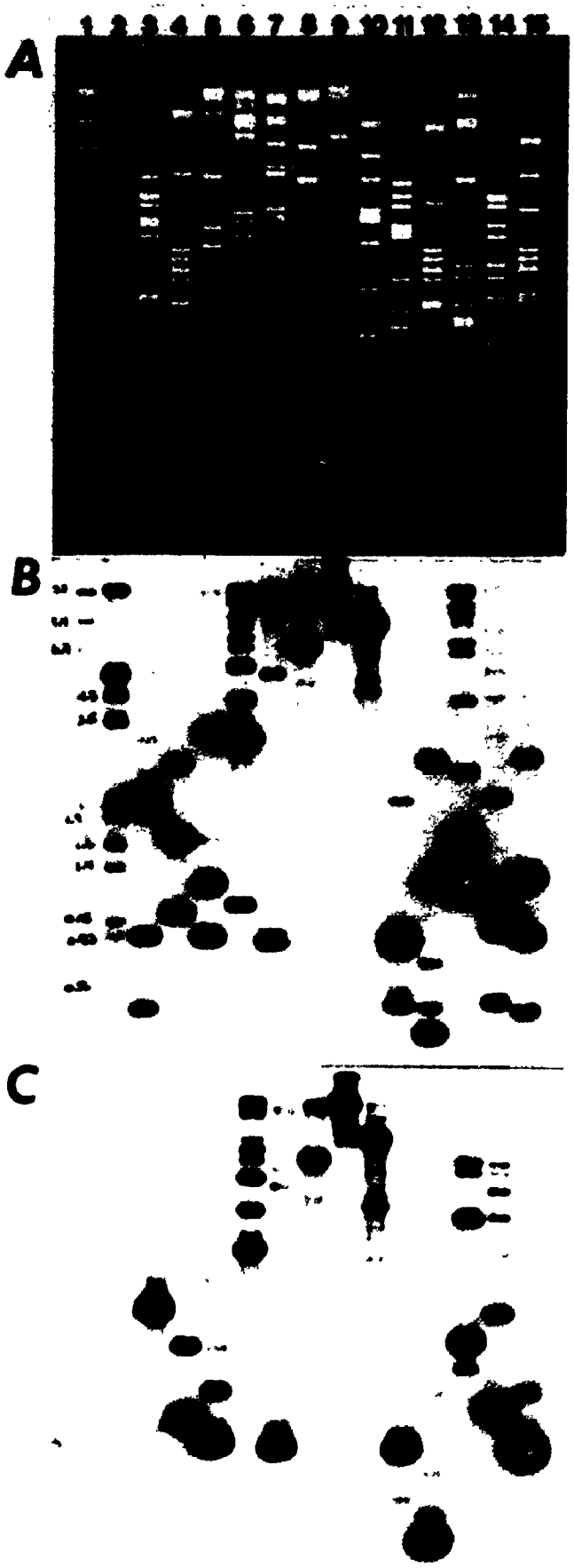


Figure 5.2

Partial map of the mouse λ 9090 2ar genomic clone. The clone contains at least six exons and 15 kb of 5' flanking DNA. Exons 1, 3, and 4 have been sequenced, and exons 2, 5, and 6 (presumably single exons) have only been roughly mapped. The genomic clone does not contain exons corresponding to sequence 3' to the PvuII site in the cDNA. Small circles by restriction enzyme sites indicate that these are the most 5' sites determined. The bar below the figure indicates the PstI fragment whose sequence is shown in Fig. 5.5.

Figure 5.3

Sample Southern blot analysis of the λ 9090 2ar genomic clone. λ 9090 2ar DNA was restricted and electrophoresed (0.5 μg/lane) on a 1% agarose gel. Numbers above the lanes indicate the restriction enzymes used: 3, PvuII; 4, PstI; 5, EcoRI; 6, SphI; 7, BamHI; 8, BglII; 9, HindIII; 10, XmnI; 11, BamHI and PvuII; 12, BamHI and PstI; 13, BglII and SphI; 14, BglII and PvuII; 15, EcoRI and PstI. Lanes 1 and 2 contain molecular weight markers. The gel was stained with ethidium bromide (A), blotted, and probed with ^{32}P-labeled fragments of the 2ar cDNA corresponding to position -69 to +131 of Fig. 3.2 (containing exons 1, 2, 3, and 4)(B), or position -69 to -15 (containing only exon 1) (C).


A
B
C

minimum of six exons in this clone; it is possible that exons 2, 5, and/or 6 are composed of two small, closely spaced exons. The size of exon 6 is not known, but based on Southern blot analyses, it would be ≥174 bp and ≤425 bp (if present as a single exon). The splice junction sequences agree well with consensus sequences for exon/intron boundaries.

Southern blot analyses of the λ 9090 2ar clone and of genomic DNA probed with portions of the cDNA have been used to estimate the size of the complete gene (Fig. 5.4A). Thus, the λ 9090 2ar clone contains 4.8 kb of the gene (i.e. 3' from the start of exon 1) and is missing ≈4 kb from the 3' end of the gene.

An attempt was made to detect the homologous gene in human DNA, using part of the mouse 2ar cDNA (nt 89-642) as probe (Fig. 5.4B). There did appear to be human genomic fragments of 7.0 kb (EcoRI), 7.1 kb (HindIII) and 18 kb (PvuII) giving a weak hybridization signal with the mouse probe. If these signals are indeed from the homologous human gene, then the restriction map of the human 2ar gene is apparently different from that of the mouse gene. The lack of a strong hybridization signal is not unexpected, based on a report of limited sequence conservation between rat and human osteopontin cDNAs (Fisher et al., 1989).

5.2.2 Identification and Sequence Analysis of the 2ar Promoter

The transcriptional start site was determined by an S1 mapping experiment (Fig. 5.5A). The probe was a 998-bp fragment of the genomic clone labeled at the 3' end, at the StyI site just within exon 1. A fragment of 79 (± 3) nt was protected fron S1 nuclease by poly(A^+) RNA from TPA-treated JB6 cells. The sequence of the DNA just upstream of this

TABLE 5.1

PARTIAL DETERMINATION OF EXON BOUNDARIES OF 2AR

Intron /	Exon[1]	/ Intron	Exon Size (nt)
- -93 /AGCAG.....	exon 1	-15 .....CCAAG/gtaagcctgc	79
n. d.[2] -14 /GACTA.....	[exon 2][3]	54 .....TCCCG/ n. d.	[68]
ctttctgttttaaag/GTGAA..... 55	exon 3	90 .....AGAAG/gtaagcacct	36
ct_{15}gaaaattaag/CTTTA..... 91	exon 4	171 .....CACAG/gtattgtgtt	81
n. d. 172 /AATGC..	[exon 5]	(211) .likely GCAAG/ n. d.	[40-48][4]

Consensus Splice Junction Sequences[5]

(t) c C a
$(c)_n$xtag/G.....AAG/gtgagt

[1]Numbers indicate position in the mRNA with respect to initiation of translation.
[2]Not determined (n. d.).
[3]Square brackets indicate that the exon was only roughly located by restriction enzyme mapping and is presumed to be a single exon.
[4]The 3' boundary of exon 5 was deduced to be somewhere between nt 211 and 220. (This is based on the presence of an XmnI site in the cDNA which is absent in the genomic clone, and is presumably formed by the joining of exons 5 and 6.)
[5]From Mount (1982); of the >130 sequences examined, the underlined bases were 100% conserved, compared with ≈60-85% for the other bases.

Figure 5.4

Southern blot analysis of mouse and human 2ar genes.

A. Summary of restriction fragment composition and size estimation of mouse 2ar gene.

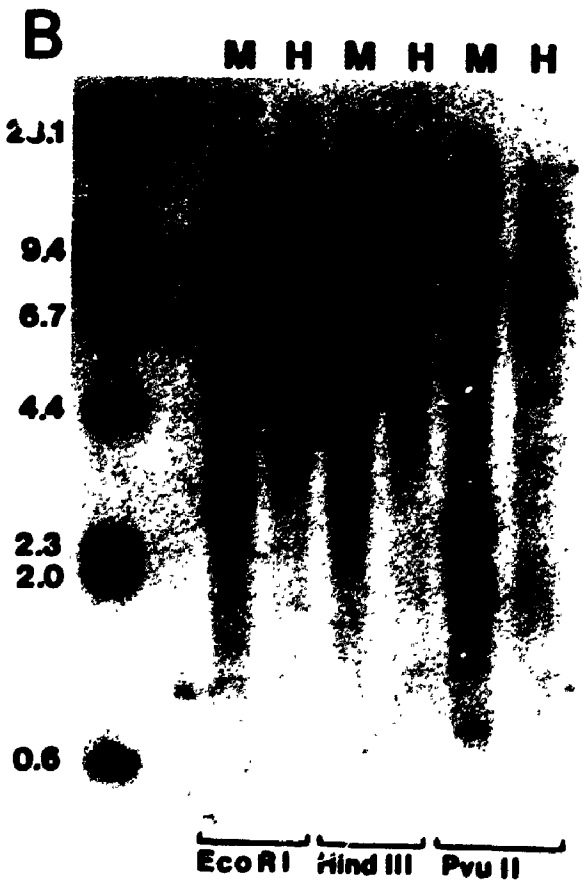
B. Southern blot analysis of mouse (M) and human (H) DNA probed with a portion of the 2ar cDNA. Nuclear DNA from mouse JB6 epidermal cells or human IMR-90 fibroblasts was restricted with the indicated enzymes, electrophoresed (10 μg/lane), blotted, and probed with a ^{32}P-labeled 2ar cDNA fragment (nt 89-642 in Fig. 3.2). The blot was washed under relatively non-stringent conditions (42°C in 2X SSC as described in the methods). Small circles indicate the positions of faint signals which may be due to hybridization of the mouse 2ar cDNA probe to fragments of human DNA.

A

Restriction enzyme	Sizes of fragments comprising 2ar gene, 5' to 3' (kb)[1]	Size of 2ar gene (kb)
HindIII	15	≤ 15
PstI	1.0 + 1.6 + 2.7 + 2.3 [+ 2.3][2]	≤ 9.9
PvuII	2.0 + 0.5 + 0.8 + 2.3 + 4.0	≤ 9.6
BamHI	0.7 + 8.7	≤ 9.4
EcoRI	0.7 + 1.2 + 7.1	≤ 9.0

[1]Determined by Southern blot analyses of genomic DNA and from the analysis of the λ 9090 2ar clone.
[2]The 2.3 kb band gave a strong hybridization signal and may represent two species.

B



putative transcriptional start site contains elements similar to the consensus TATA box (TTTAAAA) and CAT box (in reverse orientation; ATTGG) (Fig. 5.5B). TATA and CAT boxes are found in similar locations in many eukaryotic genes transcribed by RNA polymerase II (Breathnach and Chambon, 1981). Two further lines of evidence support the designation of this region of the gene as the 2ar promoter. Restriction fragments spanning 6.5 kb upstream of exon 1 did not hybridize to 2ar mRNA by Northern blot analyses (data not shown), consistent with the proposal that this is indeed exon 1. A small fragment of DNA surrounding the proposed transcriptional start site was able to direct expression of a fused marker gene; i.e. this region of DNA demonstrated promoter activity in a transient transfection assay (see below).

The complete sequence of a 1-kb PstI fragment containing the 2ar promoter was determined from a nested set of deletion derivatives in M13 phages, generated with the IBI "Cyclone" methodology (Fig. 5.6). Features distinguishable in this sequence include: the consensus TATA and CAT boxes (-27 tc -22; and -53 to -49, respectively); a 9-bp direct repeat (-758 to -741); and a number of elements similar to sequences which are known to modulate transcription in other genes (Table 5.2). A consensus sequence identical to the nuclear factor III binding site (GNTAATGAPuAT; Pruijn et al., 1986) is present at -200 to -190 of the 2ar gene. Sequences identical to the Sp1 recognition sequence (GGGCGG; Dynan et al., 1986), the steroid-response element (TGTTCT; Ham et al., 1988) or the immunoglobulin enhancer (ATTTGCAT; Singh et al., 1986), or similar to the serum response element (GATGTCCATATTAGGACATC; Treisman, 1986) were not found in this region of the 2ar gene.

Figure 5.5

Determination and sequence of the 2ar transcriptional start site.

A. S1 mapping analysis of 2ar transcriptional start site. The 998-bp probe rom the 2ar genomic clone was 5' end-labeled at the StyI site (S) of exon 1 (hatched box), as shown at the bottom. Other restriction enzyme sites indicated are: B, BamHI; E, EcoRI; P, PstI; Pv, PvuII; Sp, SphI. Probe was annealed to cytoplasmic poly(A^+) RNA from TPA-treated JB6 cells (lane 1) or yeast tRNA (lane 2). The annealing mixture was treated with S1 nuclease and protected fragments were visualized on a denaturing 8% polyacrylamide gel. Molecular weight marker sizes are given in nt. The protected fragment is 79 (±3) nt.

B. DNA sequence of the 2ar genomic clone surrounding the transcriptional start site (open arrow). An autoradiogram of the products of dideoxy-nucleotide sequencing reactions with an M13 subclone is shown. Sequence (5' to 3') is read upwards. The positions of the TATA-like box and CAT box (reverse complement) are indicated.

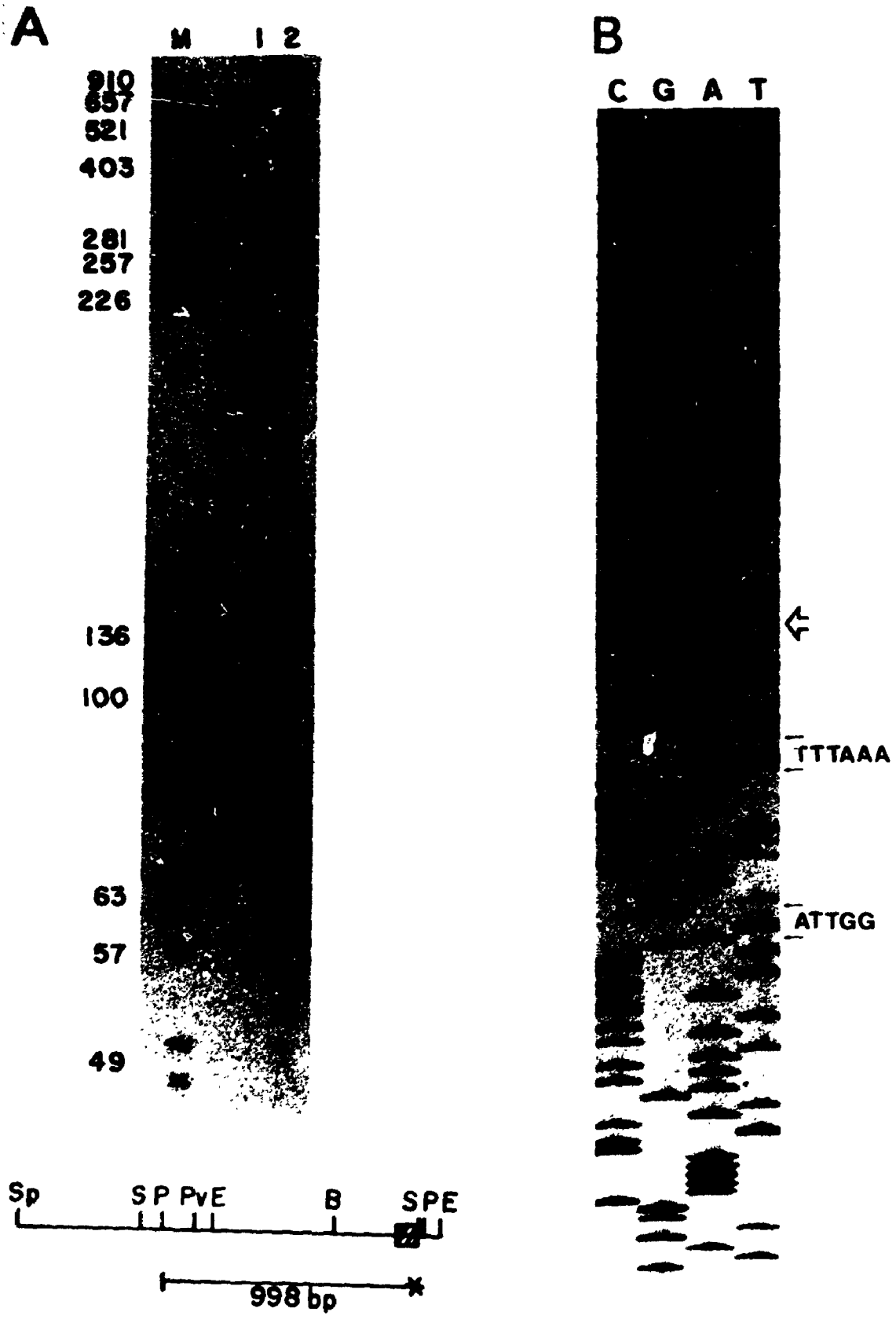
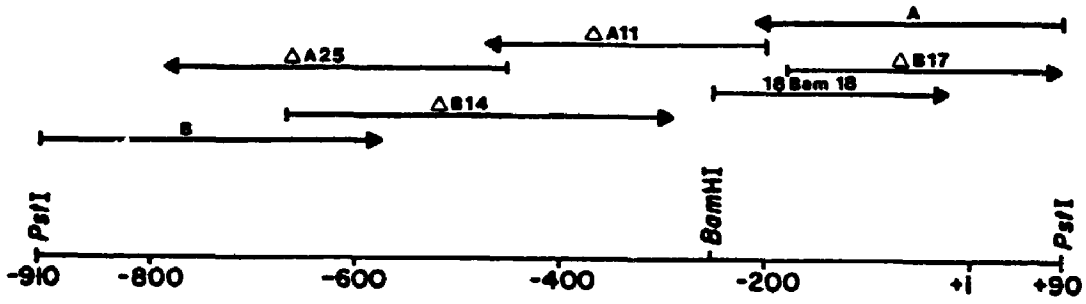

A
M
1
2
310
657
521
403
281
257
226
136
100
63
57
49
Sp
S P PvE
B
SPE
998 bp
B
C G A T
TTTAAA
ATTGG

Figure 5.6

Nucleotide sequence of the 2ar promoter region.

Top: sequencing strategy. Sequence was obtained from M13 subclones using the dideoxynucleotide methodology. Subclones used contained the BamHI-PstI fragment (18Bam18), the 1-kb PstI fragment (A and B), or deletions thereof made with the IBI Cyclone System.

Bottom: nucleotide sequence. Numbers above the line indicate the nucleotide position relative to the transcriptional start site. The TATA-like box, CAAT box (reverse complement), and a direct repeat are underlined. Arrows above the sequence indicate the 5' endpoints of segments tested for promoter activity (Figs. 5.7 and 5.8); the 3' endpoint for all is at the first exon/intron boundary.



-910
CTGCAGCTTCCCCACCCAGCAAGTATTCCAGTCTCACAAACTGCTTGCTTAGGCGAGCTCTATCTGGAGT

-840
AACCACCTCTTCTGCTCTATATGGCCATTATAGTTTAGATAGCATCAGAACCATCACTCCCAGCTGCTCC

-770
AACAGAGCAACAAGGTTCACGAGGTTCACGTCTCTAAAGGTCAGTGGAGGCAGGAGAGGAATTCAGGGTC

-700
ACTGTGTGGTTTGCACAAAGAGCAAGCGGCTGCATAGTTCAAGTGTCAAAACTGAGATGTTACTTTGTTT

-630
CGTTTTGTTGTGA GGTTTTGTTTCTTTTGTGAGAGGGTCTCACTCTCTGTTGCCCCAACTGACCTGGAAC

-560
ACAGTATGGAGCCCAGGCTAGCCTCAAACTCATGGTGATCTTCCAGAGTACAAAACAGAGCCACTGTGGC

-490
CTGGCCAACCTAAGCTACCGAATACAAAGGCAAAAGGGAGGGTCTGAAAGTTCTGCCGAGACAGCAGTTT

-420
TCTAGATTTAAGTAAGTCTGAGAGAATCAAATTGTGTATCCATGTGGCCTTTATCTGTAACTTAGATAGG

-350
AGAATCCATACCTTTCATCCCCACTGATGTTTTTCTACTAATTCAGTAACTAGAAACAAGGTCTCTGTGA

-280
GGGTGATCTACTCTTCCTTTCCTTATGGATCCCTGATGCTCTTCCGGGATTCTAAATGCAGTCTATAAAT

-210
GAAAAGGGTAGTTAATGACATCGTTCATCAGTAATGCTTTGTGTGTGTTTCCTTTTCTTCCTTTTTTTTT

-140
TTTTAACCACAAAACCAGAGGAGGAAGTGTAGGAGCAGGTGGGCCGGGTAGTGGCAAAAACCTCATGACA

-70 CAAT box TATA box
CATCACTCCACCTCCTGATTGGTGGAGACTGTCTGGACCAGCATTTAAATTCTGGGAGGTCTGAGCCACC

1
AGCAGCTCACACTGAAGAAGCATCCTTGCTTGGGTTTGCAGTCTTCTGCGGCAGGCATTCTCGGAGGAAA

71 Intron 1
CCAGCCAAG GTAAGCCTGCAG

TABLE 5.2

SEQUENCE ELEMENTS WHICH MAY MEDIATE TPA INDUCTION OF 2AR

Sequence Element in 2ar 5' Region	Related Element[1]
(-9)TGAGCCA(-3) (-75)TGACACA(-69) (-312)TAATTCA(-306) (-398)AGAATCA(-392)	TGA(G/C)TCA AP-1 binding site
(+48)CGGCAGGC(+55) (-485)GGCCAGGC(-492)[2] (-550)GCCGAGGC(-543) (-901)CCCCACCC(-894)	CCCCAGGC AP-2 binding site
(-95)GGGTAGTGGCAAA(-82) (-451)GGGTCTGAAAG(-438)	GGGTGTGGAAAG AP-3 binding site
(-58)AGGTGGAGTG(-57)[2] (-497)CTGTGGCCTG(-488) (-697)GTGTGGTTTG(-688)	CTGTGGAATG AP-5 binding site

POTENTIAL RESPONSE ELEMENTS IN THE 2AR GENE TO AGENTS OTHER THAN TPA

Sequence Element in 2ar 5' Region	Related Element
AP-2 elements above	cAMP response element[3]
(-195)TGACATCG(-188) (-745)TCACGTCT(-738)	TGACGTCA cAMP-response element[4]
(-153)AGAAAAGGAAACA(-165)[2] (-117)CCTCCTCT(-124)[2]	(A/G)GAAANNGAAA(C/G)(C/T) TCTCCTCTC IFN-response elements[5]
(-463)AAAGGCAAA(-457)	AA(G/A)CCAAA potential epidermal-specific element[6]
(-149)AGGAAGAAAAGGAAA(-163)[2]	AGGA(A)GAAAGAAA(A)G potential 1,25(OH)$_2$vitD$_3$-response element[7]
(-704)GGTCACTGTGTGG(-692) (-502)AGCCACTGTGGC(-490) (-291)GGTCTCTGTGAGG(-279) (-13)GGTCTGAGCCACC(-1) (+5)GCTCACACTGAAG(+17)	GGTCACAGTGACC estrogen-response element[8]
(-564)GAACACAGTATGG(-552)	GAACACAGTGTTC glucocorticoid-response element[8]
(-201)AGTTAATGACAT(-190)	AGGTCATGACCT retinoic acid-response element[9]

[1]Reviewed in: Jones et al. (1988)
[2]These elements are present in reverse orientation.
[3]Imagawa et al. (1987)
[4]Roesler et al. (1988)
[5]Hug et al. (1988)
[6]Blessing et al. (1987)
[7]Yoon et al. (1988)
[8]Metzger et al. (1988)
[9]Desono et al. (1988)

5.2.3 Inducible Expression of a Fused Marker Gene from the 2ar Promoter

Portions of the 2ar gene surrounding the transcriptional start site were cloned into pSV0CAT, a plasmid carrying the chloramphenicol acetyltransferase (CAT) gene, but lacking a eukaryotic promoter. The cloning was done such that transcription from the 2ar promoter through part of exon 1 (to +79) would continue through the CAT gene. The CAT gene (derived from the E. coli transposable element Tn9) is a useful marker since mammalian cells are free of endogenous chloramphenicol acetyltransferase activity, and rapid sensitive assays for CAT activity are available (Gorman et al., 1982). pSV2CAT, which carries the SV40 promoter to drive CAT expression (Gorman et al., 1982), was used as a positive standard.

Three segments of the 2ar gene differing in the 5' endpoint (-777, -543, and -253) were assayed for the ability to drive expression of the CAT gene in transient transfection assays in JB6 epidermal cells (Fig. 5.7). The quantitative data in Fig. 5.7 represents the results of three independent experiments. All three 2ar-CAT constructs supported high level expression of the CAT gene (16-56% relative to the SV40 promoter in the pSV2CAT construct). A comparison of the relative activities of the three constructs suggests that there is a positive transcription element between -253 and -543 (indicated by a 2.7-fold increase in activity). There also appears to be a negative transcription element between -543 and -777 (indicated by a 3.5-fold decrease in activity).

In the case of all three constructs, treatment with 10 ng/ml TPA resulted in a ≈2-fold increase in activity (relative to pSV2CAT). This result indicates that there are sequences in the short region of DNA between -253 and +79 that are able to mediate TPA-induction of a fused marker gene. An interesting phenomenon was the greater induction (3-fold)

Figure 5.7

Transient transfection analysis of 2ar promoter activity in JB6 epidermal cells: effect of TPA. In three independent experiments, cells were transfected with 1, 2, or 5 μg of the indicated plasmids and treated with TPA 12 h prior to harvest. The region of the 2ar promoter present in the p2arCAT constructs is given relative to the transcriptional start site. Equal volumes of cell lysates were assayed for chloramphenicol acetyltransferase (CAT) activity. A representative autoradiogram showing the chromatographic separation of the chloramphenicol substrate (lower spot) from mono- and di-acetylated products (upper spots) is shown. Activity was normalized to the amount of protein in each cell lysate and to the activity of pSV2CAT-transfected cells (100%). The average activity of each construct (± standard deviation) from the three experiments is indicated below the figure.

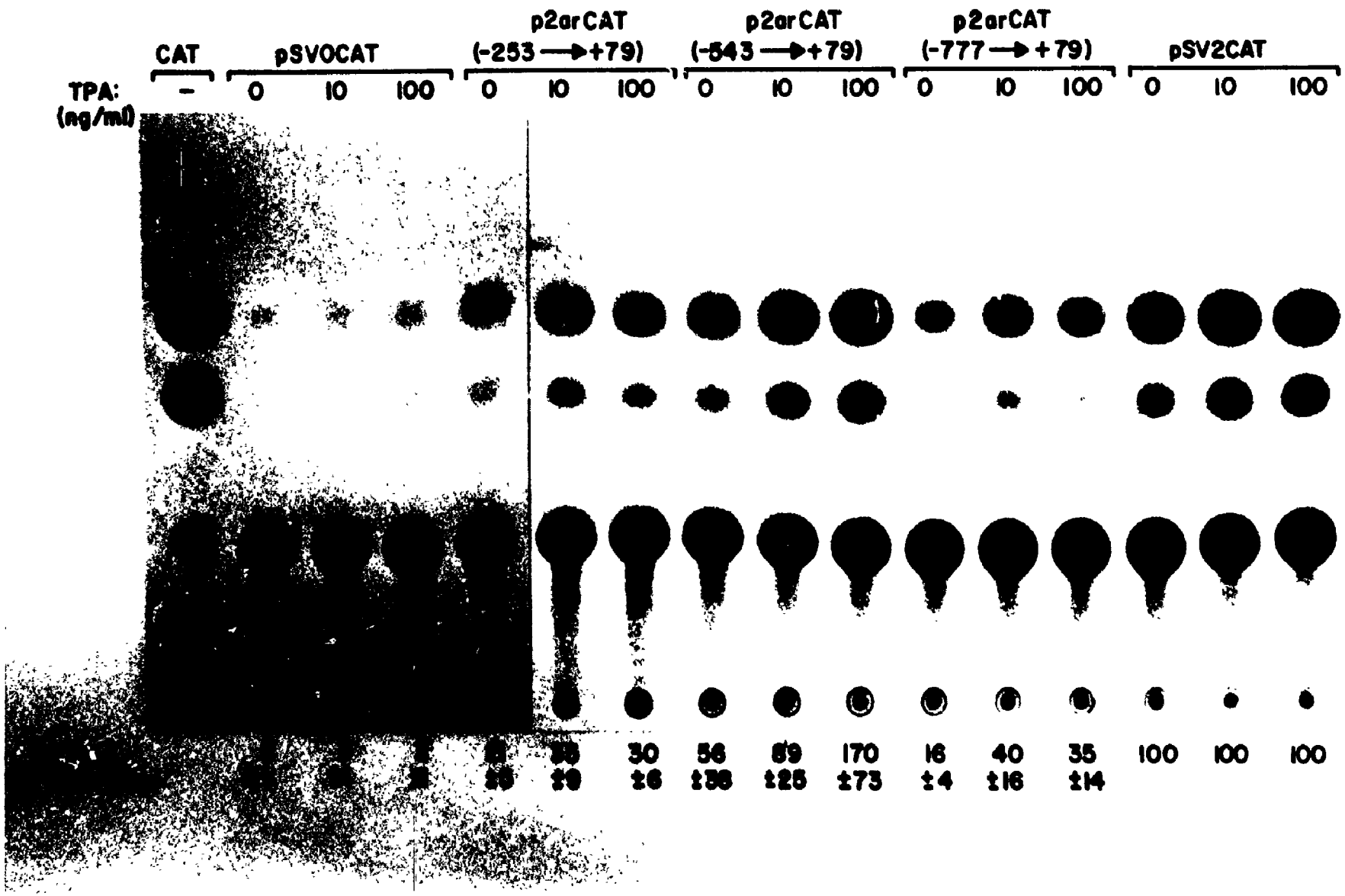
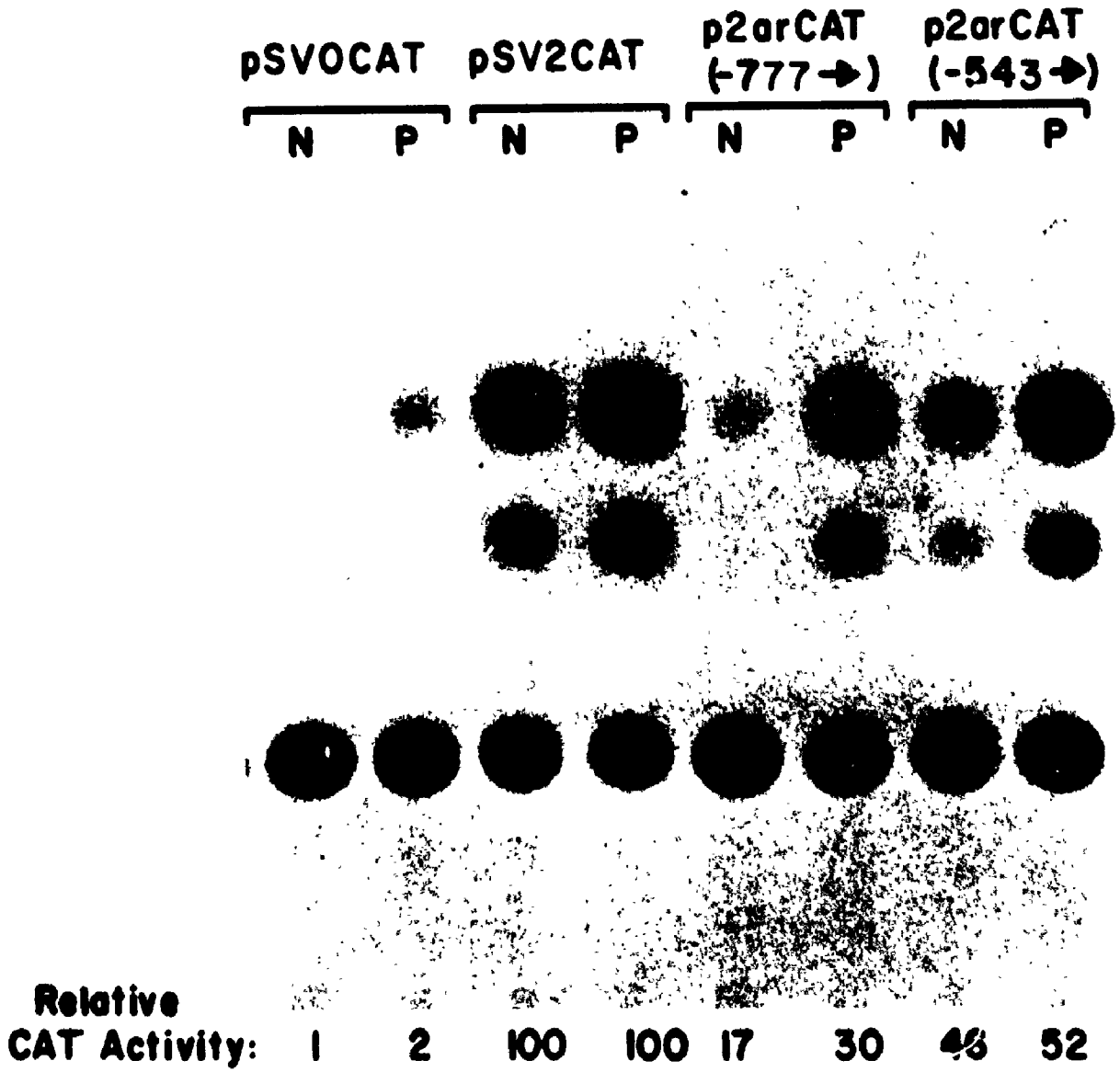

CAT
pSVOCAT
p2arCAT (-253 → +79)
p2arCAT (-543 → +79)
p2arCAT (-777 → +79)
pSV2CAT
TPA: (ng/ml)
–
0
10
100
0
10
100
0
10
100
0
10
100
0
10
100
0
10
100
30 ±6
56 ±38
89 ±25
170 ±73
16 ±4
40 ±16
35 ±14
100
100
100

Figure 5.8

Transient transfection analysis of 2ar promoter activity in NIH3T3 fibroblasts (N) and ras-transformed PAP2 cells (P). Cells were transfected with 10 μg of the indicated plasmid, harvested 48 h later, and assayed for chloramphenicol acetyltransferase (CAT) activity. The p2arCAT constructs contained DNA from the 2ar promoter region (-777 or -543 to +79, with respect to the transcription initiation site). An autoradiogram showing substrate (lower spot) and products (upper spots) of the CAT reaction is shown. The promoter activity of each construct relative to that of pSV2CAT (100%) and normalized to the amount of protein assayed is given below the figure (these are the results of a single experiment).


pSVOCAT
pSV2CAT
p2orCAT (-777→)
p2orCAT (-543→)
N P N P N P N P
Relative CAT Activity: 1 2 100 100 17 30 46 52

of the 622 bp construct, but not of the shorter 332 bp or longer 856 bp constructs, by the higher concentration of TPA.

Two of the three 2ar-CAT constructs were also assayed for expression in NIH3T3 and PAP2 cells (Fig. 5.8). PAP2 cells are tumorigenic, metastatic cells derived by transfection of NIH3T3 cells with the T24 H-ras oncogene (Hill et al., 1988). The PAP2 cells exhibit elevated expression of 2ar compared with the parental NIH3T3 cells (see Chapter 4). Relative to pSV2CAT, expression from the 856 bp segment of the 2ar promoter was 1.8-fold higher in PAP2 cells compared with NIH3T3 cells. However, in this experiment, expression from the 622 bp segment of the 2ar promoter was approximately the same in both cell lines. These results suggest that there is a ras-responsive element between -777 and -543 of the 2ar promoter (but it should be emphasized that these results are from a single experiment).

5.2.4 Effect of Cell Density on 2ar Expression

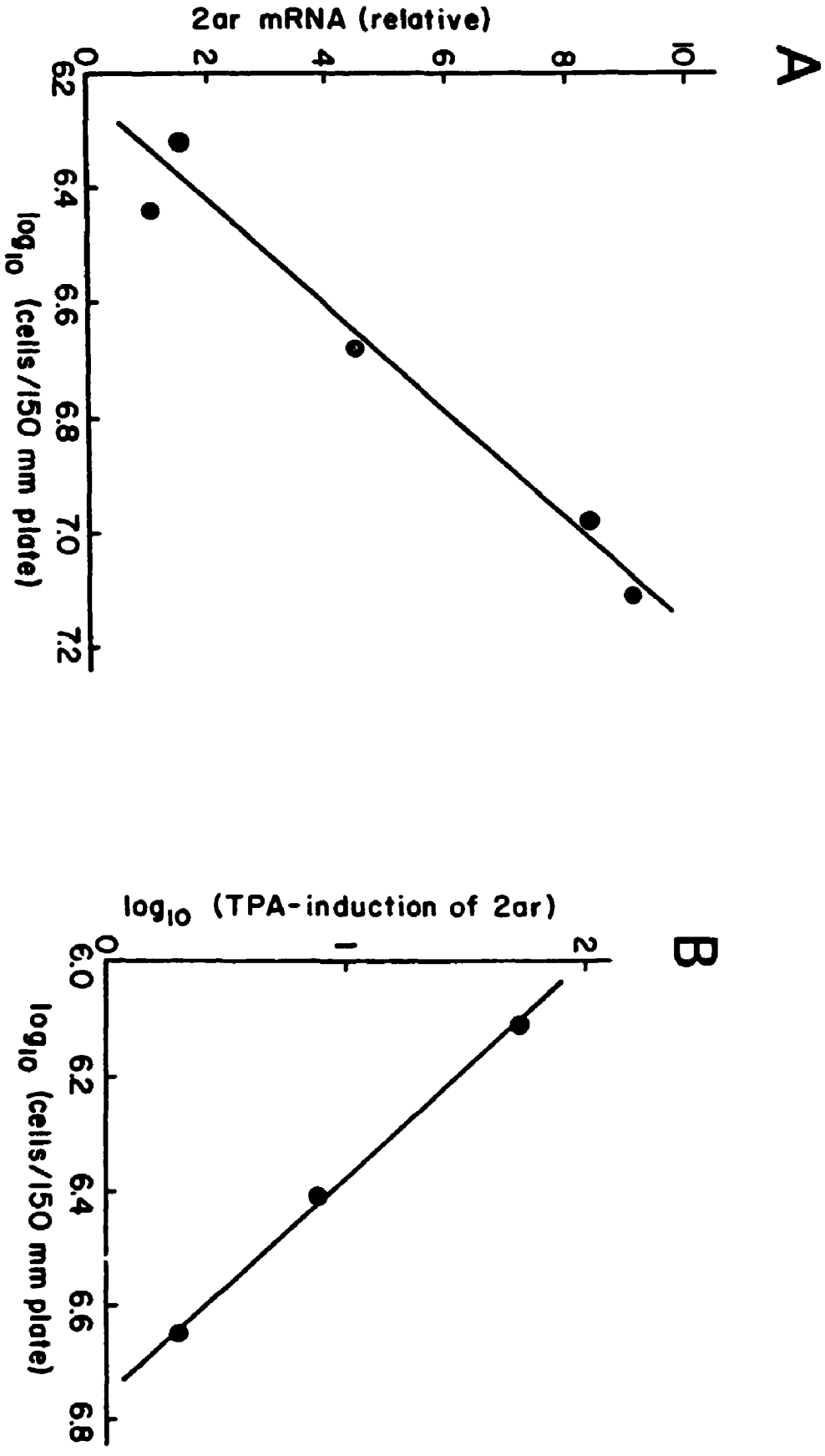
The effect of cell density on 2ar expression in JB6 cells (as reported by Smith and Denhardt, 1987) was further investigated. The effect of cell density on constitutive expression of 2ar was determined. Cell density in this experiment was manipulated both by seeding cells at different densities, and by variation in culture times for cells seeded at a single density. The relative level of 2ar mRNA was found to increase with increasing cell density (Fig. 5.9A). In contrast to the constitutive expression, the sensitivity of 2ar to TPA-induction (measured 6 h after addition of the tumor promoter) decreased with increasing cell density (Fig. 5.9B). In both cases, the effects of cell density were great,

Figure 5.9

Effect of cell density on 2ar mRNA constitutive expression and TPA-induction in JB6 epidermal cells.

A. Effect of cell density on constitutive expression of 2ar mRNA. Cells were either plated at different densities, or plated at a single density and grown for different periods of time. The steady-state levels of 2ar mRNA were determined by densitometric scanning of Northern blots probed with the 2ar cDNA.

B. Effect of cell density on TPA-induction of 2ar mRNA. Cells were plated at different cell densities, treated with DMSO alone or TPA in DMSO for 6 h, and harvested. 2ar mRNA levels were determined by densitometric scanning of Northern blots, and the ratio in TPA-treated versus control cells (fold-induction) was calculated for each cell density.


A
2ar mRNA (relative)
0
2
4
6
8
10
log10 (cells/150 mm plate)
6.2
6.4
6.6
6.8
7.0
7.2
B
log10 (TPA-induction of 2ar)
0
1
2
log10 (cells/150 mm plate)
6.0
6.2
6.4
6.6
6.8

involving 10- to 50-fold differences in steady state 2ar mRNA levels over a ≤10-fold change in cell density.

2ar message stability in JB6 cells was estimated by Northern blot and densitometric analysis of 2ar mRNA levels at several timepoints (2, 6, 12, and 24 h) after addition of 5 μg/ml actinomycin D, which shuts off transcription. A 5-fold variation in cell density (2 X 10^6 versus 1 X 10^7 cells/150 mm plate) and the presence or absence of TPA had no significant effect on the apparent 2ar mRNA stability, which in all cases was ≈15 h (data not shown).

5.3 DISCUSSION

5.3.1 2ar Gene Structure and Promoter Activity

The complete mouse 2ar gene spans ≈9 kb and contains at least 7 exons. The partial analysis of the first 6 exons revealed no evidence of alternate splicing or similar complex regulatory mechanisms. There appears to be a single transcriptional start site; 5'-terminal mRNA homogeneity is common in genes containing a TATA box at ≈-25 (Breathnath and Chambon, 1981). The TTTAAAT box at -27 to -21 in the 2ar gene varies from the consensus sequence TATA(A/T)A(A/T). The human thrombospondin promoter also contains a TATA box with a variant T at the second position (TTTAAAA; Donoviel et al., 1988). The sequence ATTGG (the inverse complement of CAATT) is able to interact with a multiplicity of nuclear proteins to stimulate and otherwise modulate gene transcription (Dorn et al., 1987). This sequence is present in the 2ar gene at -53 to -49.

The identification of this region of the 2ar gene as the promoter was confirmed by transient transfection assays of the 2ar promoter linked to the CAT marker gene. A small region of the 2ar promoter (-253 to +79)

contains sufficient information to direct a high rate of transcription in JB6 cells. In fact, expression from all of the 2ar-CAT constructs in JB6, NIH3T3 and PAP2 cells was high for a cellular promoter (compared with pSV2CAT). A high level of constitutive transcription was surprising, considering the relatively low levels of 2ar mRNA expressed by the endogenous 2ar promoter under these conditions. These results suggest that transcription from the 2ar promoter is normally repressed, perhaps by negative enhancers, or silencers, elsewhere in the gene, or by conformational effects. The cloned 2ar promoter, due to its strength in the three cell lines assayed, is potentially useful for directing expression of other sequences in mammalian cells, i.e. as part of a mammalian expression vector.

Based on the expression assays of the 2ar promoter in JB6 cells, there appears to be a positive enhancer between -253 and -543, and a negative enhancer between -543 and -777. The existence of the putative negative transcription element is supported by the expression assays in the NIH3T3 cells. Thus, the negative element appears to be active in both epidermal and fibroblast cells. It is possible that the direct 9-bp repeat at -758 to -741 is responsible for inhibition of transcription.

As described in the introduction, 2ar expression is modulated by a number of hormones, tumor promoters, and tissue-specific factors, in many cases at least partly at the transcriptional level. There are sequence similarities in the 2ar gene to elements which mediate transcriptional regulation of other genes (Table 5.2). These <u>cis</u>-acting DNA sequence elements, or motifs, bind <u>trans</u>-acting protein factors to form transcriptional regulatory modules (reviewed by Wingender, 1988). Combinations of overlapping or adjacent modules often act synergistically to enhance

transcription (Jones et al., 1988). Mammalian enhancers, which were identified as sequences able to confer position- and orientation-independent stimulation of transcription from a linked promoter, are generally ≈200 bp long, and can be composed of combinations of different transcription factor-binding elements, or multiple copies of a single element (Schaffner et al., 1988).

In many cases the degree of variation from the consensus DNA element that will still result in transcription factor-binding and modulation of activity is not known, and so sequences in the 2ar gene with only 6/8 or 9/13 bp in common with the consensus sequences were considered as potential regulatory elements. Transcription factor OBP100 was found to bind to remarkably degenerate octamer motifs through interactions with flanking sequences (Baumruker et al., 1988). For example, the sequence GGCATCTCATTACC functioned as a binding site for OBP100, even though it has only 3/8 (or 4/14) bp in common with the proposed consensus sequence, AGTATGCAAAGCAT. Such flexibility in sequence recognition by OBP100 and other DNA-binding proteins (e.g. Costa et al., 1988) indicates that, while consensus sequences are of some predictive value, functional studies are required for identification of the regulatory elements of a promoter.

5.3.2 Regulation of 2ar Gene Expression Associated with Carcinogenesis

In the 2ar promoter region, there are a number of potential binding sites for activator proteins AP-1, AP-2, AP-3 and AP-5, which may mediate TPA-induction. The binding sites for these factors were initially identified in the human collagenase gene (Angel et al., 1987b; Lee et al., 1987) and the SV40 enhancer (Chiu et al., 1987; Imagawa et al., 1987; Jones et al., 1988). Sites for AP-1 and AP-2 binding have since been

found in many cellular genes. The AP-3 binding site is very similar to the binding site for NFκB, another transcription factor which mediates gene induction by TPA (Nelsen et al., 1988). Expression from pSV2CAT (carrying the SV40 enhancer) is apparently TPA-inducible in some but not all cell lines (Imbra and Karin, 1986). Based on absolute values of CAT activity per μg of protein, pSV2CAT did not exhibit significant TPA-induction in JB6 cells, and was used as a standard to control for differences in transfection efficiencies. An ideal control for assays of promoter activity, i.e. a promoter which directs a constant easily detectable level of transcription under all circumstances, does not seem to exist (which is not surprising given the synergistic effects of multiple elements in creating a strong promoter).

All three 2ar promoter segments tested conferred ≈2-fold TPA-induction on the CAT gene, relative to expression from the SV40 promoter in pSV2CAT. The results indicate that the region -253 to +79 of the 2ar promoter contains sequences able to mediate TPA-induction. This conclusion, along with the tendency of enhancers to be composed of several closely spaced modules, suggests that the potential AP-3, AP-1 and AP-5 binding sites clustered just 5' to the CAT box and the potential AP-1 site just 3' to the TATA box may be most important in TPA-induction of 2ar. The potential binding sites for these six factors are within a 92-bp fragment (-95 to -2). The potential AP-1 site at -75 to -69 is in the same relative position as the active AP-1 binding sites in the rat transin and human collagenase promoters (-72 to -64, and -73 to -65, respectively; Angel et al., 1987a). The AP-1 binding site in the human collagenase gene conferred TPA-induction on the heterologous thymidine kinase promoter, but the extent of induction depended on the cell type (Angel et al.,

1987a). High basal expression and only a 2-fold TPA-induction was observed in mouse and rat cells, whereas in human cells (GM637 or HeLa), the basal expression was much lower and the TPA-enhancement much greater (25- or 250-fold, respectively). If this effect were also observed with the 2ar promoter, it would more strongly implicate AP-1. Identification of cell lines in which the TPA-enhancement was greater than 2-fold would greatly facilitate the identification of TPA-responsive DNA sequence elements in the 2ar promoter.

Transcription factor AP-1 is particularly interesting with respect to carcinogenesis. TPA increases the DNA-binding activity of AP-1 (Chiu et al., 1987). The AP-1 complex contains the product of the c-jun oncogene, which is similar to the yeast transcription factor GCN4 (Bohmann et al., 1987), and is itself inducible by TPA (Lamph et al., 1988). Jun binds to the fos oncogene product via interdigitating leucine residues, and a complex containing Jun and Fos stimulates transcription from the TPA-responsive element (Chiu et al., 1988; Sassone-Corsi et al., 1988; Schuermann et al., 1989). AP-1 actually appears to be composed of multiple protein complexes, containing other Jun-related proteins (e.g. JunB) and Fos-related proteins (e.g. Fra-1) (Cohen et al., 1989). It is unclear whether Fos is absolutely required for TPA-induction mediated by AP-1 binding sites (Chiu et al., 1988; Schönthal et al., 1988), but Fos clearly stimulates AP-1-dependent transcription. TPA treatment may enhance Jun/Fos complex formation, DNA binding or interaction with the transcriptional machinery, perhaps through phosphorylation of Fos.

Not only TPA, but enhanced expression of the oncogenes v-src, c-Ha-ras, activated c-Ha-ras, and v-mos can stimulate transcription from the AP-1 binding site (Schönthal et al., 1988). This result linked the

effects of several oncogenes and the tumor promoter TPA to a single pathway of gene activation. The induction of 2ar by T24-H-ras in the PAP2 cells and in the 10T1/2 derivatives (Chapter 4) may be mediated, at least in part, by the potential AP-1 binding sites in the 2ar gene. Further expression studies of 2ar-CAT constructs in the PAP2 versus NIH3T3 cells could lead to a determination of specific DNA sequences involved. It is interesting that the H-ras promoter region itself was TPA-inducible in stable transfection studies and contains potential AP-1 binding elements (Spandidos et al., 1988). PDGF- and EGF-induction of the transin gene appeared to be mediated by the AP-1 binding site (Kerr et al., 1988). This suggests that growth factor induction of gene expression may occur by the same mechanism as TPA induction, through changes in Fos, Jun, and/or other components of AP-1. Therefore, the same DNA elements may be responsible for induction of 2ar by TPA, H-ras and growth factors. This would be consistent with the proposal that these agents all act along a common pathway involving activation of PKC (see Fig. 1.1).

The TPA-induction of 2ar was only ≈2-fold in the transient transfection assays and approximately the same in a run-on transcription assay (Smith and Denhardt, 1987), but >50-fold in the cytoplasmic steady-state mRNA levels and levels of secreted metabolically labeled protein (Fig. 4.1). Although these methods are not directly comparable, the results suggest that post-transcriptional effects may contribute to the TPA-induction of 2ar mRNA in JB6 epidermal cells. Post-transcriptional mechanisms of regulation could include changes in processing, transport or stability of the mRNA. The mRNA stability ($t_{1/2}$ ≈ 15 h), as determined by an actinomycin D chase, was not altered by TPA treatment. However, it does not seem possible that a message with a half-life of 15 h could

produce the transient TPA-induction pattern reported by Smith and Denhardt (1987). The use of the drug actinomycin D may have affected the half-life of the 2ar mRNA, resulting in an artificially high estimate. This could occur, for example, by actinomycin D inhibition of the synthesis of a factor which promotes the degradation of the 2ar message. Another possibility is that the 2ar mRNA may be degraded more quickly at a lower cell density (1 X 10^6 cells/150 mm plate as in Smith and Denhardt, 1987) than at the two cell densities studied here (2 X 10^6 or 1 X 10^7 cells/150 mm plate).

An effect of cell density on the sensitivity of the 2ar message to TPA-induction has been reported before (Smith and Denhardt, 1987). Here it was shown that this effect is not all-or-none in subconfluent versus confluent cells, but that the extent of TPA-induction varied inversely with cell density, at least in the range of 10^6-10^7 cells/150 mm plate. Over the same cell density range, the basal expression of 2ar increased ≈10-fold. Smith and Denhardt (1987) reported only a ≈2-fold induction in basal expression over this cell density range; the reason for this discrepancy is not known. Thus, as basal 2ar expression increased, TPA-induction decreased, both as a result of cell density in JB6 cells and also in NRK cells compared with other cell lines. Some of the same or overlapping sequence elements may mediate both high constitutive expression of 2ar and TPA-induction. Collagen production in cultured bovine aortic smooth muscle cells is also regulated by cell density, increasing with the time in culture (Stepp et al., 1986). The significance of the cell density effect with respect to normal <u>in vivo</u> production of 2ar is not clear. TPA-induction of proliferation in skin and tumor cell interactions may contribute to the enhanced expression of 2ar associated

with carcinogenesis. It is interesting that cells grown to higher density in culture tend to be more metastatic (A.F. Chambers, personal communication), again consistent with a correlation between 2ar expression and metastic ability.

5.3.3 Hormonal Regulation of 2ar Gene Expression

Parathyroid hormone decreased the rate of osteopontin transcription in rat osteoblast-like cells, and this effect appeared to be mediated by an increase in cAMP (Noda and Rodan, 1989). There are two classes of cAMP-response elements identified in other genes, known as CRE and AP-2 elements (Roesler et al., 1988). Sequences similar to both of these consensus elements are present in the 2ar promoter region. They have been found to confer cAMP-mediated transcriptional stimulation in other genes, rather than inhibition. However, the arrangement of these elements with respect to the binding sites of other transcriptional factors may determine whether the effect of cAMP binding is stimulatory or inhibitory. For example, the binding of glucocorticoid to an element which is generally responsible for transcriptional stimulation prevented the binding of another transcriptional factor to an overlapping element in the human glycoprotein hormone α-subunit gene, leading to glucocorticoid-inhibition of transcription (Akerblom et al., 1988). One of the potential CREs at -190 in the 2ar promoter region overlaps with a potential nuclear factor III binding site, and thus binding of a transcription factor to this CRE could result in decreased transcription. Transfection studies of this small region of the 2ar gene linked to a heterologous promoter, and DNaseI or methylation protection studies to determine factor binding sites could be used to test this hypothesis.

Transcription factor AP-2 mediates the response to two different signal transduction pathways; activation of protein kinase C by TPA or activation of protein kinase A by cAMP independently appeared to lead to AP-2 enhancement of transcriptional activity (Imagawa et al., 1987). The enhancement of transcription was not accompanied by a change in DNA-binding activity of AP-2 (Imagawa et al., 1987). It would be interesting if AP-2 activation were involved in TPA-induction of 2ar in epidermal cells and cAMP-repression in osteoblastic cells, although this seems unlikely.

The 2ar gene is regulated by steroid hormones. 1,25-Dihydroxy-vitamin D_3 induced the message in osteosarcoma cells, likely at the transcriptional level (Prince and Butler, 1987). Retinoic acid and the synthetic glucocorticoid dexamethasone inhibited TPA-induction of 2ar in JB6 epidermal cells (Smith and Denhardt, 1987). Dexamethasone also inhibited osteopontin expression in osteosarcoma cells (Yoon et al., 1987). The abundance of 2ar mRNA in the skin of pregnant and lactating mice suggests a stimulatory effect of estrogens and/or progesterone, although progesterone did not induce the message in cultured fibroblasts (Nomura et al., 1988). In summary, 2ar is induced by vitamin D_3 and likely estrogen and/or progesterone, and repressed by retinoic acid and glucocorticoid.

Gene activation by steroid hormones is fairly direct, since the hormone-receptor complex itself binds to DNA to activate transcription (Mader et al., 1989). There are sequences similar to the palindromic response elements for estrogen, glucocorticoid and retinoic acid in the 2ar promoter region (see Table 5.2 for specific sequences). The potential retinoic acid response element overlaps the potential nuclear factor III

binding site, suggesting a possible inhibitory mechanism (as suggested above for cAMP-mediated inhibition). It has been proposed that the response element of the vitamin D_3 receptor is similar to that of the retinoic acid receptor (Mader et al., 1989); this would be interesting given the opposing effects of these two agents on 2ar expression. An unrelated sequence has also been proposed as a vitamin D_3 responsive element based on studies of the osteocalcin and calbindin genes (Yoon et al., 1988); the reverse complement of this sequence is present in the 2ar promoter (Table 5.2).

Sequences identical to interferon-response elements were found in the 2ar promoter (Table 5.2). It is not known whether these sequences are active, since modulation of 2ar expression by interferons has not been studied. Induction by interferons might be consistent with a role for 2ar in the immune response, as suggested by the secretion of 2ar by macrophages and elevated expression associated with gram-negative sepsis (Senger et al., 1988) and the possible allelism with the Ric gene encoding resistance to scrub typhus (Fet et al., 1989). However, the elevated 2ar expression was specific to bacterial and not viral infection (Senger et al., 1988).

The mechanism of modulation of gene expression by TGFß is largely unknown. The differential effects on 2ar expression suggests the existence of cell-specific factors which mediate TGFß induction and repression; it would be interesting to determine whether a common DNA sequence element is responsible. This hypothesis and the others proposed here could now be tested using the mouse λ 9090 2ar genomic clone and appropriate expression systems.

CHAPTER 6

FUTURE PROSPECTS

In summary, 2ar mRNA is inducible by the tumor promoter TPA in mouse epidermis, and encodes the murine homolog of rat osteopontin, a 41.5 kDa bone phosphoprotein. 2ar is the major phosphoprotein secreted by all rodent cell lines studied, and is apparently identical to a transformation-associated phosphoprotein, or pp69. By agreement with several groups studying various aspects of this protein, it has been given the formal name "secreted phosphoprotein 1" (SPP) and the designation spp.

In developing bone and in the inner ear, SPP appears to act to anchor the cells to the calcified matrix, as suggested by its ability to bind hydroxyapatite and to bind cells via the ArgGlyAsp sequence. SPP may also mediate cell adhesion to non-mineralized matrices. The abundance of SPP in the kidney could be related to a function in mineral metabolism, since SPP does bind free Ca^{2+} at low affinity. SPP mRNA was localized to the medulla of adult mouse kidney, and appears to be expressed in the thin segments of Henle's loop. The production of SPP by macrophages and its presence milk, blood, uterus, ovary, decidua, placenta, and skin of pregnant and lactating mice suggests that this protein may have an entirely different function than those indicated above. Possibilities that could be tested include anti-bacterial activity, mitogenicity, determination of cell position during development, inhibition of protein phosphatase activity (as suggested by PEST sequences in the polypeptide), and induction or development of female-specific characteristics. Expression of the full-length cDNA could facilitate isolation of large amounts of the protein and would make possible site-directed mutagenesis studies to determine functional contributions of specific amino acids. Up- or

down-regulation of SPP in mammalian cells (using the expression vectors constructed here) or in specific tissues in transgenic mice (depending on the promoter used) could be used to determine more precisely the physiological function of SPP.

An association of SPP expression with carcinogenesis was established. SPP was induced by tumor promoters, growth factors, and the activated H-ras oncogene, and was constitutively expressed at high levels in epidermal carcinomas. SPP expression in several tumorigenic cell lines correlated roughly with the ability of the cells to induce experimental and spontaneous metastases. The proposal that SPP facilitates metastasis is amenable to more direct experimentation by the use of the sense and antisense expression constructs, and immunoprecipitations or Western blot analyses with the anti-cro-ß-galactosidase-SPP sera to monitor SPP levels. If a direct stimulatory effect of SPP on metastasis in cultured mouse cells or in transgenic mice can be demonstrated, then further questions could be asked, such as which stage of the metastatic process is affected by SPP. Mutational analyses could be used to determine which features of the primary structure are required for enhancement of metastasis. For example, an obvious experiment would be to mutate the ArgGlyAsp site and determine whether or not high level expression of the mutant protein was still able to stimulate metastasis.

This is the first report of the isolation and characterization of the SPP promoter in any species. The mouse SPP gene spans approximately 9 kb, and is located on chromosome 6. The mouse SPP genomic clone described here contains 16 kb upstream of the transcriptional start site, and 4.8 kb downstream, including at least the first 6 exons. The sequence from -910 to +91 with respect to the transcriptional start site contains

a number of elements similar to transcription factor-binding sequences which may mediate the regulation of SPP expression by TPA, growth factors, H-ras, parathyroid hormone (via cAMP), and several of the steroid hormones.

Transient transfection assays of portions of the SPP promoter linked to the chloramphenicol acetyltransferase marker gene in JB6 epidermal cells and NIH3T3 fibroblasts indicated that a small region (-253 to +79) was sufficient to direct high level expression. The same region conferred TPA-inducible expression, although induction was only 2-fold. It seems likely that a cluster of potential AP-1, AP-3 and AP-5 binding sites surrounding the TATA and CAT boxes is responsible for the TPA-induction. Comparisons between different constructs indicated the presence of a positive transcriptional element between -543 and -253 and a negative element between -777 and -543. In a preliminary study, the region between -777 and +79 directed enhanced transcription as a result of T24 H-ras expression. Many of the factors which modulate SPP expression are inhibitory rather than stimulatory. Based on potential overlapping DNA sequence elements, it was proposed that parathyroid hormone (via cAMP) and retinoic acid inhibition of SPP expression could occur by inhibition of nuclear factor III binding.

Short DNA sequence elements responsible for SPP regulation were not directly identified in this work, but the groundwork has been done to make such experiments possible. Further experiments could involve more transient transfection expression studies, using oligonucleotide enhancers or specific mutations in the SPP promoter, as well as gel mobility assays and DNA footprinting analyses. Two of the most interesting unresolved questions in gene expression are the mechanisms whereby a protein such as

SPP is expressed in a specific limited set of tissues in vivo, and how the pattern of expression changes with development. Expression in transgenic mice of portions of the SPP promoter linked to a marker gene such as ß-galactosidase would lead to the identification of DNA sequence elements responsible for tissue-specific expression. It might then be possible, using the relevant portions of the SPP promoter linked to the full-length cDNA in the antisense orientation, to specifically down-regulate SPP expression in either bone or kidney or ovary, for example, and thus come to a better understanding of the physiological function of SPP.

APPENDIX

2AR CLONES GENERATED DURING THIS WORK

A. cDNA Clones

AMC#	Designation/Features	Insert (nt in Fig. 3.2)	Vector
1	pEX2-2arN ("2-9"); expression of cro-β-galactosidase-2arN (Fig. 3.5A)	XmnI-PvuII (216 to 642)	pEX2 (SmaI)
2	pEX2-2arC ("445-7"); expression of cro-β-galactosidase-2arC (Fig. 3.5A)	PvuII-HindIII/Klenow (643 to 1079)	pEX2 (BamHI/Klenow)
3	pBR-0.12 HindIII-XmnI	HindIII-XmnI (89 to 215)	pBR322 (HindIII-PvuII)
4	pSP65-551 2ar H-P ("2-7")	HindIII-PvuII (89 to 642)	pSP65 (HindIII-PvuII)
5	pGEM3-2arH s ("3-4"); 2ar sense expression from SP6 promoter	HindIII (89 to 1079)	pGEM3 (HindIII)
6	pGEM3-2arH as ("3-5"); 2ar antisense expression from SP6 promoter	HindIII (89 to 1079)	pGEM3 (HindIII)
7	pGEM4-2arH s ("4-2"); 2ar sense expression from T7 promoter	HindIII (89 to 1079)	pGEM4 (HindIII)
8	pGEM4-2arH as ("4-1"); 2ar antisense expression from T7 promoter	HindIII (89 to 1079)	pGEM4 (HindIII)
9	5' 2ar cDNA (by primer extension) (Fig. 3.1)	-69 to 131 (& G-C tails & PstI ends)	pBR322 (PstI)
10	pGEM3-2ar Sty-Hind (-18 to 1079) (recombined 2ar) (Fig. 4.8)	StyI/Klenow-BstXI partial of 5' 2ar cDNA	pGEM3-2arH s (XbaI/ Klenow-BstXI)
11	pGEM4-2ar Sty-HincII (-18 to 880) (Fig. 4.8)	SmaI-HincII fragment of above construct	pGEM4 (HincII)
12	pGEM4-2ar Sty-Hind (-18 to +1079); whole coding region ("whole 2ar") (Fig. 4.8)	HincII-HindIII (881 to 1079)	above construct (HincII)
13	pNMT-2ar s; 2ar sense expression from metallothionein promoter (Fig. 4.8)	BamHI of above construct (-18 to 1079)	pNMT (BamHI)
14	pNMT-2ar as; 2ar antisense expression from metallothionein promoter (Fig. 4.8)	as above	pNMT (BamHI)

15	pCGH-2ar s; 2ar sense expression from cytomegalovirus promoter	as above	pCGH (BamHI)
16	pCGH-2ar as; 2ar antisense expression from cytomegalovirus promoter	as above	pCGH (BamHI)
17	M13mp19-0.12 XmnI-HindIII	XmnI-HindIII (seq. 89 to 215)	M13mp19 (SmaI-HindIII)
18	M13mp18-2ar Hind(89)-Sph	HindIII-SphI (seq. 89 to 594)	M13mp18 (HindIII-SphI)
19	M13mp18-2ar Hind(1079)-Sph	HindIII-SphI (seq. 1079 to 591)	M13mp18 (HindIII-SphI)
20	M13mp19-2ar Sph-Hind(1079)	SphI-HindIII (seq. 591 to 1079)	M13mp19 (SphI-HindIII)
21	M13mp18-2arH1	HindIII (seq. 1079 to 89)	M13mp18 (HindIII)
22	M13mp18-2arH5	Hi dIII (seq. 89 to 1079)	M13mp18 (HindIII)
23	M13mp18-2arP5	PvuII (seq. polyA to 643)	M13mp18 (SmaI)
24	M13mp18-2arP1	PvuII (seq. 643 to polyA)	M13mp18 (SmaI)
25	M13mp19-2ar 5'cDNA-3	PstI (seq. 5'G-C tail & -69 to 131 & 3'G-C tail)	M13mp19 (PstI)
26	M13mp19-2ar 5'cDNA-8	PstI (seq. 3'G-C tail & 131 to -69 & 5'G-C tail)	M13mp18 (PstI)
27	M13mp19-2ar Alu-Pst-6 from 5'cDNA	AluI-PstI (seq. 90 to 131 & 3'G-C tail)	M13mp19 (SmaI-PstI)
28	M13mp19-2ar Alu-Pst-9 from 5'cDNA	AluI-PstI (seq. 77 to -69 & 5'G-C tail)	M13mp19 (SmaI-PstI)
29	M13mp18-BamHI-XmnI from whole 2ar	BamHI/StyI-XmnI (seq. 5' linker & -18 to 131)	M13mp18 (BamHI-SmaI)
30	M13mp19-XmnI-BamHI from whole 2ar	XmnI-StyI/BamHI (seq. 131 to -18 & 5' linker)	M13mp19 (SmaI-BamHI)

B. Genomic Clones

AMC#	Designation/Features	Insert (exon #: E)	Vector
31	λ 9090 2ar; whole genomic clone (Fig. 5.2)	E1-6 + 16 kb 5'DNA (≈20 kb + λ arms)	Charon 4A
32	pBR-λ2ar 3.3 kb Eco	EcoRI (E3-6)	pBR322 (EcoRI)
33	pBR-λ2ar 0.8 kb Pvu	PvuII (E4)	pBR322 (PvuII)
34	pBR-λ2ar 2.0 kb Pvu-Eco	PvuII-EcoRI (E5-6)	pBR322 (EcoRI-PvuII)
35	pBR-λ2ar 0.5 kb Eco-Pvu	EcoRI-PvuII (E3)	pBR322 (EcoRI-PvuII)
36	pBR-λ2ar 3.1 kb Sph (double insert)	SphI (E1-3)	pBR322 (SphI)
37	pGEM4-λ2ar 6.7 kb Bgl	Bgl II (E1)	pGEM4 (BamHI)
38	pGEM4-λ2ar 1.0 kb Sph	SphI (E4)	pGEM4 (SphI)
39	pGEM4-λ2ar 1.0 kb Pst; #7: EcoRI →5'genomic3'→ HindIII #6: EcoRI →3'genomic5'→ HindIII	PstI (E1)	pGEM4 (PstI)
40	pSV2arCAT (-777 to +79); CAT expression from 2ar promoter	PvuII-StyI/Klenow (E1)	pSVOCAT (SmaI)
41	pSV2arCAT (-543 to +79); CAT expression from 2ar promoter	NheI/Klenow-StyI/Klenow (E1)	pSVOCAT (SmaI)
42	pSV2arCAT (-253 to +79); CAT expression from 2ar promoter	BamHI/Klenow-StyI/Klenow (E1)	pSVOCAT (SmaI)
43	M13mp18-λ2ar 0.8 kb Pvu-1	PvuII; seq. 3'→5' (E2)	M13mp18 (HincII)
44	M13mp19-λ2ar 0.8 kb Pvu-2	PvuII; seq. 5'→3' (E4)	M13mp19 (SmaI)
45	M13mp18-λ2ar 3.3 kb Eco-3	EcoRI; seq. 5'→3' (E3-6)	M13mp18 (EcoRI)
46	M13mp18-λ2ar 3.3 kb Eco-4	EcoRI; seq. 3'→5' (E3-6)	M13mp18 (EcoRI)
47	M13mp18-λ2ar 0.7 kb BamHI-18	BamHI; seq. 5'→3' (E1)	M13mp18 (BamHI)
48	M13mp18-λ2ar 0.7 kb BamHI-17	BamHI; seq. 3'→5' (E1)	M13mp18 (BamHI)
49	M13mp18-λ2ar 0.7 kb Pst-Eco	PstI-EcoRI (E1)	M13mp18 (PstI-EcoRI)
50	M13mp19-λ2ar 0.7 kb Eco-Pst	EcoRI-PstI (E1)	M13mp19 (EcoRI-PstI)
51	M13mp19-λ2ar 1.0 kb Pst-A	PstI; seq. 3'→5'(E1)	M13mp19 (PstI)

S2	ΔA: deletion derivatives of above (Fig. 5.6)	ΔA11 (-203 to -910) ΔA25 (-456 to -910) (& more uncharacterized)	M13mp19; IBI Cyclone kit
S3	M13mp19-λ2ar 1.0 kb Pst-B	PstI; seq. 5'→3' (E1)	M13mp19 (PstI)
S4	ΔB: deletion derivatives of above (Fig. 5.6); inserts (EcoRI-StyI) could be cloned into pSVOCAT to test for 2ar promoter activity	ΔB14 (-667 to +88) ΔB17 (-178 to +88) (& more uncharacterized)	M13mp19; IBI Cyclone kit

Note: Unless otherwise indicated, the restriction enzyme sites used for cloning can be found in the sequence in Fig. 3.2 (cDNA clones) or in the map in Fig. 5.2 (genomic clones). Seq. refers to the direction of sequence obtained from the M13 subclones, in cases where there are two possible orientations. For the genomic M13 subclones, seq. 5'→3' indicates that sequence was read in the direction from exon 1 towards exon 6, and thus the clone contains DNA of the template strand. The endpoints of the ΔA and ΔB deletion derivatives are given relative to the transcription initiation site.

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