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Transcription Factor Sp1 Is Important for Retinoic Acid-Induced Expression of the Tissue Plasminogen Activator Gene during F9 Teratocarcinoma Cell Differentiation

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The induced differentiation of F9 cells by retinoic acid (RA) and cyclic AMP (cAMP) activated transcription of the tissue plasminogen activator (t-PA) gene. This differentiation-responsive regulation of the t-PA promoter was also observed in transient assays. Multiple sequence elements within 243 bp of t-PA DNA contributed to the high level of transcription in retinoic acid- and cyclic AMP-differentiated cells. To investigate the factors involved in controlling t-PA transcription upon differentiation, we used F9 cell extracts to examine proteins that bind two proximal promoter elements. These elements (boxes 4 and 5) are homologous to GC boxes that are known binding sites for transcription factor Sp1. Mobility shift assays in the presence and absence of anti-Sp1 antibodies demonstrated that the proteins which bound to this region were immunologically related to human Sp1. The proteins also had a DNA-binding specificity similar to that of a truncated form of Sp1. Mutations of the GC motif within boxes 4 and 5 that interfered with Sp1 binding reduced in parallel the binding of the F9 cellular factors and lowered transcription in vitro as well as in vivo. Although this proximal region of the t-PA promoter was active in vivo only in differentiated cells, the Sp1-like binding proteins were present in equal concentrations and had similar properties in extracts of both stem and differentiated cells. These data suggest that other cellular elements participate with this Sp1-like factor in controlling differentiation-specific expression.

The F9 teratocarcinoma cell system is a useful model for investigating mechanisms controlling mammalian differentiation and development. F9 cells, which biochemically are similar to inner cell mass cells of the early mouse embryo (41, 61), can be maintained as a homogeneous population of stem cells in vitro (3). Treatment of F9 cells with retinoic acid (RA) causes their differentiation into a tissue type that resembles extraembryonic primitive endoderm of the embryo (64). Like authentic primitive endoderm (13), RA-differentiated F9 cells are bipotential: elevation of the intracellular levels of cyclic AMP (cAMP) produces parietal endoderm (63), whereas aggregation leads to the formation of visceral endoderm (24). This system therefore has characteristics that make it attractive for the study of gene regulation during mammalian embryogenesis (62).

Tissue plasminogen activator (t-PA) is one gene product that is not expressed in F9 stem cells but becomes induced upon differentiation with RA and further increased by cAMP (40, 63). With both inducers, regulation of t-PA expression occurs in part at the transcriptional level (53). Other genes that are induced upon differentiation in a similar two-stage manner have been identified in F9 cells (36, 42, 69). Thus t-PA is a member of a set of genes activated in response to RA- and cAMP-induced differentiation. Since RA influences gene transcription by mechanisms similar to those of steroid hormones (17, 50), it is possible that some of these genes are direct targets for the RA receptor (68). Alternatively, the transcriptional stimulation of these genes may be a secondary event effected by a product directly induced by the RA receptor.

To define elements that control the expression of the t-PA gene in response to differentiation, we have previously isolated the 5′-flanking sequences. Using a series of 5′-3′ deletion mutants, it was shown that multiple elements contribute to the expression of the t-PA promoter in RA- and cAMP-differentiated F9 (RA/cAMP-F9) cells. The elements necessary for the full level of expression in transient assays reside within 243 bp of t-PA sequences (mutant designated Δ11). Further deletions demonstrated that a promoter containing only 107 bp of t-PA sequences exhibited a decreased level of expression relative to that observed for Δ11, but differentiation-specific expression was still maintained. This mutant (Δ16) is of interest because an additional deletion of 64 bp almost completely abolished expression (54). These analyses established that a very small region of the promoter was sufficient for regulated transcription.

Here we document that a factor which binds to this region recognizes two GC-box motifs directly upstream from the start sites of transcription. Several lines of evidence suggest that the protein is closely related to the human transcription factor Sp1 and is likely to be its murine homolog. Although factor binding is required for regulated expression in vivo, the factor is present in apparently equal concentrations in both F9 and RA/cAMP-F9 cells. Therefore, the differentiation-specific transcription of the t-PA gene in the F9 system probably involves an Sp1-like factor in conjunction with as yet undefined cellular elements.

MATERIALS AND METHODS

Extract preparation. F9 cells were cultured and differentiated as described elsewhere (A. L. Darrow, R. J. Rickles, and S. Strickland, Methods Enzymol., in press). Whole-cell

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extracts were prepared by the method of Manley et al. (39). Nuclear extracts were obtained as described previously (51), with the following modifications. Cells were lysed by vortexing in buffer A containing 0.4% Nonidet P-40 instead of Triton X-100 (49). Proteins were extracted by gentle agitation of the nuclear suspension in buffer B containing 400 mM NaCl, with the 100 mM NaCl extraction omitted. Preparations always included the following protease and phosphatase inhibitors: 2.5 kallikrein inhibitor units of aprotinin per ml, 1 mM phenylmethysulfonylfluoride, 20 μg of soybean trypsin inhibitor per ml, 2.5 mM benzamidine, 1 μg each of leupeptin, antipain, chymostatin, and pepstatin per ml, 1 mM $NaMoO_4$, and 5 mM NaF. For both extract preparation methods, proteins were concentrated by ammonium sulfate precipitation and suspended in Manley dialysis buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol, and 2.0 mM dithiothreitol. Preparations were then dialyzed on ice with two changes for 2 to 3 h each against at least 200 volumes of Manley dialysis buffer containing the foregoing inhibitors at a 10-fold dilution. Extracts were stored under liquid nitrogen, and protein concentrations were determined by a dye-binding assay (Bio-Rad Laboratories).

Plasmids and transfections. The fragment containing the simian virus (SV40) 21-bp repeats was obtained from plasmid B50, containing the 763-bp *Taq*I-*Kpn*I fragment of SV40. A 59-bp *Nco*I-*Fok*I subfragment corresponding to nucleotides 38 to 97 (66) was isolated, flush ended by using T4 DNA polymerase, and cloned into the *Sma*I site of pBS. The tetrameric AP-2-binding site fragment was constructed by ligating synthetic double-stranded oligonucleotides having the sequence for the high-affinity AP-2 site of the human metallothionein II_A promoter (27). After a ligation reaction, multimerized oligonucleotides were flush ended and cloned into the *Sma*I site of plasmid pβe$^-$ (48). Plasmid sequencing (6) was used to establish that four tandem copies of the AP-2-binding site were present. These two competitor fragments were preparatively isolated from their plasmids by digestion with *Eco*RI and *Bam*HI.

Procedures for calcium phosphate transfections, RNase protections, and the construction of the BAL 31-generated Δ11, Δ16, Δ17, and Δ18 TAMGAL series were as described previously (54). The *Hin*dIII-*Pst*I fragment of Δ16 was subcloned into the mp19 vector, and boxes 4 and 5 were mutagenized by the method of Kunkel (35). Mutations were confirmed by sequence analysis (56). Altered Δ16 promoter fragments were then cloned upstream of the *Escherichia coli* β-galactosidase gene (GAL) in pUCGALpA (54).

Plasmid Δ11 ψTAMGAL was made by digesting Δ11 TAMGAL with *Pst*I and subjecting the linearized fragments to BAL 31 exonuclease (International Biotechnology Inc.) for various lengths of time. After the ends were polished with T4 DNA polymerase, *Pst*I linkers were ligated onto the blunt-ended fragments. A series of truncated *Pst*I-*Kpn*I fragments was generated by digesting the ligated products with *Pst*I and *Kpn*I. These deleted inserts were size fractionated by electrophoresis, purified, and subcloned back into the *Pst*I-*Kpn*I vector fragment of Δ11 TAMGAL. Plasmid sequencing indicated that Δ11 ψTAMGAL contains an internal deletion of 36 bp at the TAM (tissue activator mouse)-GAL gene fusion.

DNase footprinting. Footprint analyses were performed as described by Jones et al. (28). The truncated t-PA promoter insert of Δ16 TAMGAL was end labeled at the upstream *Hin*dIII site by a fill-in reaction using Klenow fragment and [α-^{32}P]dATP (55). The Δ16 probe was then obtained by digestion with *Pst*I, followed by electrophoresis on a 2.0% low-melting-temperature agarose gel (Bethesda Research Laboratories) and purification by Elutip (Schleicher & Schuell) chromatography. Probes isolated by this method were extracted with phenol-chloroform (1:1), followed by chloroform extraction and precipitation in ethanol. The initial footprint probe, containing Δ11 sequences, was first isolated as a 284-bp *Stu*I-*Pst*I fragment and cloned into the *Sma*I-*Pst*I-cleaved vector of plasmid pBS (Stratagene). This construct was linearized with *Hin*dIII, ^{32}P end labeled, and purified as described above.

Band shift assay. The gel electrophoresis DNA-binding assays were done as described by Carthew et al. (5). Probes used were ^{32}P labeled at one end as described above, or the purified inserts were radiolabeled with Klenow fragment and [α-^{32}P]dATP. Protein samples were added to 5 to 10 fmol of probe (10^4 cpm) in the presence of 1.0 μg of double-stranded poly(dI-dC) in 12% glycerol–12 mM HEPES (pH 7.9)–60 mM KCl–5 mM $MgCl_2$–0.6 mM EDTA–0.6 mM dithiothreitol to a total volume of 15 μl. Binding reaction mixtures were incubated at room temperature for 20 to 30 min, followed by the addition of 5 μl of 4× gel shift dye containing 4× TBE, 20% glycerol, 4 mM dithiothreitol, and 0.1% bromophenol blue. Samples were then loaded onto a running 4% polyacrylamide (30:1) gel and electrophoresed at 20 to 30 mA in 1× TBE (55) at 4°C. Gels were applied to Whatman 3MM paper, dried, and autoradiographed.

DNA fragments used in competitions were excised from plasmids and purified by low-melting-temperature agarose electrophoresis and Elutip chromatography. Competitors were combined with probe before the addition of protein extract. Antibody band shift perturbation experiments (33) were performed as described above except that Sp1 antiserum (2892-E) or preimmune serum (2892-pI) was added. 2892-E is a rabbit polyclonal antiserum raised against *E. coli*-synthesized human Sp1. No difference was observed when antiserum was preincubated with extract before being combined with probe (data not shown). The amino-terminal deletion mutant of human Sp1 protein (Sp1-168C) used in these studies was purified by affinity chromatography. Sp1-168C contains 11 NH_2-terminal amino acids encoded by the *lacZ* gene of pUC118 fused to the COOH-terminal 168 amino acids of Sp1 (29).

Interference binding analysis of protein-DNA contacts. Contact point analysis of the lower strand was carried out by using the Δ16 footprint probe partially methylated with dimethyl sulfate (59). The probe used to examine contacts on the upper strand was made by subcloning the *Hin*dIII-*Pst*I Δ16 fragment into pBS. This plasmid was then cleaved with *Xba*I and labeled with Klenow fragment by using [α-^{32}P] dATP, and the end-labeled insert was obtained by a *Hin*dIII digest, followed by isolation as described for other probes. The upper-strand probe was then partially modified with diethyl pyrocarbonate (DEPC) as described previously (65).

Protein-DNA binding reactions were scaled up 10-fold except that the amount of chemically modified probe was increased by 50-fold (5×10^5 cpm). After preparative electrophoresis, the shifted complexes and unbound probe were excised, eluted, and piperidine treated as described previously (2). Equal amounts of recovered radioactivity were electrophoresed on 8 M urea–12% polyacrylamide (19:1) gels. Samples of both chemically modified probes subjected to piperidine treatment directly were run beside the gel-isolated samples. In addition, the products of purine or pyrimidine strand scission (43) were included for sequence

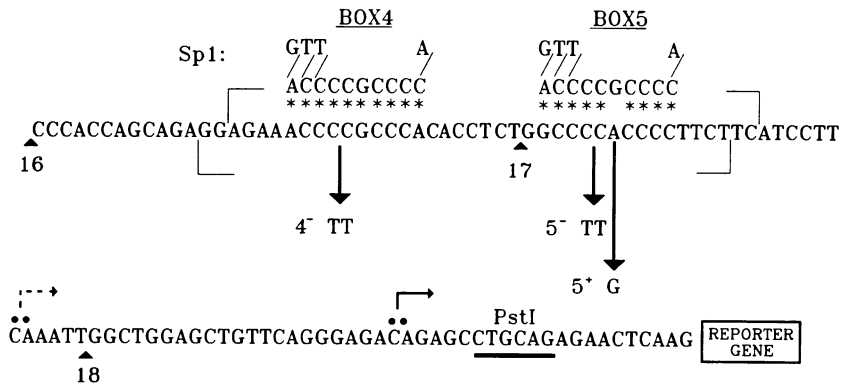


FIG. 1. Salient features of the Δ16 promoter region. The t-PA sequence is shown from the BAL 31-generated Δ16 5′ endpoint, where a *Hin*dIII linker was added (not indicated). The 3′ end of the sequence terminates at a flush-ended *Sac*I site, where the chloramphenicol acetyltransferase or GAL reporter gene is fused. The *Pst*I restriction site illustrated is used in combination with the 5′ *Hin*dIII site to excise the insert. Brackets indicate the region of protection from DNase I digestion as determined by footprint analysis of the upper (⌈⌉) and lower (⌊⌋) strands. Boxes 4 and 5 are labeled, and their homologies to the GC-box consensus sequence binding site of transcription factor Sp1 are shown (30). Changes in the nucleotide sequence introduced by site-directed mutagenesis are indicated, as is the designation of the mutant. Also shown are the 5′ endpoints of two other deletions within this region (Δ17 and Δ18). The two predominant in vivo start sites mapped previously (54) are indicated by angled arrows. Only the proximal start site (solid arrow) initiates transcripts in vitro (Fig. 5).

ladders. After electrophoresis, gels were autoradiographed at −70°C with intensifying screens.

In vitro transcription assays. Transcription reactions were performed as described by Heberlein et al. (21), with a few modifications. Supercoiled DNA templates were incubated in cell extracts at 30°C for 1 h in a total volume of 25 μl. Reactions were supplemented with 10 U of RNAguard (Pharmacia) and 10 mM creatine phosphate. Transcriptions were terminated with 275 μl of stop buffer containing 0.5% sodium dodecyl sulfate, 10 mM EDTA, 300 mM NaCl, 100 mM Tris hydrochloride (pH 7.5), and 70 μg of tRNA per ml and were phenol-chloroform extracted twice. Nucleic acids were precipitated with ethanol two times, rinsed with 70% ethanol, and dried by vacuum. Primer extensions were performed (44) by using a GAL-specific primer in order to detect TAM-GAL RNA transcripts synthesized in vitro. Extended products were fractionated by electrophoresis on an 8 M urea–6% polyacrylamide (19:1) gel and visualized by autoradiography.

RESULTS

Factor(s) binding to GC boxes in the t-PA promoter. To identify protein factors binding to the t-PA promoter, the Δ11 fragment was subjected to DNase I footprint analysis (12). Binding activities were observed over two distal sequence elements (data not shown). One, located at −175 to −168 nucleotides relative to the distal start site, has homology to the cAMP response element. The other, positioned at −148 to −135, matches the CTF/NF-I consensus sequence (54). A large footprint was also observed at −45 to −8, overlapping two sequence motifs previously termed boxes 4 and 5 or GC boxes (54). Protection from DNase I over these regions was detected by using extracts prepared from both F9 stem and RA/cAMP-F9 cells (A. Darrow, unpublished results). The t-PA sequence and location of the DNase footprint over boxes 4 and 5 within the Δ16 region are displayed in Fig. 1.

To investigate the nature of the binding activity to the two GC boxes, we resorted to the more sensitive mobility shift assay (11, 14). The ^{32}P-labeled Δ16 insert was incubated with increasing amounts of whole-cell extract from either F9 stem or differentiated cells and then subjected to electrophoresis. The mobility of the probe in the gel was retarded by increasing amounts of extract (Fig. 2). The shifted material migrated as three predominant bands and a fourth weaker, lower band. As observed in the DNase footprint assay, binding activities were equivalent in extracts prepared from both cell types. In addition, the patterns of the shifted probe were identical whether F9 stem or RA/cAMP-F9 extracts were analyzed. Similar results were obtained when nuclear extracts were used (data not shown).

We performed a series of competitions in an attempt to determine the specificity of the shifted complexes (Fig. 3). Competition with a 50-fold molar excess of unlabeled Δ16 insert reduced the amount of shifted material. Complete competition was observed when a 500-fold excess of this homologous fragment was used (data not shown).

Because of the similarity of the sequences within boxes 4 and 5 to those of GC boxes (30), a portion of the SV40 21-bp repeats was used as a competitor. This fragment contained five GC boxes, a subset of which interacts with transcription factor Sp1 (16). When incubated with the radiolabeled Δ16 probe, the GC-box-containing fragment efficiently competed at a 200-fold molar excess.

Since transcription factor AP-2 is also capable of binding to the SV40 21-bp repeats (45), we performed competitions by using a DNA fragment containing four tandem copies of an AP-2-binding site (27). No alterations in the complexity of the shifted patterns resulted at even a 1,000-fold molar excess of cold competitor. This result indicates that the factors responsible for the complexes do not recognize a high-affinity binding site for AP-2.

Finally, to determine whether protein-DNA interactions occurring in more proximal regions of the promoter could be generating any of the complexes, a subfragment of Δ16 that lacks boxes 4 and 5 was used in competitions. This 28-bp insert, termed Δ18 (Fig. 1), failed to compete with the Δ16 probe for the formation of the shifted bands, suggesting that these complexes form as a consequence of interactions restricted to the box 4 and 5 footprinted region. As observed earlier, no significant difference was seen when extracts prepared from the F9 or RA/cAMP-F9 cells were analyzed.

Mutagenesis of boxes 4 and 5 affects both binding and transcription. The foregoing data suggested that the murine

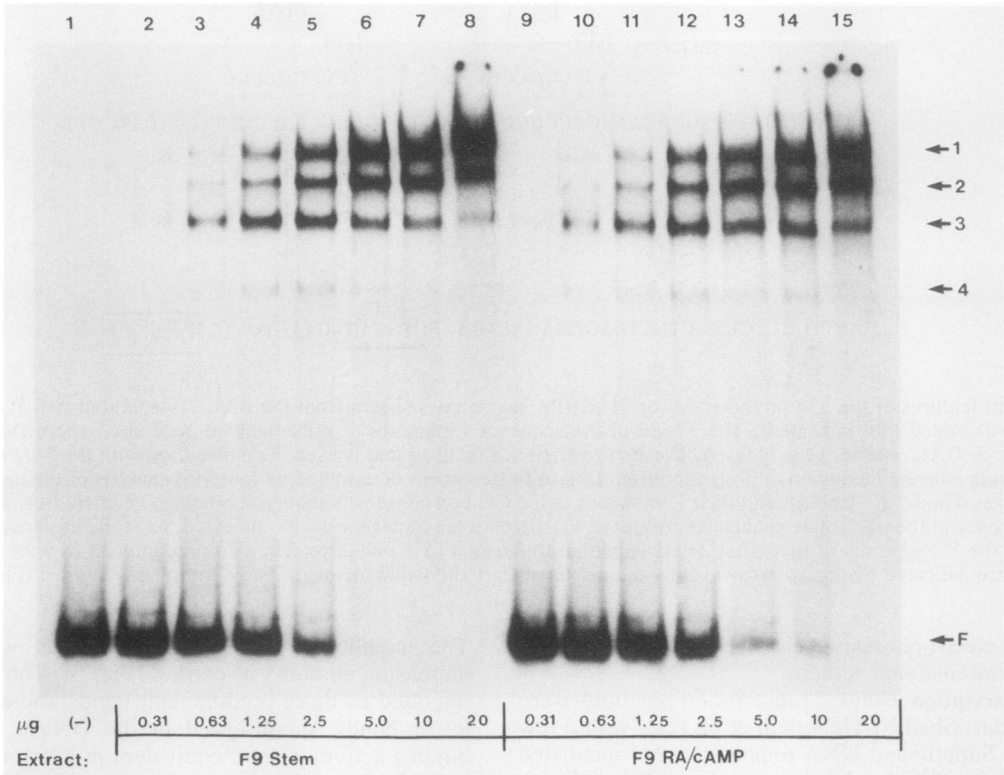


FIG. 2. Band shift analysis of the box 4 and 5 region. The 94-bp Δ16 insert was ^{32}P labeled and incubated in the absence of extract (lane 1) or in the presence of increasing amounts of whole-cell extract prepared from either F9 stem (lanes 2 to 8) or RA/cAMP-F9 (lanes 9 to 15) cells before electrophoresis. The positions of free probe (F) and complexes (1 to 4) are shown on the right.

homolog of the transcription factor Sp1 might be involved in binding to a critical region of the t-PA promoter. To investigate the role of this binding in promoter activity, we performed site-specific mutagenesis of boxes 4 and 5 to determine whether correlations exist between protein binding and the transcriptional capacity of the promoter fragment. The mutations (Fig. 1) were designed to investigate the possibility that transcription factor Sp1 binds to boxes 4 and 5, since the residues within GC boxes that are contacted by Sp1 have been established (15, 25). To disrupt the GC motif in each box, two neighboring cytidine residues were changed to thymidines in box 4 (mutant 4^-), in box 5 (mutant 5^-), or in both boxes (double mutant $4/5^-$). The sequence within box 4 corresponds perfectly to the GC box consensus, whereas the box 5 homology deviates at a single residue (Fig. 1). Thus, a neutral mutant termed 5^+ was constructed in box 5 to give this element a perfect homology to an Sp1 site by replacing the central adenine with a guanine.

The effects of these mutations on protein binding were examined by using the mobility shift assay (Fig. 4). The neutral mutation 5^+ had no effect on protein binding. Mutations in either box (4^- or 5^-) that altered the GC motif abolished the two higher bands (complexes 1 and 2; Fig. 2) seen with the wild-type promoter. No complexes were observed with the double mutant $4/5^-$, suggesting that all of the shifted bands are dependent on at least one functional GC box. Again, no difference in the pattern of the mutant promoters was observed when the extracts used were derived from stem or differentiated cells. Thus, it is likely that the stem cell factors interacting with boxes 4 and 5 have binding specificities identical to those in differentiated extracts.

We used cell extracts from F9 and RA/cAMP-F9 cells to analyze transcription in vitro from the t-PA promoter and the effects of the mutations in boxes 4 and 5 on this transcription. The mutants were again compared with the wild-type Δ16 promoter and two 5′-3′ deletions within this region (Δ17 and Δ18; Fig. 1). For these experiments, the promoters were inserted upstream of GAL (20). The supercoiled templates were added to nuclear extracts obtained from either F9 stem or RA/cAMP-F9 cells, and the transcripts synthesized in vitro were then analyzed by primer extension. A reference construct, Δ11 ψTAMGAL, which gave rise to a product that could be readily distinguished from those of the test constructs, was included in each fraction for normalization. The in vitro transcriptional analysis indicated that although the TAM promoter is regulated in vivo, regulation was not maintained in vitro (Fig. 5), since t-PA gene transcription was observed when stem cell extracts were used. Furthermore, the effect of the various mutations on the in vitro transcription mirrored what was observed for protein binding in that the 5^+ mutation had no effect, the 4^- and 5^- mutants had decreased activity, and the $4/5^-$ double mutant had essentially no activity.

To determine the correlation of binding with transcriptional activity in vivo, expression of the mutant promoters was analyzed in transient transfection assays. The site-directed mutants of box 4 and 5 were tested along with the wild-type Δ16 promoter as well as the two deletion mutants Δ17 and Δ18 in RA/cAMP-F9 cells. A reference plasmid containing the human α-globin gene (67) was cotransfected with each TAM-GAL construct to normalize for differences in transfection efficiency. The levels of accurately initiated TAM-GAL and α-globin transcripts, generated from the

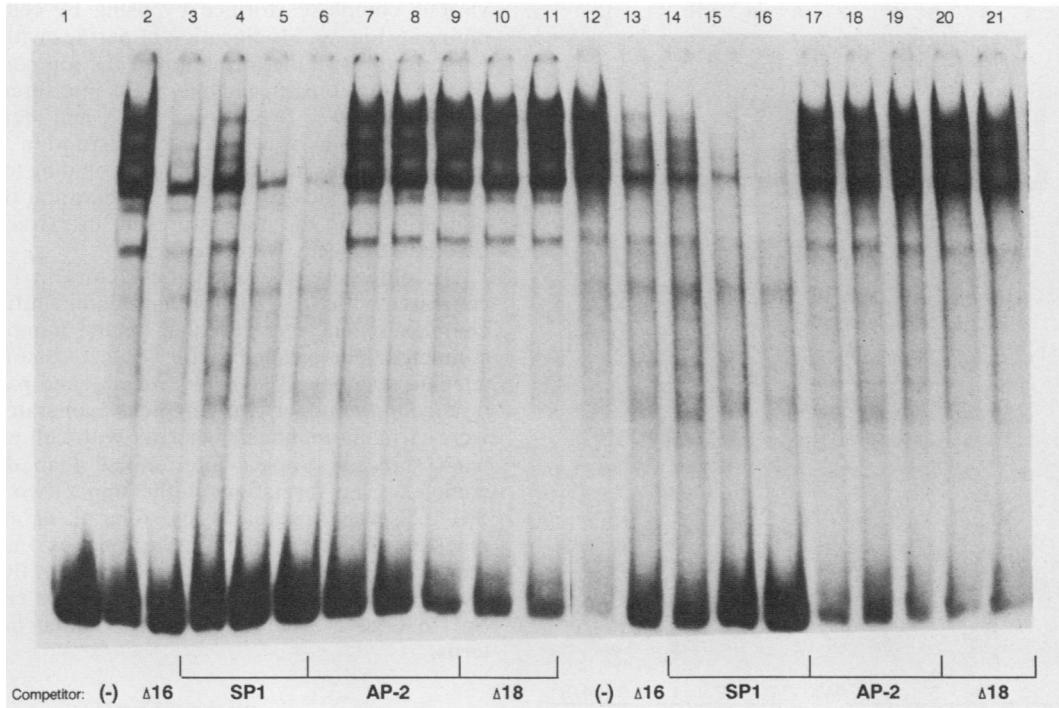


FIG. 3. Competition analysis of the box 4 and 5 band shift. The Δ16 ^{32}P-labeled probe was incubated in the absence of extract (lane 1) or in the presence of 7.5 μg of whole-cell extract from either F9 stem (lanes 2 to 11) or RA/cAMP-F9 (lanes 12 to 21) cells before electrophoresis. Binding reactions performed in the absence of specific competitor DNA (lanes 2 and 12) are indicated as (−). The unlabeled DNA fragments, coincubated with probe, that were used as cold competitors were as follows: a 50-fold molar excess of the Δ16 insert (Δ16) (lanes 3 and 13); a 10 (lanes 4 and 14), 50 (lanes 5 and 15), and 200 (lanes 6 and 16) molar excess of a DNA fragment containing the SV40 21-bp repeats (SP1); a 50 (lanes 7 and 17), 200 (lanes 8 and 18), and 1,000-fold (lanes 9 and 19) molar excess of a tetramerized oligonucleotide having the sequence of a high-affinity AP-2-binding site (AP-2); and a 500 (lanes 10 and 20) and 1,000 (lanes 11 and 21) molar excess of the Δ18 insert fragment (Δ18).

transfected constructs, were then determined by RNase protection.

The wild-type Δ16 construct generated TAM-GAL transcripts in differentiated cells that were not detected in stem cells transfected in parallel (Fig. 6). This finding indicates that in transient assays the Δ16 promoter is differentially regulated, as shown previously (54). Truncations within this DNA that remove box 4 (Δ17) or both boxes 4 and 5 (Δ18) reduced the level of transcription in vivo. Expression of the box 4 and 5 site-directed mutants correlated with protein binding in that the 5^+ mutant transcribed to a similar extent as the wild-type Δ16 promoter. The mutations that affected protein binding (4^-, 5^-, and $4/5^-$) dramatically lowered the level of transcription in differentiated cells. Thus, the combined results from the site-directed mutants paralleled what was observed with the 5′ deletion mutants and further support the possibility that an Sp1-like factor is involved in t-PA transcription.

The factor(s) binding box 4 and 5 is related to Sp1. The observation that proteins interacting with Δ16 have binding specificities similar to that of Sp1 led us to examine whether the factor is the murine homolog of Sp1. To address this question, we used polyclonal antibodies directed against purified human Sp1 in a mobility shift perturbation assay. The Δ16 probe was incubated with F9 cell extracts in the presence or absence of Sp1-specific antiserum before electrophoresis. Shifts produced by both F9 stem and RA/cAMP-F9 extracts were perturbed by Sp1-specific antiserum but not by preimmune serum (Fig. 7). Control shifts were done by using a radiolabeled probe containing the adenovirus E1A enhancer site A (4), which lacks GC motifs (data not shown). With use of this probe, the pattern of shifted complexes was unaltered in the presence of Sp1 antiserum, indicating that the perturbation of the Δ16 complex is a specific effect.

We also investigated whether an affinity-purified preparation of Sp1 would bind to the Δ16 promoter. For these experiments, we used a truncated form of human Sp1 containing the carboxyl-terminal 168 amino acids expressed in *E. coli* (Sp1-168C) (8, 29). A comparison between the wild-type Δ16 mobility shift obtained by using F9 whole-cell extracts and increasing amounts of Sp1-168C shows that in both cases, the probe became complexed as four bands of retarded mobility (Fig. 8). Mobility shifts of the box 4 and 5 mutant probes with use of Sp1-168C also looked strikingly similar to the patterns generated by the factor present in the cell extracts (compare lanes 10 to 13 of Fig. 8 with the corresponding lanes of Fig. 4). As in the case of the crude extracts, mutants 4^- and 5^- reduced the pattern of four complexes down to two, and mutant $4/5^-$ was not shifted.

To determine whether a correlation exists between the four bands generated by cell extracts and those formed from purified Sp1-168C, we carried out binding interference assays (59). This technique relies on the fact that modification of bases which form contacts with a bound protein disfavors complex formation. Probe molecules altered at these nucleotides are thus underrepresented in the shifted complexes relative to unbound DNA. To map the points of factor-DNA contact, the Δ16 promoter fragment was ^{32}P end labeled and chemically modified. Dimethyl sulfate, which methylates the N-7 of guanines present in the major groove (43), was used to modify the probe labeled on the lower strand. Probe labeled

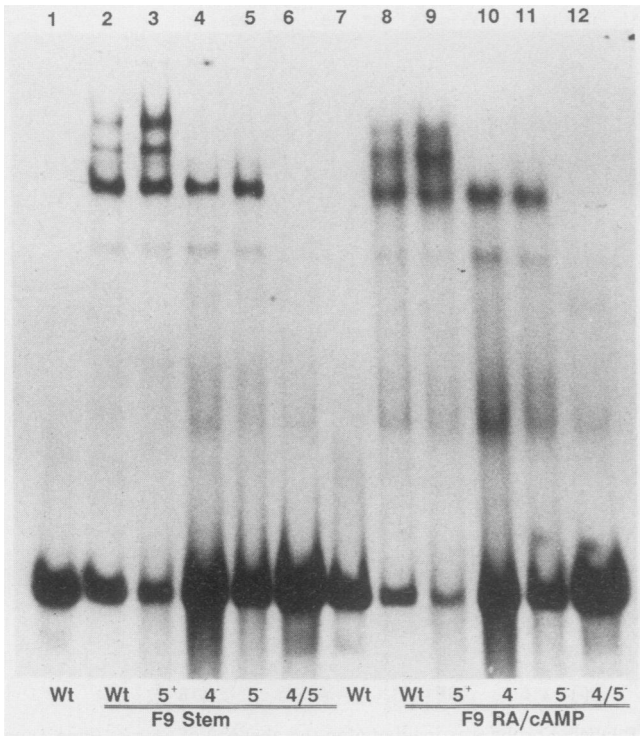


FIG. 4. Band shift analysis of the Δ16 box 4 and 5 mutants. The indicated probes were combined with 7.5 μg of whole-cell extract obtained from either F9 stem (lanes 2 to 6) or RA/cAMP-F9 (lanes 8 to 12) cells before electrophoresis. The position of wild-type probe (Wt) run in the absence of extract is also indicated (lanes 1 and 7).

on the upper strand was treated with DEPC, which modifies predominantly adenines as well as guanines by carbethoxylation (23). These partially modified fragments were then used as probes for interference analysis.

The analysis indicated that the contact points within the various complexes formed by using F9 cell extracts were different (Fig. 9). Modification of purines within boxes 4 and 5 interfered with the formation of the top complex the most (strong interference), and only weak interference was detectable in the lower two bands, which had identical points of contact. This may result if the disruption of one site by modification has no effect on factor binding to the other site. Most notable, however, is the observation that the contact points between the factors present in extracts of stem and differentiated cells were identical.

The differences between the factors in F9 cell extracts compared with Sp1-168C were predominantly in the top two complexes and were revealed by the number of guanines contacted. The purified factor contacted more lower-strand guanine residues. Also, the strengths and patterns of interference in the upper two complexes generated by Sp1-168C were virtually identical, whereas with cell extracts the top band showed stronger interference than did the second complex. The formation of the upper two complexes by Sp1-168C was apparently less tolerant of methylated guanines on the lower strand, in both boxes 4 and 5, than was their formation with the factors present in the cell extracts. When the upper strand was analyzed, the cell extracts and Sp1-168C gave essentially identical binding interference patterns.

DISCUSSION

The following evidence implicates the murine Sp1 homolog as one factor binding to the t-PA promoter: (i) the binding sites conform to the Sp1 consensus sequence; (ii) mutagenesis of nucleotides within the consensus sequences that are known to be important for Sp1 binding abolishes complex formation, whereas mutations predicted to be neutral have no effect; (iii) antibodies specific to human Sp1 perturb the mobility shifts; (iv) the contact points that the cellular factor makes with the putative GC boxes are those predicted from work with human Sp1; and (v) in comparison with the cellular factor, a truncated form of Sp1 expressed in *E. coli* binds to the same GC boxes in the t-PA promoter,

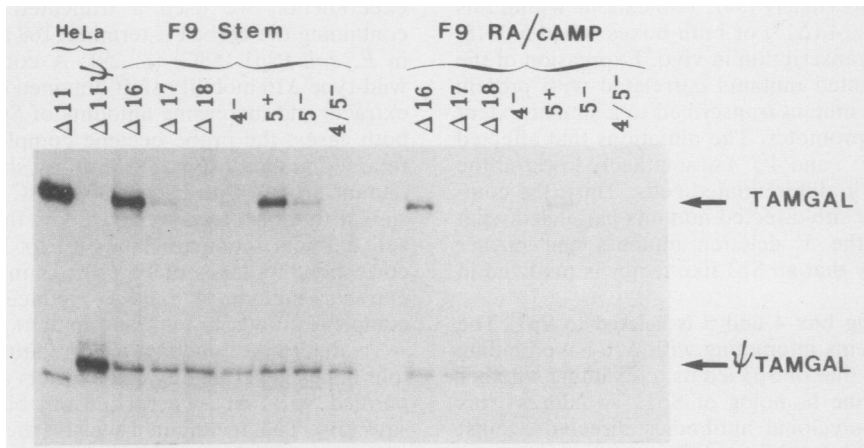


FIG. 5. Effects of box 4 and 5 mutations on transcription in vitro. In vitro transcription reactions were performed, and RNA was analyzed by primer extension. HeLa whole-cell extracts (125 μg) or nuclear extracts prepared from either F9 stem (60 μg) or RA/cAMP-F9 (60 μg) cells were used as indicated. The 5′ deletion clone Δ11 (450 ng) or Δ11 ψTAMGAL (Δ11ψ) (450 ng) was transcribed alone in HeLa extract as markers. For transcriptions performed in both types of F9 extracts, the internal control Δ11 ψTAMGAL (225 ng) was included with the indicated test templates (225 ng). Primer-extended products were resolved by electrophoresis and visualized by autoradiography. Positions of the extended products from both the test gene (TAM-GAL) and reference template (ψTAMGAL) are shown at the right. The 5′ ends of these extensions map to the proximal start site adenine residue of the t-PA gene. The smaller primer-extended product detected in the Δ11 HeLa lane was produced by a low level of transcripts initiating in the reporter gene sequences (L. Pecorino, unpublished results).

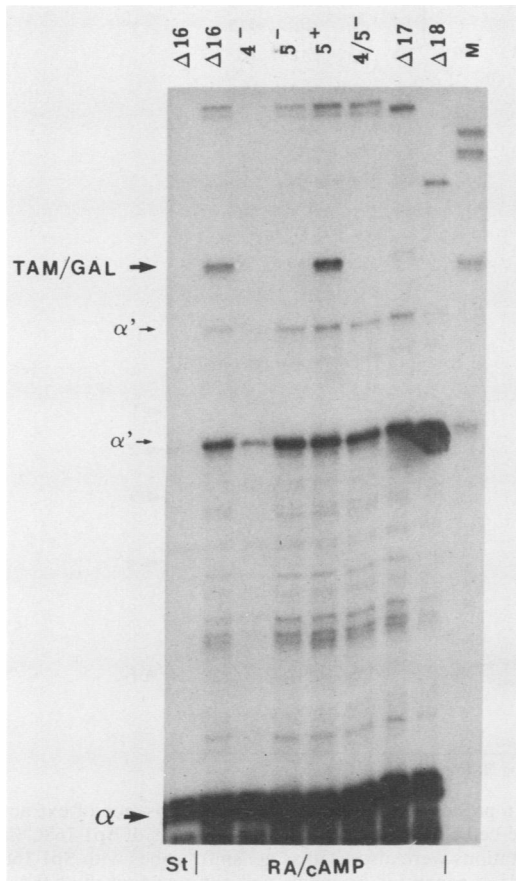


FIG. 6. Effects of mutations in boxes 4 and 5 on transcription in vivo. Shown is an autoradiogram of an RNase protection assay of 30 μg of total RNA isolated from either F9 stem (St) or differentiated (RA/cAMP) cells transfected with the indicated TAM-GAL constructs. Two uniformly ^{32}P-labeled antisense RNA probes were used in the analysis: a TAM-GAL probe and a human α-globin probe. Positions of RNase-resistant probe fragments protected by correctly initiated TAM-GAL and α-globin (α) transcripts are indicated on the left. Aberrant α-globin probe-specific bands (α') resulted from either improper initiation or splicing of α-globin gene transcripts. At this exposure, only TAM-GAL transcripts initiating at the proximal start site are observed. Lane M shows a subset of ØX174-*Hae*III size markers.

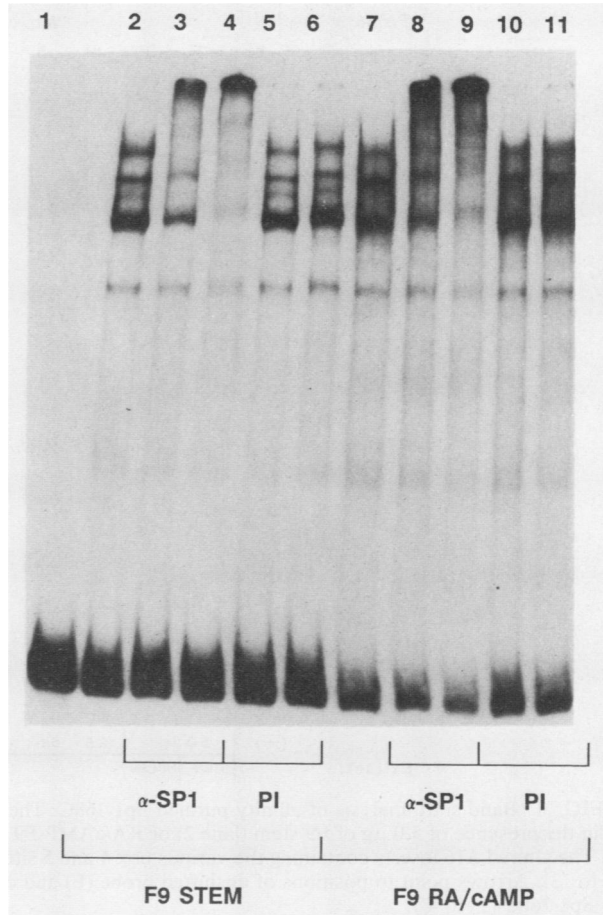


FIG. 7. Demonstration that the factor(s) interacting with boxes 4 and 5 is immunologically related to human Sp1. The Δ16 probe was incubated in the absence (lane 1) or presence of 7.5 μg of whole-cell extract from the indicated sources. Binding reactions were carried out in the presence of either serum directed against purified human Sp1 (α-SP1) or preimmune serum (PI). The amounts of sera added in each case were 0.5 μl (lanes 3, 5, 8, and 10) and 1.5 μl (lanes 4, 6, 9, and 11).

forms a similar pattern of complexes, is sensitive to the same mutations, and has similar contact points.

The exact identification of *trans*-acting factors is complicated, since several different isoforms of various transcription factors exist (19, 47, 57, 58) and it is known that particular DNA sequences may be occupied by factors having little similarity except for recognition specificity (10, 26, 45, 70). Therefore, it is possible that the F9 cellular factors which bind the GC boxes are not homologous to Sp1, although they are immunologically related. Competitions of the mobility shifts (Fig. 3) suggest that the factor AP-2 (45) is not involved. The interference data indicate that the F9 proteins do not contact the same GC box nucleotides as does transcription factor MTF-1 (70) or LSF (26). Transcription factor Zif268 binds to related G+C-rich sequences that are distinct from the actual GC-box consensus site (7). Although we present no evidence excluding the possible involvement of factor ETF (31) or GCF (32), the band shift perturbation experiment suggests that a protein immunologically related to Sp1 complexes with Δ16 (Fig. 7). It is not known whether this antiserum cross-reacts with these two other proteins (ETF and GCF).

The interference assays indicate that the F9 cell factors in crude extracts contact nucleotides similar to those contacted by the purified preparation of Sp1-168C, but the contact points are not identical. This difference might indicate that the F9 cell factors are separate and distinct from Sp1. There are, however, other plausible explanations: (i) a species divergence between the two polypeptides may create a slight difference in DNA-protein interaction, (ii) posttranslational modifications on the factors obtained from F9 cells not present on the bacterially expressed mutant protein may create subtle differences in nucleotide apposition, (iii) differences in protein contacts could occur as a consequence of comparing proteins in a crude extract with those in an affinity-purified preparation, and (iv) gross truncations in the Sp1 polypeptide (168 versus 778 amino acids) may alter the affinity of the protein for its cognate element. Evidence for this last possibility has been obtained (29), since amino-terminal deletions affect the salt sensitivity of the Sp1-DNA interaction.

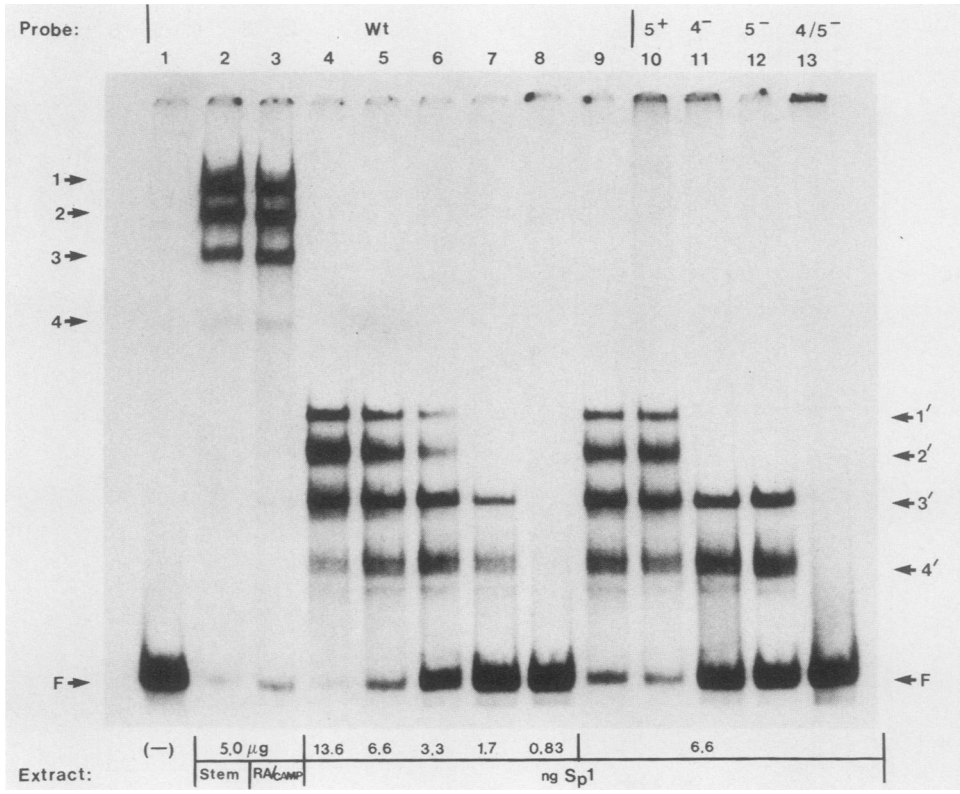
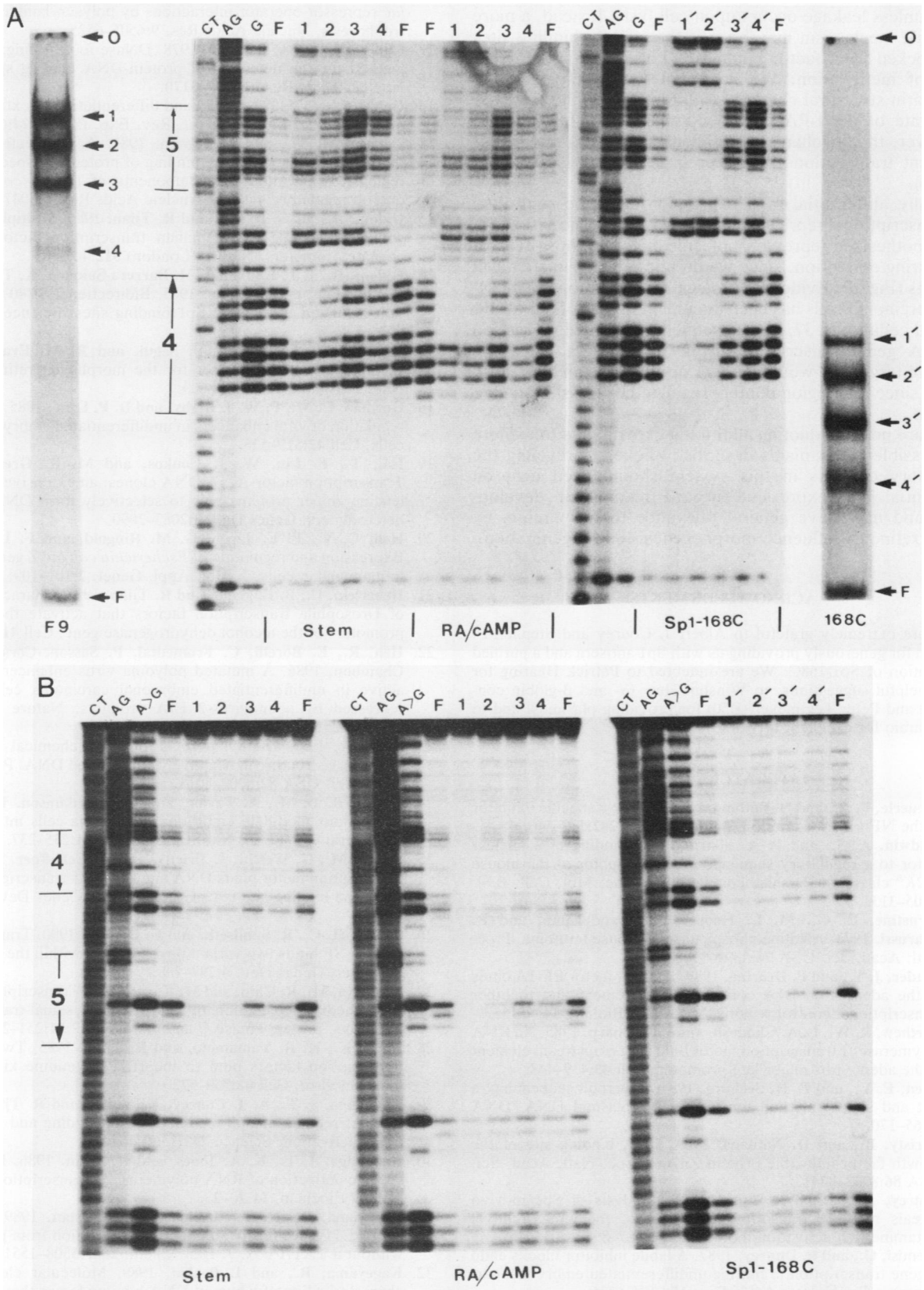


FIG. 8. Band shift analysis of affinity-purified Sp1-168C. The wild-type Δ16 probe (Wt) was incubated in the absence of extract (lane 1) or in the presence of 5.0 μg of F9 stem (lane 2) or RA/cAMP-F9 (lane 3) whole-cell extract or the indicated amounts of Sp1-168C (lanes 4 to 9). The altered Δ16 inserts containing the various box 4 and 5 site-directed mutations were also used as gel shift probes with Sp1-168C (lanes 10 to 13). Arrows point to positions of unshifted probe (F) and complexes (1 to 4) generated by whole-cell extracts as well as those formed by Sp1-168C (1′ to 4′).

Using whole-cell or nuclear extracts, we have detected proteins binding to the t-PA promoter cAMP response element, NF-I, and Sp1 consensus sequences. The results suggest that the factors are present in approximately equal abundance in extracts prepared from either F9 stem or RA/cAMP-F9 cells. This finding is in contrast to the binding activity of AP-1/PEA1, which is known to be induced upon the differentiation of F9 cells (34). We cannot exclude the possibility that an undetected binding activity is induced (or diminished) upon differentiation. We performed mobility shift experiments under a variety of conditions, and no significant differentiation-specific difference in the banding pattern was observed (data not shown). Thus, altering the binding conditions did not give rise to a differentiation-specific mobility shift. The inability to detect a particular binding activity could also be due to limiting amounts of the factor in our preparations. However, with these considerations in mind, it would appear that the differentiation-specific cues may not activate the t-PA gene by the induction of transcription factor binding. Instead, differential gene regulation might be achieved by other mechanisms. For example, the transcription factors that we detect in both stem and differentiated cell extracts might bind constitutively but become transcriptionally active in response to differentiation. Posttranslational modifications that can regulate transcriptional activity may be too subtle to detect in mobility shift assays.

Regulation upon differentiation could also be due to the inaccessibility of factors present in stem cells to the promoter elements in vivo. For example, upon induction, factors might be directed toward nuclear binding sites from a sequestered active or inactive form, as has been shown for other regulatory systems (1, 38, 52). Since we detect equivalent binding activities in nuclear extracts from both stem

FIG. 9. Contact point analysis of Δ16 shifted complexes. (A) The Δ16 probe, end labeled on the lower strand, was partially methylated and used for binding interference. Two representative band shifts obtained by using either F9 whole-cell extract (F9) or Sp1-168C (168C) are included to illustrate the relative mobilities of the shifted complexes (1 to 4 or 1′ to 4′) and free probe (F) from the origin (O) of the gel. Unbound probe (F) and DNA within complexes (1 to 4 and 1′ to 4′) was purified and reacted with piperidine, and the products were fractionated on a sequencing gel. Sequencing markers (AG and CT) were generated by partial cleavage of the end-labeled probe at purines and pyrimidines (43). Methylated probe not incubated with extract was subjected to cleavage with piperidine directly and electrophoresed in parallel (G). (B) The upper strand of Δ16 was end labeled, and the probe was treated with DEPC for binding interference as in panel A. A sample of the DEPC-modified probe was cleaved with piperidine directly (A>G) and analyzed in parallel with the free (F) and complexed samples (1 to 4 and 1′ to 4′) as well as sequence markers (CT and AG). The source of F9 extract (Stem or RA/cAMP) or purified Sp1-168C (Sp1-168C) used for each analysis is indicated. The locations of boxes 4 and 5 are shown in both panels.


A
CT AG G F 1 2 3 4 F F 1 2 3 4 F
CT AG G F 1´ 2´ 3´ 4´ F
0
1
2
3
4
F
5
4
1´
2´
3´
4´
F9
Stem
RA/cAMP
Sp1-168C
168C
B
CT AG A>G F 1 2 3 4 F
CT AG A>G F 1 2 3 4 F
CT AG A>G F 1´ 2´ 3´ 4´ F
4
5
Stem
RA/cAMP
Sp1-168C

and differentiated cells, compartmentalization seems unlikely unless leakage occurs upon cell lysis. Instead, a more plausible mechanism may be that the cellular binding sites are blocked from factor binding by chromatin structure or state of methylation. We have not yet investigated the chromatin structural changes or differences in the methylation state of the t-PA gene accompanying differentiation. However, this mechanism is difficult to reconcile with our transient transfection data wherein regulation is observed (Fig. 6).

Finally, differential t-PA expression could be controlled by transcriptional repression in F9 stem cells. The activity of the hypothesized repressor molecule might be short-lived or lost during extraction, since we do not detect it in stem cell extracts (Fig. 5). Evidence for negative regulation in embryonal carcinoma cells has been documented for both viral (18, 22) and cellular (9, 37, 46, 60) sequences. Should repression of t-PA gene transcription occur in F9 stem cells, the putative target site would lie over or downstream of boxes 4 and 5, since this region confers regulated expression in vivo (Fig. 6).

We are now conducting additional experiments to explore the possible mechanisms described above. Unraveling the regulatory controls in this system should shed light on differential gene expression in early mammalian development and may have general relevance to the circuits by which retinoids influence morphogenesis and regeneration.

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