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Constitutive synthesis of activator protein 1 transcription factor after viral transformation of mouse fibroblasts

(growth control/cAMP/tumor promoter/NIH 3T3 cells/*jun* oncogene)

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ABSTRACT **Transcription factor activator protein 1 (AP1) interacts with the promoter region of a number of genes that are stimulated by growth factors present in serum or by agents such as phorbol 12-myristate 13-acetate (PMA) that partially mimic their action. To investigate the possible role of AP1 in the control of cellular growth and in transformation of mammalian cells, we monitored its activity by binding to a specific DNA probe in normal or transformed mouse NIH 3T3 cells. A 3- to 4-fold increase in the DNA-binding activity was found after serum stimulation of quiescent NIH 3T3 cells. A 2- to 3-fold activation was found after treatment with PMA or dibutyryl-cAMP, suggesting that different signal-transducing pathways could activate AP1 factor in these cells. PMA stimulation was dependent on new protein synthesis. In contrast to normal cells, a high serum-independent AP1 DNA-binding activity was found in NIH 3T3 cells transformed by simian virus 40. These results suggest that constitutive AP1 synthesis may be a crucial step in cellular transformation.**

Mouse 3T3 fibroblasts provide a model system for the study of growth control and transformation of mammalian cells. In the absence of serum growth factors the cells become quiescent in the G_0 phase of the cell cycle. Addition of fresh serum or defined mitogens leads to resumption of DNA synthesis and cell division. Ample evidence indicates that transformed cells lose at least partially the dependence on growth factors (1).

It is generally assumed that stimulation of quiescent cells by growth factors ultimately results in the activation of specific transcription factors, which could in turn activate the genes involved in the many aspects of cellular proliferation (2). This stimulation would occur through signal-transducing pathways, such as the protein kinase C pathway, which can be artificially stimulated by the phorbol ester phorbol 12-myristate 13-acetate (PMA) (3). Another pathway involves activation of protein kinase A by an increase in intracellular cAMP concentration. cAMP levels can be artificially increased by addition of N^6,$O^{2'}$-dibutyryl-cAMP (Bt_2cAMP) to the medium, by addition of forskolin (an activator of adenylate cyclase), or by addition of 3-isobutyl-1-methylxanthine (iBuMeXan; an inhibitor of the phosphodiesterase) (4).

Transcription of nuclear protooncogenes, such as c-*fos* or c-*myc*, is transiently activated after growth stimulation of quiescent NIH 3T3 cells, and this activation does not require *de novo* protein synthesis (5). Thus, a first step in growth stimulation could involve post-translational modification, perhaps phosphorylation, of preexisting transcription factors such as the serum-responsive factor (SRF) that interact with the c-*fos* or β-actin promoter (6–8). These factors could activate the transcription not only of c-*fos* or c-*myc* but of a whole class of immediate early or competence genes, some of them encoding factors needed for the expression of delayed early genes. The expression of this second class of genes, which could include still another group of transcription factors, would precede DNA synthesis (9).

The transcription factor activator protein 1 (AP1), recently characterized in HeLa cell extracts, may play a role in growth control. AP1 interacts with the enhancer of the human metallothionein gene and that of simian virus 40 (SV40) (10). The mouse counterpart of this protein, which we have previously termed PEA1 (polyoma enhancer A binding protein 1), interacts with the polyomavirus, SV40, and c-*fos* gene enhancers (11). AP1 or PEA1 sites were recently found in the transcription control sequences of many genes that are stimulated by serum, growth factors, or oncogenic transformation (refs. 12 and 13; S. Saragosti and J.P., unpublished data). A minimal AP1 binding site was shown to enhance transcription from a heterologous promoter *in vitro* and to confer PMA responsiveness to a heterologous promoter *in vivo* (12, 13). As PEA1 and AP1 probably belong to the same family of factors, we will deal in the following text with AP1 exclusively, designating a factor or family of factors with the sequence specificity of AP1.

The AP1 site present in the α domain of the polyomavirus enhancer is crucial both for replication and early promoter activity of this virus (J.P. and W. R. Folk, unpublished data). Moreover, the absence of activity of the polyomavirus A enhancer in embryonal carcinoma cells has been correlated with the absence of detectable DNA-binding activity of AP1 in these cells (14). Cotransfection of polyomavirus with v-Ha-*ras* activates the polyomavirus enhancer in embryonal carcinoma and myeloma cells; the same effect is also obtained in myeloma cells after PMA treatment (15).

To investigate the possible role of AP1 in growth control of 3T3 fibroblasts we have analyzed its DNA binding activity in different growth conditions in normal or transformed NIH 3T3 cells. We show here that AP1 activity is low in quiescent 3T3 cells and that it is stimulated by the addition of serum, PMA, or cAMP or by viral transformation. The stimulation of its activity in normal cells requires *de novo* protein synthesis.

MATERIALS AND METHODS

Cell Culture. NIH 3T3 or SV40-transformed NIH 3T3 cells [SVTT1, a subclone of the SVLTR I cell line (16)] were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum. For the stimulation experiments, the cells were grown to confluence and then incubated for another 2 days in 0.5% serum. Stimulation took place by replacing the medium with medium containing the agents

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Abbreviations: Bt_2cAMP, $N^6O^{2'}$-dibutyryl-cAMP; iBuMeXan, 3-isobutyl-1-methyl-xanthine; AP1, activator protein 1; NF-I, nuclear factor I; PMA, phorbol 12-myristate 13-acetate; SV40, simian virus 40.

indicated in the legend of Fig. 2. Cells were collected after 2 hr of stimulation.

Preparation of Cell Extracts. Nuclear extracts were prepared as described in ref. 17. Briefly, the cells were rinsed twice with phosphate-buffered saline and kept on ice. The content of 20 dishes (9 cm in diameter) was resuspended in 5 ml of buffer A (10 mM Hepes, pH 8/50 mM NaCl/0.5 M sucrose/1 mM EDTA/0.5 mM spermidine/0.15 mM spermine/0.5% Triton X-100/1 mM phenylmethylsulfonyl fluoride/7 mM 2-mercaptoethanol). The cells were disrupted in a Dounce homogenizer by 20 strokes with an A pestle, and the nuclei were collected by centrifugation for 10 min at 1000 × *g*. The nuclear pellet was rinsed twice with 5 ml of buffer A and resuspended in 2 ml of buffer B containing 100 mM NaCl [buffer B is 10 mM Hepes, pH 8/25% (vol/vol) glycerol/0.1 mM EDTA/0.5 mM spermidine/0.15 mM spermine/1 mM phenylmethylsulfonyl fluoride/7 mM 2-mercaptoethanol]. After 15 min of gentle agitation the nuclei were centrifuged at 1000 × *g* and resuspended in 2 ml of buffer B containing 500 mM NaCl. After 30 min of gentle agitation, the nuclei were centrifuged at 2000 × *g* and the supernatant was brought to 45% saturation with $(NH_4)_2SO_4$. Proteins were precipitated by centrifugation for 30 min at 10,000 × *g*. The pellet was resuspended in 100 μl of buffer B containing 100 mM NaCl.

Whole cell extracts were prepared as described in ref. 18.

Gel Retardation Experiments. Gel retardation experiments were performed as described in ref. 11. The amounts of protein indicated in the figures were incubated on ice with 1.0 μg of poly(dI-dC)•poly(dI-dC) in 10 mM Hepes, pH 8/17.5% (vol/vol) glycerol/0.1 mM EDTA/20 mM NaCl/4 mM $MgCl_2$/2 mM dithiothreitol/4 mM spermidine in a total volume of 20 μl. After 10 min approximately 0.025 pmol of 3′-^{32}P-labeled double-stranded oligonucleotide was added and the incubation was continued for another 10 min at 20°C. The reaction mix was immediately loaded on an 8% polyacrylamide gel and subjected to electrophoresis at 160 V in 0.25× TBE (1× TBE is 89 mM Tris/2.5 mM EDTA/89 mM boric acid). The gel was subsequently dried and autoradiographed. The retarded bands were cut out and their radioactivities were measured to give an estimation of the amount of probe bound by the protein extract.

RESULTS

Oligonucleotide Probe for Assay of Transcription Factor AP1. In the present work we wished to analyze the effect of serum or mediators of signal transduction on the DNA-binding activity of the transcription factor AP1 in NIH 3T3 cells. To do this we developed a sensitive assay to quantify AP1 in different cell extracts. We synthesized a double-stranded oligonucleotide that we named PK, which contains a short palindromic consensus sequence, CTGACTCAG, derived from known AP1 sites (refs. 10 and 11; Fig. 1 *a* and *b*). Since it is possible that base pairs adjacent to the consensus could have an influence on the strength of the DNA–protein interaction, we chose to include several base pairs present in the c-*fos* enhancer at both sides of the consensus (11). The presence in nuclear extracts of proteins binding to this radioactively labeled oligonucleotide was monitored by the gel retardation assay (refs. 19 and 20; Fig. 1*c*). We obtained with this probe a retardation pattern identical to that with the A2$^-$ oligonucleotide, which contains the AP1-binding site of polyomavirus (11). Moreover, binding to this A2$^-$ oligonucleotide is blocked by an excess of nonlabeled PK oligonucleotide. Similarly, binding to the PK probe is blocked by the A2$^-$ oligonucleotide but not by the A1$^-$ oligonucleotide, which contains a mutated AP1 site. No band in this position was obtained when A1$^-$ was used as probe (result not shown). Thus, we conclude not only that AP1 interacts with the PK probe but also that no other factor interacts in a stable fashion with this probe. However, the binding experiments cannot exclude that AP1 is a mixture of several related polypeptide chains that recognize the same target sequence.

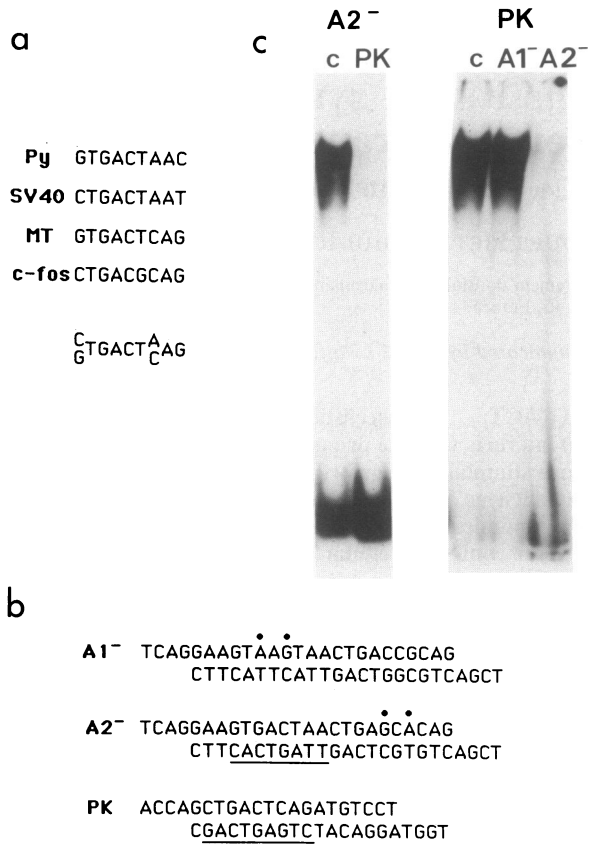

FIG. 1. Construction of an AP1-specific probe. (*a*) AP1-binding sites of the polyomavirus (11), SV40 (10, 11), c-*fos* gene (11), and human metallothionein IIa gene (10) enhancers are shown as well as the derived consensus sequence. (*b*) Oligonucleotides used in the gel retardation experiments. The AP1-binding site is underlined. The mutations that destroy the AP1-binding site in A1$^-$ and the PEA2-binding site in A2$^-$ are indicated by dots (PEA2 is polyoma enhancer A binding protein 2) (11). The 5′ protruding ends were chosen to facilitate subsequent cloning or fill-in labeling. (*c*) Competition experiments. The A2$^-$ oligonucleotide was used as labeled probe in the first two lanes, the PK oligonucleotide in the next three lanes. A 100-fold excess of unlabeled PK, A1$^-$, or A2$^-$ oligonucleotide was added during the preincubation where indicated; c indicates control without competitor. In each lane 3T6 nuclear extract was used at 0.4 μg/ml.

Effect of Serum, PMA, or cAMP on AP1 Activity. Nuclear extracts were prepared from quiescent NIH 3T3 or from the same cells stimulated for 2 hr with 15% serum, with PMA at 100 ng/ml, which activates protein kinase C (21), or with 1.0 mM Bt_2cAMP in the presence of 0.5 mM iBuMeXan. iBuMeXan increases intracellular cAMP by inhibiting the phosphodiesterase that degrades cAMP (4). AP1 activity was measured in gel retardation assays by binding to the PK probe. The amount of retarded probe is a good indication for the concentration of active factor, as this amount varied linearly with the protein concentration in the conditions of probe excess used here (results not shown). The specificity of the interaction was checked for each extract by absence of binding to the A1$^-$ probe (results not shown). A low activity was detected in quiescent NIH 3T3 cells (Fig. 2*b*, lane a). A 3- to 4-fold increase in the amount of retarded probe was reproducibly observed after serum stimulation (lane b). A weaker 2- to 3-fold stimulation was observed after PMA

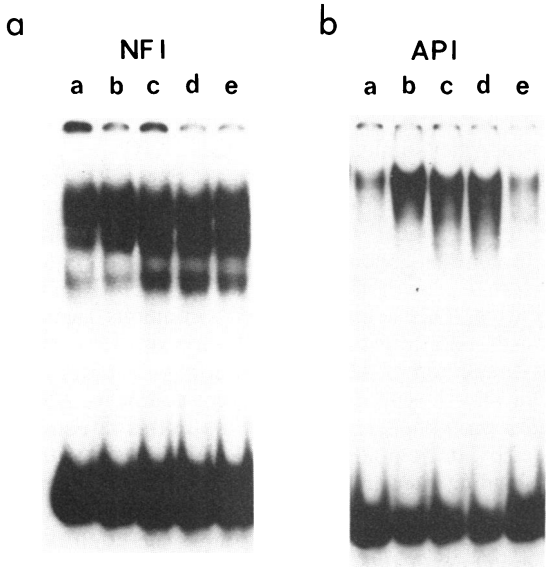

FIG. 2. Serum, cAMP, and PMA effect on AP1 activity. The stimulation experiments, extract preparation, and gel retardation experiments were performed as described in *Materials and Methods*. The nuclear factor I (NF-I) probe was used in *a* and the PK probe in *b*. NF-I contains the NF-I-binding site of adenovirus—i.e., nucleotides 20 to 49. Extract was used at 0.2 mg/ml for the NF-I probe and at 0.4 mg/ml for the PK probe. Extracts were from serum-starved cells in lanes a, from cells stimulated with 15% newborn calf serum in lanes b, from cells stimulated with 1 mM Bt_2cAMP and 0.5 mM iBuMeXan in lanes c, from cells stimulated with PMA at 100 ng/ml in lanes d, and from cells stimulated with PMA at 100 ng/ml in the presence of 10 μM anisomycin in lane e.

treatment (lane d) or Bt_2cAMP plus iBuMeXan treatment (lane c). AP1 activation is not merely the result of a preferential nuclear localization of the factor after stimulation of quiescent cells, since we found the same increase in activity when whole cell extracts were analyzed (results not shown). Stimulation of transcription factors as revealed by increased DNA binding is not general after mitogenic treatment; indeed, only a marginal increase in the activity of NF-I as detected by gel retardation was observed in the different nuclear extracts (Fig. 2*a*). NF-I is a ubiquitous factor involved in the transcription and replication of a number of genes and viruses (22). Some variation was noticed in the relative intensity of the different bands constituting the NF-I retardation pattern; however, the overall amount of retarded oligonucleotide was nearly identical in the different samples of one experiment. The stimulation values for AP1 binding given in Table 1 were corrected for these small variations. We conclude that AP1 DNA-binding activity is rapidly stimulated after serum treatment of quiescent NIH 3T3 cells. This activation is partially reproduced by agents stimulating either the protein kinase C pathway (PMA) or the protein kinase A pathway (Bt_2cAMP and iBuMeXan).

Effect of Inhibition of Protein Synthesis on AP1 Stimulation. Some early events after stimulation of quiescent cells, such as the induction of transcription of the so-called competence genes such as c-*fos* or c-*myc*, can occur in the absence of protein synthesis (5). It has been assumed that modification of preexisting transcription factors could play a role in this induction. To determine if AP1 belongs to this class of factors, we repeated the stimulation experiments in the presence of the protein synthesis inhibitors anisomycin or cycloheximide. As shown in Fig. 2*b*, lane e, the presence of anisomycin abolished the increase in DNA-binding activity upon PMA addition. Cycloheximide had the same effect (results not shown). We conclude, therefore, that in at least one step of the activation process in NIH 3T3 cells, *de novo* synthesis of proteins is required. This can be either the synthesis of new AP1 molecules or the synthesis of a modifying enzyme that will activate preexisting inactive AP1 molecules. These results are in contrast with those obtained in HepG2 or HeLa tk$^-$ cells, in which AP1 activation was not sensitive to translational inhibitors (refs. 12 and 13; see *Discussion*).

Table 1. Quantitative measure of the relative increase in the AP1 DNA-binding activity

Cells	Additions to medium	Activity relative to serum-starved NIH 3T3		Activity ratio, AP1/NF-I
		AP1	NF-I	
NIH 3T3	15% serum	3.4	1.2	2.8
	Bt_2cAMP + iBuMeXan	2.8	1.5	1.9
	PMA	2.6	1.5	1.7
	PMA + anisomycin	0.9	1.4	0.6
SVTT1	0.5% serum	2.2	1.1	2.0
	15% serum	2.5	1.1	2.3

To quantify the increase in AP1 activity, the gel areas corresponding to the retarded band of the experiments given in Figs. 2 and 3 were excised and the radioactivity retained was measured in a scintillation counter. The values obtained with the NIH 3T3 or SVTT1 cells were divided by the value obtained with serum-starved NIH 3T3 cells. These numbers are given in the third column for the AP1 probe and in the fourth column for the NF-I probe. The last column gives the increase in AP1 activity normalized to the changes observed for NF-I activity—i.e., the AP1 values are divided by the NF-I values.

Constitutive AP1 Activity in Transformed NIH 3T3 Cells. If the level of AP1 transcription factor plays an important role in the regulation of cell proliferation, the question arises as to whether cellular transformation will modify the regulation of its synthesis. In other words, do transformed NIH 3T3 cells display a high, serum-independent, level of AP1-binding activity? To answer this question, we prepared nuclear extracts from NIH 3T3 cells stably transformed by SV40 that were serum starved (0.5% serum) for 2 days, or stimulated by 15% serum for 2 additional hr. As shown in Fig. 3 and Table 1, AP1 activity is high in transformed cells, and this high level is not strongly decreased in conditions of serum starvation. No difference was observed in NF-I activity between serum-starved NIH 3T3 cells and virally transformed cells either in the absence or in the presence of serum.

DISCUSSION

AP1 is involved in the transcriptional regulation of a large number of cellular genes, many of which are subject to control by growth or transformation (10–13). The experiments described here deal with the regulation of AP1 activity. We show that the DNA-binding activity of the AP1 factor is increased in quiescent NIH 3T3 cells by serum mitogens. On the other hand, the concentration of transcription factor NF-I, as detected by DNA binding, is almost independent of serum concentration, suggesting that the increase in AP1 transcription factor is directly correlated with increased gene activity. The effect of serum on AP1 activity can be partially mimicked by phorbol esters or by an increase in intracellular cAMP. Although PMA and cAMP have different effects on cellular metabolism, they work synergistically to promote cellular division in Swiss 3T3 cells (23). Phorbol esters are believed to activate protein kinase C, while cAMP probably activates a different class of protein kinases—e.g., protein kinase A (3). Both classes of kinases may be involved in stimulating AP1 activity. Alternatively, PMA could increase intracellular cAMP by an unknown mechanism. It should be

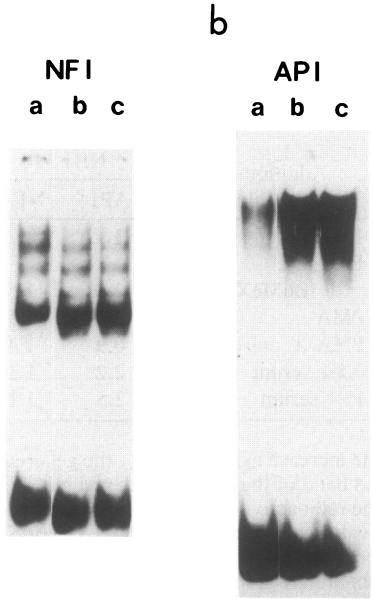

FIG. 3. Constitutive AP1-binding activity in transformed NIH 3T3 cells. The NF-I probe was used in *a* and the PK probe in *b*. Each time extract was used at 0.5 mg/ml. Extracts from serum-starved NIH 3T3 cells were used in lanes a, from serum-starved SVTT1 cells in lanes b, and from serum-stimulated SVTT1 cells in lanes c.

stressed, however, that we cannot exclude that two different factors with the same sequence specificity are induced by PMA and cAMP, respectively. It was shown recently that another transcription factor, activator protein 2, can be activated by either PMA treatment or increase in intracellular cAMP (24).

The block of AP1 activation by translational inhibitors seems to argue against activation by direct modification of a preexisting factor as suggested for NFκB transcription factor activation in pre-B cells (25). Early translation-independent events after serum stimulation, like c-*fos* induction, may be a prerequisite for AP1 activation after *de novo* protein synthesis. The partial overlap of the AP1 site with the serum-responsive element mapped by Treisman in the c-*fos* promoter suggests that AP1 may participate in a later step of c-*fos* transcriptional regulation (11, 26). AP1 may also be involved in the delayed activation of genes such as that for ornithine decarboxylase, an activation that requires new protein synthesis (27). In contrast to our observations with NIH 3T3 cells, AP1 activation by PMA was not sensitive to translational inhibitors in human HeLa tk^- or HepG2 cells (12, 13). AP1 synthesis may be already constitutive in these cells, which were originally derived from tumors. The DNA-binding activity of AP1 would then be modulated by post-translational modifications. On the other hand, the regulation in NIH 3T3 cells could occur at the transcriptional level or at the level of RNA stability or processing. However, we cannot exclude that mitogen induction in NIH 3T3 cells involved covalent modification in addition to *de novo* synthesis.

Since AP1 activity seems to be directly related to cellular proliferation it is of interest to monitor its activity in transformed cells. We showed that in SV40-transformed NIH 3T3 cells this activity is constitutively higher than in NIH 3T3 cells and is independent of serum concentration. Even in the presence of 0.5% serum in the growth medium these cells contain almost the same level of AP1 DNA-binding activity as normal NIH 3T3 cells stimulated with 15% serum. Cellular transformation is a complex multistep process, and a constitutive expression of AP1 may represent one of the crucial requirements for cellular transformation (28). The fact that PMA or cAMP by itself does not induce fibroblasts to divide suggests also either that an increased AP1 level by itself is not decisive and requires the collaboration of other factors to induce cell division or that a threshold level of AP1 is necessary, a level that can be reached only by a cooperation between both signalling pathways. Indeed serum activation leads to a higher stimulation than either cAMP or PMA alone.

The results presented here suggest that AP1 could be a competence factor for cellular division. Its intracellular concentration appears to be regulated by mitogens in normal cells. In contrast, oncogenes that alleviate the requirement for external growth factors would render AP1 synthesis constitutive. This could occur by continuous signal transduction in cells transformed by *ras* or *src*, or perhaps by direct stimulation of AP1 transcription with tumor (T) antigen. One may thus predict that the gene coding for AP1 factor is itself a protooncogene. Some hints in this direction were provided by the homology of the AP1-binding site to the yeast GCN4-binding site, and the existence of an oncogene, *jun*, displaying a striking sequence homology to the GCN4 DNA-binding domain (29–31). This hypothesis was confirmed after the completion of this work by Bohmann *et al.* (32), who could show that the major component in AP1 factor purified from HeLa cells is identical to the product of the human protooncogene c-*jun*.

Finally, the recent studies suggest that the AP1 factor defined biochemically by its ability to bind to a certain number of enhancers and promoters and to stimulate transcription may include several related polypeptide chains. The factor purified from HeLa cells by oligonucleotide affinity chromatography contains a group of polypeptides ranging in size from 40 to 47 kDa (12, 13). Mouse AP1 factor that we purified by the same approach contains two major and several minor related polypeptide chains (unpublished observations). In addition to the mouse c-*jun* gene product, it may include a polypeptide corresponding to a cDNA clone isolated from serum-stimulated BALB/c 3T3 cells by Ryder *et al.* (33), since this cDNA shares extensive sequence homology with the DNA-binding domain of c-*jun*. The gene coding for this polypeptide, named *jun-B*, is distinct from the mouse c-*jun* gene (33). Further experiments with nucleic acid probes or with antibodies specific for the different members of this family will be required to examine whether all or only a subclass of these polypeptides are induced by serum, PMA, or cAMP or are synthesized constitutively in transformed cells. It will be important to establish the precise role of the different members of this family in the control of expression of the different genes that contain AP1 sites in their regulatory regions.

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