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# Molecular Cloning of a Tumor Promoter-Inducible mRNA Found in JB6 Mouse Epidermal Cells: Induction Is Stable at High, but not at Low, Cell Densities

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From the mouse JB6 epidermal cell line C122 we have isolated a cDNA clone representing a 1.6-kilobase mRNA, called 2ar, that exhibits a biphasic induction in response to 12-O-tetradecanoyl-phorbol-13-acetate (TPA). The first phase of induction in subconfluent cells is transient, peaking at 6 h after the addition of TPA and returning to noninduced levels by 24 h. When the cells reach plateau density, in the continued presence of TPA, this mRNA is reinduced and remains so upon continued exposure to the tumor promoter. Serum and certain growth factors also induce 2ar mRNA in serum-deprived quiescent fibroblasts. In vitro nuclear "run-on" transcription experiments indicate that the induction of 2ar mRNA is controlled at the transcriptional level.

**Key words: TPA, tumor promotion, JB6 cells, transcriptional induction, inducible mRNA, 2ar mRNA, mRNA induction, serum-inducible, JB6, TPA, tumor promoter, nuclear "run-off" transcription, gene expression, colony screening, mRNA, growth factors, competence, 3T3 fibroblasts, protein kinase C**

The biochemical events that contribute to the development of a tumor are of considerable interest. Although the evidence is good that most human cancers arise in the first instance as the result of an "initiating" genetic change in the DNA of a normal cell, it is nevertheless also evident that the development of a full fledged malignancy is inevitably accompanied by additional genetic and epigenetic changes in the cell; loosely speaking, the terms tumor promotion and tumor progression refer to

Abbreviations used: TPA, 12-O-tetradecanoyl-phorbol-13-acetate; DMSO, dimethylsulfoxide; $p^+$, sensitive to promotion; $p^-$, not sensitive to promotion; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; SomC, somatomedin C; SDS, sodium dodecyl sulfate; FBS, fetal bovine serum; ssDNA, single-stranded DNA.

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this process. Tumor progression occurs as the result of the selection of cell variants in the developing tumor that have a growth advantage [1]. Tumor promotion usually refers to a process by which tumorigenesis in initiated cells is facilitated by substances that are not by themselves tumorigenic for cells that have not been initiated [2].

The pre-eminent tumor promoter is 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a potent activator of protein kinase C [3]. When mouse skin, provided that it has been previously initiated, is treated repeatedly with TPA, papillomas and ultimately carcinomas result [4]. We have chosen to study the changes in gene expression that occur in an in vitro paradigm of this system with the belief that from these studies we will gain a better understanding of important events occurring during tumor promotion. The in vitro model is based on the promotable ($p^+$) and non-promotable ($p^-$) clones of the nonclonal JB6 cell line derived from BALB/c mouse epidermal cultures developed by Colburn et al [5,6]. The $p^+$ derivatives, compared to $p^-$ derivatives, more readily acquire the ability to grow in soft agar and to form tumors in animals, when treated with TPA. In contrast to the $p^+$ lines, the $p^-$ lines show a decrease in trisialoganglioside synthesis [7], do not release fibronectin when treated with TPA [8], and do not contain active *pro* genes [9].

The strategy we adopted was based on the hypothesis (which is no longer a hypothesis, given the many examples) that TPA causes changes in gene expression, and that it is this altered gene expression that contributes, in part at least, to tumor promotion. There are numerous and diverse examples of effects of TPA on gene expression. Depending upon the specifics of the cell type, differentiation or maturation can be induced or blocked [reviewed in 10]. Gene expression can be affected at various levels. Transcription of c-*fos* [11], JD15 [12], 16C8 [13], and MEP [14], to name a few genes, is enhanced by TPA. Numerous reports have documented increases in the cytoplasmic abundance of a large number of mRNAs, possibly owing to new transcription, to altered processing/transport of the RNA, or to increased cytoplasmic stability [15–17]. Modification by phosphorylation [18,19] and poly(ADP)ribosylation [20] and enhanced secretion [21] of certain proteins have been detected. The research reported here is the initial report of a project designed to yield molecular clones of mRNA species that in response to TPA treatment are expressed exclusively (or at least to greater extents) in promotable JB6 (eg, clone 22) clones as compared to nonpromotable (eg, clone 30) controls. The particular mRNA identified here, although inducible by TPA, is, however, induced to a similar extent in nonpromotable cells.

## MATERIALS AND METHODS

### Cell Culture

The JB6 cell lines C121 and C122 (promotable) and C130 (nonpromotable), generously provided by Dr. N. Colburn, were grown in Eagles medium containing 8% fetal bovine serum (FBS) (Gibco Labs). Stock cell cultures were always maintained below plateau density. For experiments, cells were seeded at $1 \times 10^5$ cells/150-mm plate and grown for 5 days to obtain confluent cultures; details of specific experiments are given in the legends. TPA (LC Services Corp., Woburn, MA) dissolved in DMSO (Alfa products) was added to 10 ng/ml. PDGF was from Bioprocessing Ltd.; EGF was from Collaborative Research Inc. Swiss 3T3 cells were cultured and treated as described previously [22].

## RNA Isolation and Analysis

RNA was isolated as previously described [23]. For Northern blots the RNA was electrophoresed through 1.1% agarose gels in a 40 mM morpholinopropanesulphonic acid (MOPS)-acetate buffer (pH 7.0) containing 2.2 M formaldehyde [24]. RNA was transferred to nitrocellulose, and hybridization and autoradiography were performed as described [23]. Nick-translated DNA ($10^8$ cpm/$\mu$g) generated from either purified inserts or cDNA-containing plasmids was used to probe blots. When blots were reused, the previous probe was removed by incubation in hybridization buffer at 75°C for 5 min. RNA slot-blotting was performed as described [13]. Quantification of hybridization signals was accomplished by determining the amount of annealed radioactive DNA in a scintillation spectrometer.

## Preparation of the cDNA Library and Isolation of 2ar

The cDNA library was prepared using a modified Okayama-Berg procedure as described by Kowalski et al [25] using poly(A)RNA from C122 cells that had been grown for 5 days in the presence of 10 ng/ml TPA, reseeded again at $1 \times 10^5$ cells/150-mm plate, and grown for a further 5 days under the same conditions. The vector pSS24 [25] contains the origin of replication of the filamentous ssDNA bacteriophages, which enables the generation of ssDNA copies of the library when the latter is superinfected with mutant phage IR1. pSS24 serves as a vector primer for the cloning of the mRNA in a known orientation. This strategy permits the generation of clones carrying inserts with known polarity. Tailing of the first cDNA strand synthesized and linker-dependent recircularization of these clones optimizes the probability of obtaining full- to near-full-length cDNA clones. The "linker" used to recircularize the vector after cDNA synthesis was a synthetic oligonucleotide, 5′AGCTTGGGGGGG3′, synthesized for us by F. Graham, McMaster University. (A more detailed presentation of this methodology is in reference [25].) The cDNA clones were obtained by transforming *Escherichia coli* strain R4 with the DNA preparation [26].

The library, consisting of $10^5$ clones in a single-stranded DNA form, was enriched for sequences that should be relatively more abundant in the TPA-treated C122 ($p^+$) cells by employing three rounds of "cascade" hybridization to a Rot value of 500–1,000 against a 200-fold mass excess of poly(A) RNA from C130 cells ($p^-$) that also had been treated with TPA for $2 \times 5$ days. The inserts in the single-stranded DNA were complementary to the mRNA. The hybridization was performed in 0.24 M phosphate buffer, pH 6.8, 0.4% SDS, at 65°C in a 40-$\mu$l volume. RNA/DNA hybrids were separated from nonhybridized nucleic acids on a hydroxyapatite column. After each round of hybridization the nonhybridizing single-stranded DNA was rendered double-stranded by annealing to a 1.9-kb *Xmn*I/*Pvu*II restriction fragment from pBR322, "filling in" with the *E coli* DNA polymerase large fragment, covalently closing with T4 DNA ligase, and amplifying by transfection into *E coli* RR1 [25]. The final nonhybridizing fraction in a double-stranded form was then used to make an enriched sublibrary in *E coli* RR1. To eliminate clones with no or small inserts, a plasmid preparation of the sublibrary was size-selected by agarose gel electroelution and transformed into *E coli* R4 [25].

The size-selected and enriched sublibrary was screened by probing duplicate colony-blots [23] on nitrocellulose with $^{32}$P-labeled cDNA probes made from

poly(A)RNA derived from C122 and C130 cells, both treated with TPA. Colonies that gave differential signals after three independent screenings were used to make plasmid preparations. The plasmids from these clones were nick-translated and used to probe Northern blots or slot-blots of RNA preparations from TPA-treated and control cells.

### Nuclear "Run-On" Transcription

Nuclear "run-on" transcription assays were performed with isolated nuclei, from TPA-treated or untreated cells, according to Greenberg and Ziff [11] with a number of modifications [13]. Purified plasmid preparations representing specific mRNAs were blotted onto nitrocellulose (1 $\mu$g/slot) [27] and used to analyze the $^{32}$P-labeled nuclear "run-on" preparations. The results were analyzed by autoradiography.

## RESULTS

### Isolation of 2ar

Differential colony screening of the size-selected sublibrary (enriched for sequences present in TPA-treated C122 cells compared to TPA-treated C130 cells, see "Materials and Methods") with $^{32}$P-labeled cDNAs complementary to mRNA from C122 and C130 cells, both treated with 10 ng/ml TPA, yielded the strongly hybridizing cDNA clone 2ar. Seven colonies were picked out in the initial screen that gave a stronger signal when hybridized to the C122 probe versus the C130 probe. Nick-translated plasmid preparations from these seven isolates hybridized to a mRNA species in the same region of the gel and gave similar intensity patterns with various RNA preparations tested; restriction analysis and cross-hybridization of these clones confirmed that they represented at least five different individual cDNA clones of this mRNA (results not shown). 2ar was chosen because it was the longest cDNA, 1.4 kb.

To our surprise, the corresponding mRNA was found to be induced to similar, but variable, levels in the promotable and nonpromotable lines tested (Fig. 1). It was abundant in the sublibrary, because, for reasons we do not understand, the particular preparation of poly(A)RNA that was used to prepare the original library and that was employed in making one of the cDNA probes for colony screening was substantially enriched in this mRNA species (Fig. 1, lane g).

The 2ar mRNA migrates as a unique 1.6-kb species on a formaldehyde-agarose gel (see Fig. 2). The partial DNA sequence information we have obtained so far has not revealed strong homology to sequences in 1985 versions of GenBank or the NBRF protein data bank (see Note Added in Proof). Quantitative analyses of Southern blots indicates that 2ar corresponds to a unique gene in the mouse genome (A.M. Craig, personal communication).

### Induction of 2ar mRNA

Examination of the kinetics of induction of 2ar mRNA in proliferating C122 cells, treated for various times with TPA, revealed a relatively rapid induction of the mRNA that peaked around 6 h (Fig. 2A) and that returned to basal levels by 24 h. As it had already been established that 2ar mRNA was present at an increased abundance in confluent cells treated for 10 days with TPA, this observation led us to examine TPA treatments of longer duration. RNA was prepared from cells harvested after

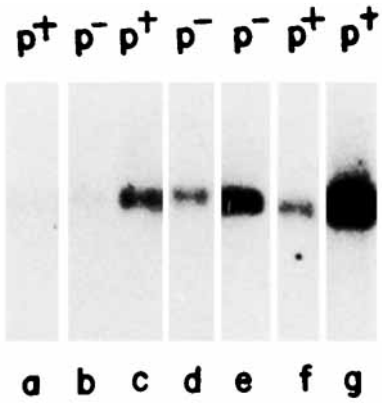


Fig. 1. Northern blot analysis of independent preparations of poly(A)RNA from different JB6 clones with or without TPA (10 ng/ml). Exposure to TPA was for a total of 10 days; after 5 days the cells were replated at $10^5$ cells/150-mm plate. Equal amounts of poly(A)RNA (1 $\mu$g per lane) were electrophoresed and transferred to nitrocellulose as described in "Materials and Methods." RNA preparations from different harvests show some variability in the relative amount of 2ar mRNA. **Lanes a** and **b** were from control cells exposed only to 0.01% DMSO; **lanes c–g** were from TPA-treated cells. The clones used were (a) C122; (b) C125; (c) C121; (d and e) C130; (f and g) C122. **Lane g** shows the preparation of poly(A)RNA used to prepare the library. The probe used was nick-translated 2ar in pSS24. $p^+$ and $p^-$ indicate whether the cells in that clone can form colonies in soft agar in the presence of TPA.

extended periods of exposure to TPA. A Northern blot analysis of these RNA preparations (Fig. 2B) disclosed that 2ar mRNA was reinduced after a period of 3 days and remained at levels of increased abundance, maintaining high levels for up to at least 8 days in the presence of TPA. TPA, therefore, induces 2ar mRNA in a biphasic manner in growing cultures. The early induction, when it takes place in cells at subconfluence, is transient in nature, whereas the later increase in confluent cells is sustained.

To ascertain whether the second phase of 2ar induction was dependent upon the duration of TPA exposure or the age or density of the cells, two sets of plates were seeded with C122 cells at low density. TPA was added to one set 1 day after seeding and to the other set 3 days after seeding. Cells from both sets of cultures were harvested at intervals, and 10 $\mu$g of total cytoplasmic RNA that was purified from each sample was immobilized on nitrocellulose using a slot-blot apparatus. The resulting blot was probed with the purified 2ar cDNA insert labeled by nick-translation. After autoradiography of the blot, the "slots" were excised, and the bound radioactivity was quantified. A graph of the level of 2ar mRNA versus time after seeding, for both sets of cultures, is shown in Figure 3A. From these data it is evident that the induction of 2ar mRNA is a function of cell concentration rather than of the duration of exposure of TPA.

A plot of the level of 2ar mRNA versus cell density (Fig. 3B) shows that the second wave of induction occurs at the point at which the control cells have reached a plateau density. In the presence of TPA, however, the cells divide one more time, and it is at this point that there is a substantial (10×) increase in the abundance of 2ar mRNA. The appearance of 2ar mRNA cannot be attributed to its having a function in

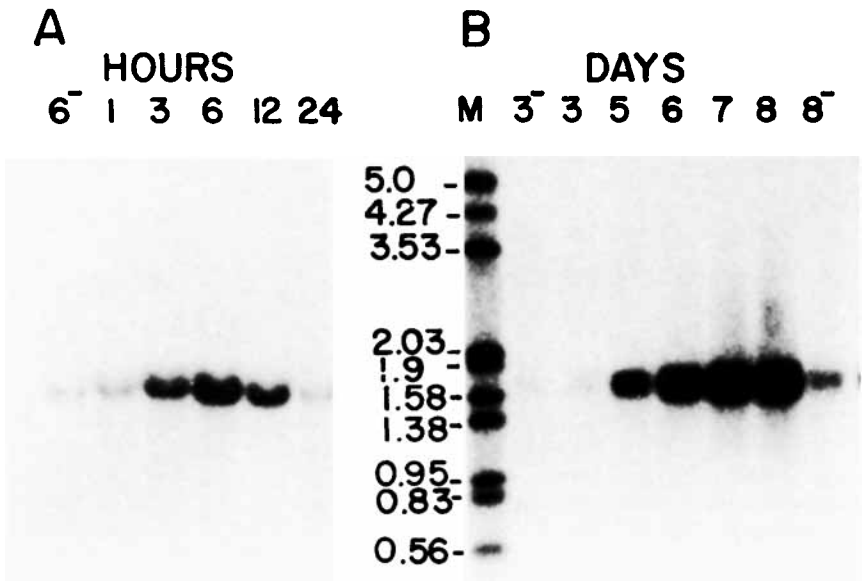


Fig. 2. Northern blot analysis of total cytoplasmic RNA (10 $\mu$g) from cells treated for various times with TPA (10 ng/ml) and probed with nick-translated 2ar. **A:** Cells were seeded at 1 $\times$ $10^6$ cells/150-mm plate, incubated 18 h, exposed to TPA for the hours indicated, and then harvested; RNA from cells exposed to DMSO alone for 6 h is shown in the lane labeled 6−. **B:** Cells were seeded at 3.5 $\times$ $10^4$ cells/150-mm plate, incubated for 24 h, and then treated with TPA. Cells from individual plates were harvested after the indicated number of days of TPA exposure; 3− and 8− are DMSO controls. The markers (denatured) are from a *Hind* III/*Eco*RI digest of λ DNA.

this round of replication only, as the level remains high even after the cells have reached a new plateau density and are no longer replicating. Induction at plateau density was also observed with C130 cells, which become confluent at a lower cell density (data not shown).

In order to ascertain the level of control of 2ar expression, nuclear "run-on" transcription analysis was carried out on isolated nuclei from untreated and TPA-treated C122 cells. Figure 4 shows that the transcription of the 2ar gene is enhanced after treatment with TPA for 4 h at a subconfluent cell density. Unfortunately the pSS24 vector alone, perhaps because of the presence of SV40 sequences, gives a positive, though clearly weaker, response. Induction is blocked by the inclusion of $\alpha$-amanitin in the in vitro assay. This is evidence that 2ar mRNA is an RNA polymerase II transcript. The MGAP (mouse glyceraldehyde phosphate dehydrogenase) and 18S rRNA clones are controls for a noninducible RNA polymerase II transcript and an $\alpha$-amanitin-resistant RNA polymerase I transcript, respectively.

### Induction of 2ar by Growth Factors in Quiescent Fibroblasts

The striking induction of 2ar mRNA in confluent cells and the growth factor-like properties of TPA led us to examine the possibility that this mRNA might be induced during the $G_0/G_1$ transition when quiescent cells are stimulated with either serum or growth factors that induce competence.

Swiss 3T3 fibroblasts were made quiescent in 0.5% serum for several days and then stimulated with fresh medium containing 10% fetal bovine serum. Subsequent to

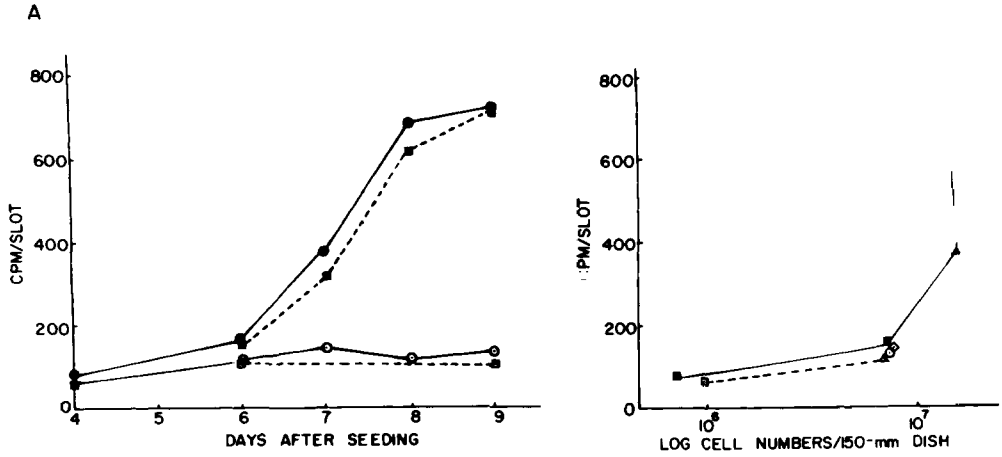


Fig. 3. Induction of 2ar as a function of TPA exposure and cell density. Cells were seeded at 3.5 × $10^4$ cells/150-mm plate. One set of plates (circles) was left for 24 h and then exposed to TPA (10 ng/ml, solid symbols) or the DMSO solvent (0.01%, open symbols) for various times. The second set (squares) was left for 72 h before being treated. Total cytoplasmic RNA was prepared from plates from each set at the indicated times. The RNA was applied to the nitrocellulose (10 μg/slot), and the resulting blot was probed with 2ar. The hybridized probe was then quantified by scintillation counting as described in "Materials and Methods." **A** shows the amount of $^{32}$P-labeled 2ar DNA bound to each "slot blot" as a function of the number of days after seeding for each set of cells. **B** is a plot of $^{32}$P-labeled 2ar DNA bound to each "slot blot" versus the $\log_{10}$ of the cell number/150-mm plate for that set of cells treated 24 h after seeding. The solid triangle, circle, and square in this plot represent the cultures at 7, 8, and 9 days after seeding.

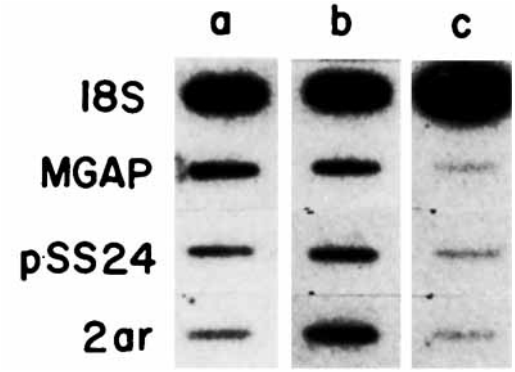


Fig. 4. Transcriptional analysis of the 2ar gene(s) in isolated JB6 C122 nuclei. Nuclei were prepared and the "run-on" transcription done as described in the "Materials and Methods." The filters with the indicated immobilized plasmid DNAs were hybridized with approximately 2 × $10^6$ cpm/filter. MGAP is a cDNA clone in pBR322 corresponding to mouse glyceraldehyde 3-phosphate dehydrogenase mRNA, whose level is not influenced by growth factors [23]. pSS24 is the vector carrying 2ar. The columns are as follows: **a,** nuclei from control cells exposed only to DMSO; **b,** nuclei from cells exposed to TPA for 4 h; **c,** nuclei from cells exposed to TPA for 4 h and then allowed to generate run-on transcripts in the presence of 2 μg/ml α-amanitin.

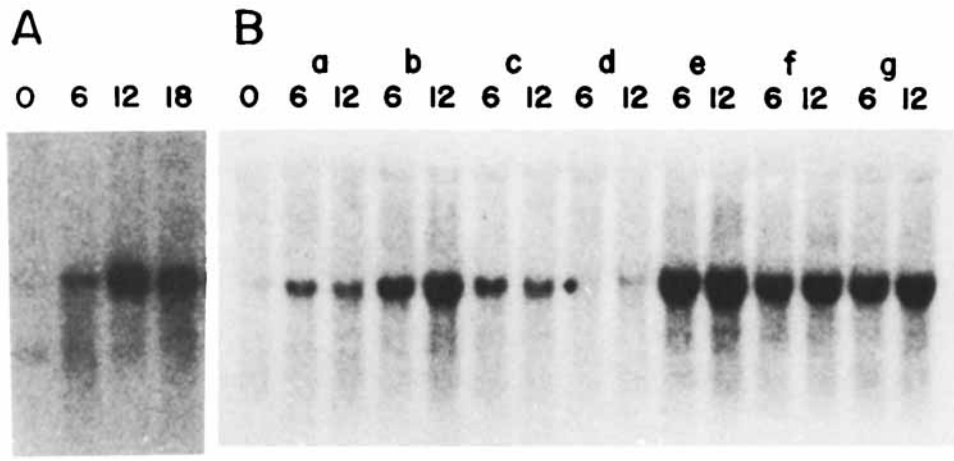


Fig. 5. A Northern blot analysis of total cytoplasmic RNA from Swiss 3T3 cells rendered quiescent and then stimulated with either 10% serum or various combinations of polypeptide growth factors for the duration indicated. The blot was probed with nick-translated 2ar. **A** shows the relative level of 2ar mRNA in quiescent cells and in cells at 6, 12, and 18 h after stimulation with 10% serum. **B** shows the induction of 2ar in quiescent 3T3 cells by treating for either 6 or 12 h with the indicated inducer(s). **Lane 0**, no induction; **a,** 10% serum; **b,** PDGF; **c,** EGF; **d,** SomC; **e,** PDGF + EGF; **f,** PDGF + SomC; **g,** PDGF + EGF + SomC. The concentrations of growth factors were as follows: PDGF, 10 ng/ml; EGF, 20 ng/ml; SomC, 26 ng/ml.

stimulation a substantial induction of 2ar mRNA levels occurred (Fig. 5A). The enhancement of 2ar mRNA levels in quiescent Swiss 3T3 cells with individual growth factors, or combinations of growth factors, is shown in Figure 5B. PDGF is a more potent inducer than serum. EGF, a poor inducer alone, has an additive effect with PDGF, whereas somatomedin C has, if anything, a negative effect on induction. It appears that 2ar belongs in the class of mRNAs that are induced when cells are made competent. Protein synthesis is required for its induction (data not shown).

## DISCUSSION

We have isolated and partly characterized a cDNA clone of a mouse 1.6-kb mRNA that does not correspond to a known protein on the basis of the sequence information we have been able to analyze so far. Also, it does not appear identical with other serum- and growth factor-inducible mRNAs that have been described [reviewed in 28]. It is inducible by TPA and other compounds (teleocidin and aplysiatoxin) with promoting activity but not by compounds lacking promoting activity (phorbol-monoacetate and ethylphenyl propiolate) (Smith and Denhardt, unpublished); these two properties correspond roughly with the ability, or lack thereof, to activate protein kinase C. However, since 2ar RNA is induced in both promotable and nonpromotable cells it clearly does not distinguish the two phenotypes.

A particularly interesting feature of this mRNA is the contrast between the transient nature of its induction in proliferating cultures and the permanent expression seen in confluent, nonproliferating cultures. We are not aware of another cellular message with this property, and it will be interesting to elucidate its molecular basis. In the subconfluent cultures, peak mRNA levels were reached about 6 h after the

addition of TPA. Protein synthesis was required, in contrast to many of the PDGF-inducible "competence mRNAs" whose induction was not inhibited, but was rather enhanced, by cycloheximide [28]. The cytoplasmic abundance of the 2ar mRNA declined slowly with an apparent half-life of roughly 6 h even though functional TPA was present in the medium. When the cells approached confluence, the cytoplasmic abundance again increased. We do not know whether this regulation is transcriptional, post-transcriptional, or at the level of mRNA turnover. In the confluent cultures in the presence of TPA the level of 2ar mRNA remained high for at least a week.

Other proteins that have been found to be expressed in a density-dependent fashion include bovine aortic smooth muscle collagenase [29] and the cytokeratins. Ben Ze'ev [30] observed that sparse monolayer cultures of Madin Darby bovine epithelial cells synthesized low levels of cytokeratins, whereas in dense cultures they were made at high levels. There was a good correlation between cytokeratin synthesis and the amount of translatable RNA, as assessed by in vitro translation in a reticulocyte lysate. We infer from hybridization analyses at moderately low stringency (single band on "Northern" blots of electrophoretically fractional mRNA) and from partial DNA sequence information (no evidence of similarities to keratins) that 2ar does not correspond to a characterized cytokeratin. Neither its characteristics nor its behaviour during induction resemble the keratin proteins studied by Toftgard et al [31] in TPA-treated mouse skin. We note, however, that keratin expression in vivo differs from that seen in cultured cells [32].

The relevance of this mRNA, if any, to tumor promotion remains to be determined. The fact that its synthesis is induced by TPA in dense, contact-inhibited cells of epithelial origin (JB6) and in serum-limited fibroblasts (3T3) is compatible with the possibility that it is relevant. To approach question more directly it will be necessary to ascertain whether the gene is expressed in mouse skin in vivo in response to TPA treatment, and, if so, whether suppression of its expression, for example with antisense RNA, affects tumor promotion.

## ACKNOWLEDGMENTS

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## NOTE ADDED IN PROOF

Comparison of the DNA sequence of 2ar with the sequence published for rat osteopontin (Oldberg A, Franzen A, Hinegard D: Proc Natl Acad Sci USA 83:8819-8823, 1986) reveals that 2ar is the murine equivalent.