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Expression of a 91-Kilodalton PEA3-Binding Protein Is Down-Regulated during Differentiation of F9 Embryonal Carcinoma Cells

M. E. MARTIN,* X. Y. YANG, AND W. R. FOLK

Department of Biochemistry, University of Missouri at Columbia, Columbia, Missouri 65212

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Proteins binding to the PEA3 enhancer motif (AGGAAG) activate the polyomavirus early promoter and help comprise the viral late mRNA initiator element (W. Yoo, M. E. Martin, and W. R. Folk, J. Virol. 65:5391–5400, 1991). Because many developmentally regulated cellular genes have PEA3 motifs near their promoter sequences, and because Ets family gene products activate the PEA3 motif, we have studied the expression of PEA3-binding proteins and Ets-related proteins during differentiation of F9 embryonal carcinoma cells. An ~91-kDa protein (PEA3-91) was identified in F9 cell nuclear extracts by UV cross-linking to a radiolabeled PEA3 oligonucleotide probe, and expression of PEA3-91 was down-regulated after differentiation of F9 cells to parietal endoderm. The c-*ets*-1 gene product binds to a sequence in the murine sarcoma virus long terminal repeat that is similar to the PEA3 motif (cGGAAG), but PEA3-91 was not cross-linked to this Ets-1-binding motif, nor did antiserum which recognizes murine c-*ets*-1 and c-*ets*-2 proteins have any effect on PEA3-binding activity in mobility shift assays. Furthermore, c-*ets*-1 mRNA was not detected in undifferentiated or differentiated F9 cells, and c-*ets*-2 mRNA levels remained high after differentiation. Antiserum against the *Drosophila* Ets-related E74A protein, however, recognized an ~92-kDa protein in F9 cells whose expression during differentiation varied in a manner identical to that of PEA3-91. These data suggest that PEA3-91 is not the product of the *ets-1* or *ets-2* genes but is likely to be the product of a murine homolog of the *Drosophila E74* gene.

Transcription factors play important roles in cellular growth and development, and alteration of their expression or function can lead to gross abnormalities, including oncogenesis (1, 2, 4, 11, 48). Two sequence motifs (PEA1 and PEA3) located in the polyomavirus enhancer are important in activating viral early and late gene expression and DNA replication (36, 40, 57, 60). Factor binding to these motifs is absolutely required for viral late gene expression, and the PEA1 and PEA3 motifs function synergistically as the viral late transcriptional initiator element (64). PEA1- and PEA3-binding activities are also capable of activating other TATA-dependent and TATA-independent promoters, and they are targets of activities induced by serum and phorbol esters and by polyomavirus middle T antigen (23, 58, 59, 61).

Factors binding to the PEA1 (AP1) motif are encoded by members of the *jun* and *fos* families of proto-oncogenes, which form homo- and heterodimers to positively or negatively regulate transcription (1, 4, 9, 24, 27, 29, 55). However, the identity of PEA3-binding protein(s) has not been well characterized. Recent reports indicate that members of the chicken *ets* family of proto-oncogenes are capable of binding to the PEA3 motif and other similar sequence motifs (22, 59). Thus, like PEA1, the PEA3 motif may be bound by multiple proteins encoded by a family of related proto-oncogenes that can exert positive and negative effects on eukaryotic transcription.

Regulation of polyomavirus gene expression in F9 embryonal carcinoma cells has been used as a model for developmental regulation of eukaryotic gene expression. The polyomavirus enhancer is not functional in undifferentiated F9 cells (uF9) because of the lack of positive-acting factors and the presence of negative-acting factors (12–14, 17, 46). PEA1-binding activity is expressed at very low levels in uF9 cells, and this deficiency may contribute to the lack of polyomavirus enhancer function in these cells (31). When F9 cells are transfected with a v-*jun* expression vector (29) or are induced to differentiate into parietal endoderm by treatment with retinoic acid (50, 51), PEA1-binding activity is increased and the polyomavirus enhancer becomes functional (13, 14, 31).

The expression of PEA3-binding proteins or *ets*-related genes during F9 cell differentiation has not been reported previously. Many developmentally regulated mammalian genes, however, have PEA3-binding motifs in their regulatory sequences, including genes involved in growth regulation and in extracellular matrix reorganization and remodeling (23, 39, 44). The chicken c-*ets*-1 proto-oncogene was first identified as one of two oncogenes transduced by the avian acute leukemia virus, E26 (3, 5, 18, 19). Subsequently, three murine *ets* homologs were identified, c-*ets*-1, c-*ets*-2, and PU.1. The c-*ets*-1 and PU.1 genes are differentially expressed in lymphoid cells (18–20, 30, 49), while the c-*ets*-2 gene is expressed in many cell types and is regulated in a cell cycle-dependent manner (5, 62). The PU.1 gene, which is the site of proviral integration in Friend erythroleukemia cells, encodes a protein which binds to a sequence (GAGGAA) very similar to the PEA3 motif (30, 38). Other *ets*-related proto-oncogenes have been identified in human cells (c-*elk* and c-*erg*), rat cells (GABP-α, -β1, and -β2), and *Drosophila* cells (*E74*), and murine homologs of these *ets* family members may exist and function as PEA3-binding proteins (5, 6, 15, 16, 41, 42, 52, 53, 62).

The *Drosophila* 74EF early puff that is induced by the steroid hormone ecdysone includes the *E74* unit that encodes two proteins, E74A and E74B, which share a carboxyl terminus but have unique amino-terminal sequences (6, 53, 54, 56). The carboxyl terminus shares a high degree of similarity with the Ets DNA-binding domain, and E74A binds to DNA sequences (C/A GGAA G/A) similar to the

* Corresponding author.

PEA3 motif (56). E74A and E74B are believed to be transcription factors important in triggering the morphogenic changes that occur at the end of the third larval instar of *Drosophila* development (6, 53, 54, 56). Ecdysone-independent bursts of *E74A* transcription are also observed during late embryogenesis and pupal development, suggesting that *E74A* is an important regulatory factor during many stages of *Drosophila* development (54).

In an effort to characterize the developmental regulation of factors that bind to the PEA3 motif and to the murine sarcoma virus (MSV) long terminal repeat (LTR) Ets-1-binding motif, we have compared factor binding to these motifs by mobility shift assays and by UV cross-linking. In this report we describe a 91-kDa protein that binds to the PEA3 motif (PEA3-91) and is down-regulated during differentiation of F9 cells into parietal endoderm. We show that PEA3-91 is not the product of any of the known murine *ets*-related genes, including c-*ets*-1, c-*ets*-2, and PU.1, but may be the murine homolog of the *Drosophila* E74 protein.

MATERIALS AND METHODS

Cell culture. Mouse 3T6 cells were obtained from F. Fujimura (Nichols Institute, San Juan Capistrano, Calif.). Mouse teratocarcinoma (F9) cells were obtained from the American Type Culture Collection and from M. Yaniv (Pasteur Institute, Paris, France). PYS-2 cells were a gift from B. Kahan (Zoology Research, University of Wisconsin-Madison). F9 and PYS-2 cells were maintained on plates treated with 0.1% gelatin and were cultured in Dulbecco's modified minimal essential media supplemented with 4.5 g of glucose per liter and 10% fetal calf serum (21, 50, 51). Mouse 3T6 cells were grown in Dulbecco's modified essential media supplemented with 10% calf serum (36). F9 cells were induced to differentiate into parietal endoderm by growth in medium containing 400 nM retinoic acid (all *trans*) (50, 51). Cell cultures were harvested by scraping into TD buffer (96 mM NaCl, 3.6 mM KCl, 0.5 mM Na_2HPO_4, 0.1% dextrose, 17 mM Tris-HCl, pH 7.4) containing 30% glycerol, and the cell pellets were stored at −70°C for further processing.

Preparation of nuclear extracts. Cells were lysed in buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 8.0], 0.5 M sucrose, 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.5% Triton X-100, 40 mM NaCl, 2.0 mM dithiothreitol) at 4°C in a Dounce homogenizer by using 10 strokes with a loose-fitting pestle and 10 strokes with a tight-fitting pestle (36). The nuclei were pelleted at 1,500 × *g*, washed with buffer A, and presoaked for 10 min in buffer B (10 mM HEPES [pH 8.0], 25% glycerol, 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 5.0 mM dithiothreitol) containing 0.1 M NaCl. The nuclei were pelleted and soaked for 30 min in buffer B containing 0.4 M NaCl. The nuclei were pelleted, and the nuclear proteins in the 0.4 M NaCl nuclear extract were precipitated with 50% ammonium sulfate. The precipitated proteins were centrifuged at 30,000 × *g* and resuspended in buffer B, and aliquots were stored at −70°C. The protease inhibitors phenylmethanesulfonyl fluoride, aprotinin, and leupeptin were added fresh to the nuclei at each step. Protein concentrations were determined by the Bio-Rad protein assay, with bovine serum albumin as a standard.

Electrophoretic mobility shift assays. Double-stranded oligonucleotide probes were end-labeled with [α-^{32}P]dGTP by incubation with the Klenow fragment of *Escherichia coli* DNA polymerase and were subsequently purified over NENSORB-20 cartridges (36). Nuclear extract (10 μg) was incubated for 10 min at 25°C with 1.0 μg of poly(dI-dC) · poly(dI-dC) in 0.02 ml of buffer C (10 mM HEPES [pH 8.0], 17.5% [vol/vol] glycerol, 0.1 mM EDTA, 0.1 M NaCl, 10 mM $MgCl_2$, 2 mM dithiothreitol). Radiolabeled probes (0.5 ng) were added, and the samples were incubated for 20 min at 25°C and subsequently fractionated on a 6% nondenaturing polyacrylamide gel (40:1 acrylamide/bisacrylamide) in 0.25× TBE (1× TBE is 0.13 M Tris base, 0.12 M boric acid, and 2.0 mM EDTA, pH 8.8). The gels were dried and exposed to X-ray film at −70°C with an intensifying screen. Sequences of oligonucleotides used in these studies are shown in Fig. 1.

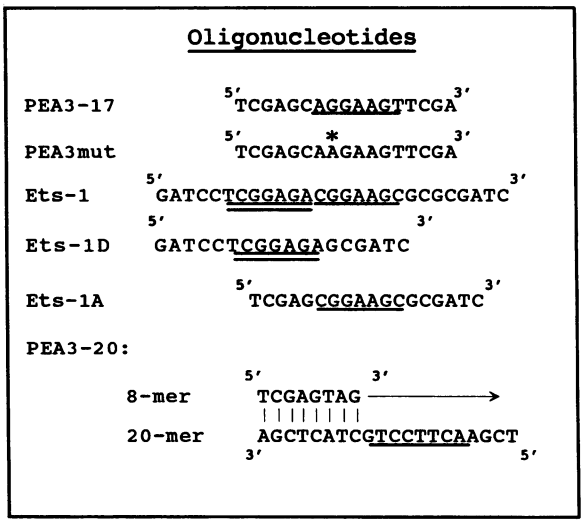

FIG. 1. Sequences of oligonucleotides used in mobility shift assays and in UV cross-linking experiments. PEA3 sequences are underlined. PEA3-like sequences that differ from the PEA3 motif at three positions are double underlined. Asterisk indicates the altered nucleotide in the PEA3mut oligonucleotide. The PEA3-20 oligonucleotide was labeled by primer extension as indicated by the arrow and was used in UV cross-linking experiments. All other oligonucleotides were labeled by end filling as previously described.

UV cross-linking of radiolabeled oligonucleotides to DNA-binding proteins. Nuclear extract (20 μg) was preincubated at 25°C for 15 min in 20 μl of buffer C containing 3.0 μg of poly(dI-dC) · poly(dI-dC), and 1.0 μg of poly(dA-dT)· poly (dA-dT) with or without unlabeled competitor oligonucleotides (500-fold molar excess). Radiolabeled oligonucleotide probe (2.0 ng, 10^6 cpm; see below) was added, and the mixture was incubated for an additional 15 min at 25°C. The mixture was then transferred to a 96-well microtiter plate and treated for 8 min with a high-intensity UV lamp (254 nm, 900 μW/cm^2 at 1 in.; Spectroline). An equal volume of 2× sample buffer was added, and the samples were boiled for 5 min and subsequently fractionated on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel (32).

The PEA3-20 oligonucleotide probe was labeled by primer extension of an 8-nucleotide oligonucleotide (8-mer) annealed to the complementary sequence of a 20-nucleotide oligonucleotide (20-mer) containing the PEA3 recognition sequence (Fig. 1). The 8-mer was extended by incubation of the annealed oligonucleotides with *E. coli* DNA polymerase (Klenow fragment) at 25°C for 10 min in the presence of [α-^{32}P]dGTP and unlabeled deoxynucleoside triphosphates. Excess unlabeled dGTP was then added, the reaction mixture was incubated for an additional 10 min, and the labeled

probe was purified over a NENSORB-20 column. The Ets-1 probe used in mobility shift assays was also used in the UV cross-linking experiments.

RNA extraction and Northern (RNA) blot analysis. Total RNA was isolated by the method of Chirgwin et al. by utilizing the CsCl gradient modification (8). RNA (20 μg) was electrophoretically fractionated on a formaldehyde-agarose gel and transferred to a nylon membrane (Biotrans; ICN). DNA probes for measuring c-*ets*-1 and c-*ets*-2 transcripts were generated by the random priming method by using cDNAs cloned into the plasmid pBluescript. The c-*ets*-1 and c-*ets*-2 cDNAs were provided by J. H. Chen (M. D. Anderson Cancer Center, Houston, Tex.) (7) and J. Hassel (McMaster University, Hamilton, Ontario, Canada), respectively. Nylon membranes were hybridized with the c-*ets*-1 or c-*ets*-2 probe in hybridization buffer (10% formaldehyde, 0.2 M $NaPO_4$ [pH 7.2], 1.0% bovine serum albumin, 1.0 mM EDTA, 7.0% SDS) at 65°C for 24 h. The membranes were washed in 40 mM $NaPO_4$ (pH 7.2)–1.0 mM EDTA–1.0% SDS at 65°C for approximately 1 h and then autoradiographed.

Immunoblot analysis of nuclear extracts. Nuclear extracts were fractionated on SDS–10% polyacrylamide gels and electrophoretically transferred to nitrocellulose filters. The filters were blocked by incubating at 30°C in TBS (20 mM Tris-HCl [pH 7.5], 500 mM NaCl) containing 3.0% gelatin for 2 h and then incubating with an E74-specific rabbit serum (1:1,000 dilution in TBS plus 0.05% Tween 20 [TTBS]) containing 1.0% gelatin for 2 h at 25°C. The filters were washed with TTBS and incubated for 1 h with goat anti-rabbit alkaline phosphatase-conjugated antiserum (1:2,000 dilution in TTBS plus 1% gelatin). After extensive washing with TTBS and then TBS, the filters were developed by incubating in alkaline phosphatase development buffer (0.1M $NaHCO_3$, 1.0 mM $MgCl_2$, 0.03% *p*-nitroblue tetrazolium, 0.015% 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt).

RESULTS

Developmental regulation of factors that bind to the PEA3 and Ets-1 motifs during F9 cell differentiation. PEA3-binding activity was first identified in mouse 3T6 cell nuclear extracts as a sequence-specific DNA-binding activity that was expressed at very low levels in mouse fibroblasts (much lower than levels of PEA1 activity, which is relatively abundant in these cells [36]). uF9 cells contain very low levels of PEA1-binding activity, which increase upon retinoic acid-induced differentiation (31, 50, 51). Remarkably, PEA3-binding activity is present at very high levels in nuclear extracts from uF9 cells, much higher than are observed in nuclear extracts from 3T6 mouse fibroblasts (Fig. 2, lanes 1 and 2). Upon differentiation of F9 cells following treatment with retinoic acid for 2 days, nuclear extracts were found to contain reduced levels of PEA3-binding activity (Fig. 2, lane 3) and PEA3-binding activity was further reduced in extracts from cells treated for 4 days (Fig. 2, lane 4) and 7 days (Fig. 2, lane 5). Identical results were observed when F9 cells were treated with retinoic acid in the presence of 1.0 mM dibutyryl cyclic AMP (35), which is reported to augment the differentiation of F9 cells to parietal endoderm (50, 51). The reduction of PEA3-binding activity was not due to proteolytic degradation of the extract, since PEA1-binding activity increased and Sp1-binding activity remained constant in differentiated F9 (dF9) cell extracts and since only minor differences in the proteins present in uF9 and dF9 cell nuclear extracts were observed in silver-stained SDS–polyacrylamide gel electrophoresis (PAGE) gels (35).

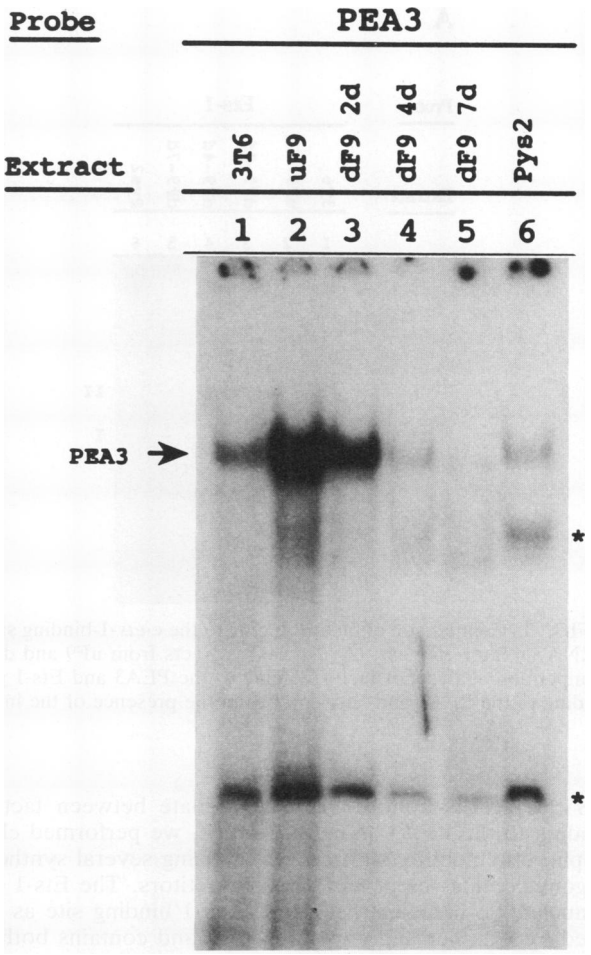

FIG. 2. Mobility shift analysis of PEA3-binding activity during F9 cell differentiation. Nuclear extract was incubated with radiolabeled PEA3 oligonucleotide, and the DNA-protein complexes were separated on a native 6% polyacrylamide gel. For comparison, nuclear extracts from 3T6 (lane 1) and PYS-2 (lane 6) cells are included. uF9 cells were induced to differentiate into parietal endoderm by treatment with retinoic acid for 2 days (lane 3), 4 days (lane 4), and 7 days (lane 5). Asterisks indicate nonspecific complexes determined by competition analysis.

To confirm that down-regulation of PEA3-binding activity accompanies differentiation into parietal endoderm, we measured PEA3-binding activity in PYS-2 cells (a parietal endoderm-derived cell line [21]). Nuclear extracts from PYS-2 cells contained similar levels of PEA3-binding activity as was observed in extracts from dF9 cells treated with retinoic acid for 4 days (Fig. 2, lane 6).

The molecular identity of the PEA3-binding protein described above is not known; however, recent reports have demonstrated that chicken c-*ets*-1 and c-*ets*-2 gene products transactivate heterologous promoters through the PEA3 motif (59). Furthermore, the murine c-*ets*-1 protein binds to a sequence important in the activation of the MSV LTR that contains two sequence elements very similar to the PEA3 motif (Fig. 1) (22). Because of the high degree of sequence similarity between the DNA-binding domains of the c-*ets*-1 and c-*ets*-2 proteins, it is likely that both proteins bind to the

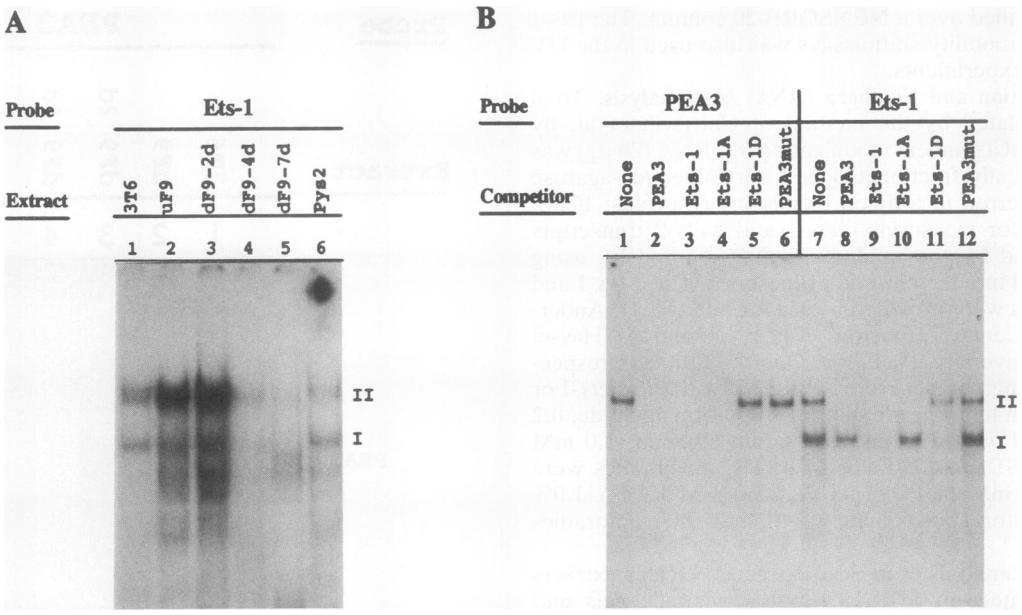

FIG. 3. Comparison of factor binding to the c-*ets*-1-binding site in the MSV LTR and to the PEA3-binding site from the polyomavirus late mRNA initiator element. (A) Nuclear extracts from uF9 and dF9 cells were analyzed by mobility shift assay as described for Fig. 2. (B) Competition analysis of factor binding to the PEA3 and Ets-1 probes. uF9 cell nuclear extracts were analyzed by mobility shift assay for binding to the PEA3 and Ets-1 probes in the presence of the indicated unlabeled (500-fold molar excess) competitor oligonucleotides.

MSV LTR Ets-1 motif. To discriminate between factors binding to the PEA3 and Ets-1 motifs, we performed electrophoretic mobility shift assays by using several synthetic oligonucleotides as probes and competitors. The Ets-1 oligonucleotide includes the entire c-*ets*-1 binding site as defined by Gunther and coworkers (22) and contains both of the PEA3-like elements (Fig. 1). The Ets-1A oligonucleotide contains the 3′ half of the c-*ets*-1-binding site (cGGAAG) which differs from the PEA3 motif (AGGAAG) by only one nucleotide. The Ets-1D oligonucleotide contains the 5′ half of the c-*ets*-1-binding site (cGGAga) which differs from the PEA3 motif at three positions (Fig. 1).

With the complete Ets-1 probe, two complexes (I and II) were observed in 3T6 and uF9 cell nuclear extract, including one (complex II) which comigrates with the PEA3 complex (Fig. 3A, lanes 1 and 2 and Fig. 3B, lanes 1 and 7). After retinoic acid-induced differentiation, both complexes were down-regulated with a time course similar to that observed for factor binding to the PEA3-binding motif (Fig. 3A, lanes 3 to 5). Factor binding to the PEA3 oligonucleotide was inhibited by excess PEA3, Ets-1, and Ets-1A oligonucleotides (Fig. 3B, lanes 2 to 4) but not by the Ets-1D oligonucleotide nor the PEA3mut oligonucleotide (Fig. 3B, lanes 5 and 6), which is altered at a residue critical for PEA3 binding (Fig. 1) (36). Similarly, the Ets-1 complex II was inhibited efficiently by excess PEA3, Ets-1, and Ets-1A oligonucleotides (Fig. 3B, lanes 8 to 10) but not by the Ets-1D or the PEA3mut oligonucleotide (Fig. 3B, lanes 11 and 12). The Ets-1A oligonucleotide formed a single complex which comigrated with the PEA3 complex in mobility shift gels, and in competition experiments the Ets-1A oligonucleotide acted in the same manner as did the PEA3 oligonucleotide, suggesting that the same factor binds to both motifs (35).

The Ets-1 complex I was inhibited by the Ets-1 oligonucleotide (Fig. 3B, lane 9) but not by the PEA3, Ets-1A, or PEA3mut oligonucleotide (Fig. 3B, lanes 8, 10, and 12). Factor binding in complex I was also inhibited by the Ets-1D oligonucleotide, although to a lesser extent than with the same amount of the Ets-1 oligonucleotide, indicating that this factor bound less tightly to the Ets-1D oligonucleotide than to the Ets-1 oligonucleotide. We were unable to demonstrate factor binding to the Ets-1D oligonucleotide probe directly in mobility shift assays (probably because of low affinity for this oligonucleotide), although at high concentrations it effectively competes for factor binding to the complete Ets-1 oligonucleotide. Furthermore, we did not observe a third Ets-1 complex which would result from the simultaneous binding of factors to both the Ets-1D and Ets-1A motifs, suggesting that factor binding to these sites is mutually exclusive.

To determine whether PEA3-binding activity contains factors related to the c-*ets*-1 and c-*ets*-2 gene products, we analyzed F9 cell nuclear extracts by using polyclonal antiserum raised against a fusion protein containing 56 amino acids derived from the v-*ets* protein (anti-Ets-A, kindly provided by D. LePrince [18, 19]). Nuclear extracts from uF9 or dF9 cells were treated with increasing amounts of the anti-Ets-A serum, but no effect on PEA3-binding activity was observed (Fig. 4). No inhibition of binding was observed, nor was a supershift complex formed with either cell extract. Similar experiments with murine c-*ets*-1 protein produced in Sf9 insect cells from a recombinant c-*ets*-1 baculovirus resulted in a supershift complex bound to the Ets-1 probe, demonstrating that the Ets-A antiserum recognizes native c-*ets*-1 protein bound to DNA (34, 35).

Expression of c-*ets*-1 and c-*ets*-2 mRNAs does not correlate with PEA3-binding activity upon F9 cell differentiation. To determine whether the c-*ets*-1 and c-*ets*-2 gene products may be candidates for uF9 cell PEA3-binding activity, we measured their mRNA levels in uF9 and dF9 cells. Total RNA isolated from 3T6 and FM3A cells contained a single 5.3-kb RNA species that was detected by the murine c-*ets*-1 cDNA

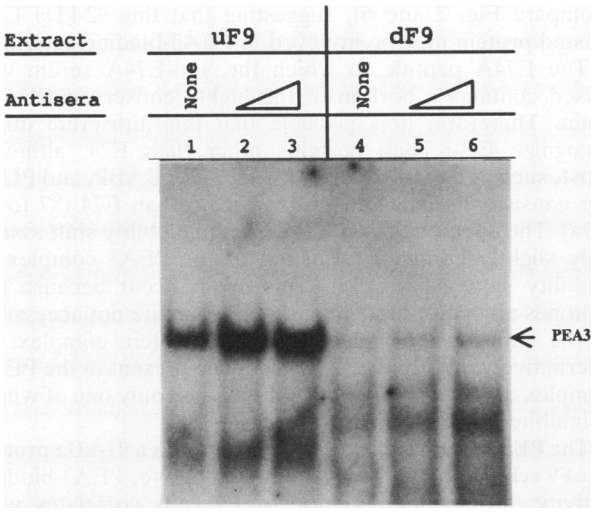

FIG. 4. Effect of anti Ets-A antiserum on PEA3-binding activity. The Ets-A antiserum (1 μl, lanes 2 and 5; 3 μl, lanes 3 and 6) was added to uF9 or dF9 cell nuclear extract 10 min prior to addition of the PEA3 oligonucleotide probe, and the resulting PEA3 complexes were analyzed by mobility shift assay.

probe, which is consistent with the reported size of the murine c-*ets*-1 transcript (Fig. 5, lanes 1 and 6) (3). In contrast, RNA isolated from two sources of uF9 cells and their differentiated derivatives did not contain detectable levels of c-*ets*-1 mRNA (Fig. 5, lanes 2 to 5), demonstrating that the c-*ets*-1 gene is not induced during F9 cell differentiation and suggesting that PEA3-binding activity in F9 cells is not likely to be the c-*ets*-1 protein. Identical results were obtained with a cDNA probe for the Ets-related PU.1 gene, indicating that PEA3-binding activity is not likely to be associated with the PU.1 protein (35).

The c-*ets*-2 cDNA probe detected a single 3.5-kb RNA species in RNA isolated from 3T6 and FM3A cells, which is consistent with the reported size of the mouse c-*ets*-2 mRNA (Fig. 5, lanes 5 and 8) (3). Similarly, uF9 cells express significant levels of c-*ets*-2 mRNA (Fig. 5, lanes 8 and 10). After retinoic acid-induced differentiation, however, c-*ets*-2 mRNA levels were slightly increased (Fig. 5, lanes 9 and 11), whereas PEA3-binding activity decreased during F9 cell differentiation. Thus, the regulation of c-*ets*-2 gene expression does not correlate with regulation of PEA3-binding activity observed during F9 cell differentiation.

In summary, the results of the mobility shift experiments, the failure of Ets-A antibodies to bind to the PEA3-DNA complex in mobility shift assays, and the absence of detectable c-*ets*-1 mRNA suggest that F9 cell PEA3-binding activity is not associated with the c-*ets*-1 gene products. Furthermore, expression of c-*ets*-2 mRNA does not correlate with PEA3-binding activity during F9 cell differentiation, suggesting that the c-*ets*-2 gene product is not a major component of PEA3-binding activity in these cells. It is possible, however, that the c-*ets*-1 and c-*ets*-2 proteins are minor contributors of PEA3-binding activity.

PEA3-binding activity correlates with expression of a 92-kDa protein possibly related to the *Drosophila* E74 protein. The *Drosophila E74* gene is located in the 74EF ecdysone-inducible puff locus of the salivary gland polytene chromosomes. Two proteins, E74A (87 kDa) and E74B (95 kDa), which share the Ets DNA-binding domain in the carboxyl terminus but have different amino-terminal sequences, are expressed from this locus (6, 53, 54). To determine whether F9 cells express an E74A-related protein, we fractionated uF9 and dF9 nuclear extracts on denaturing SDS–polyacrylamide gels and performed immunoblot analysis of these proteins by using an anti-E74 antiserum directed against the 151 carboxyl-terminal amino acids from the E74A protein which include a portion of the conserved Ets DNA-binding domain (kindly provided by C. S. Thummel) (Fig. 6). A single band which migrated with an apparent molecular mass of 92 kDa was observed in these immunoblots. The diffuse staining in the 65-kDa region is nonspecific binding to a contaminant in the loading buffer (35). The amount of the

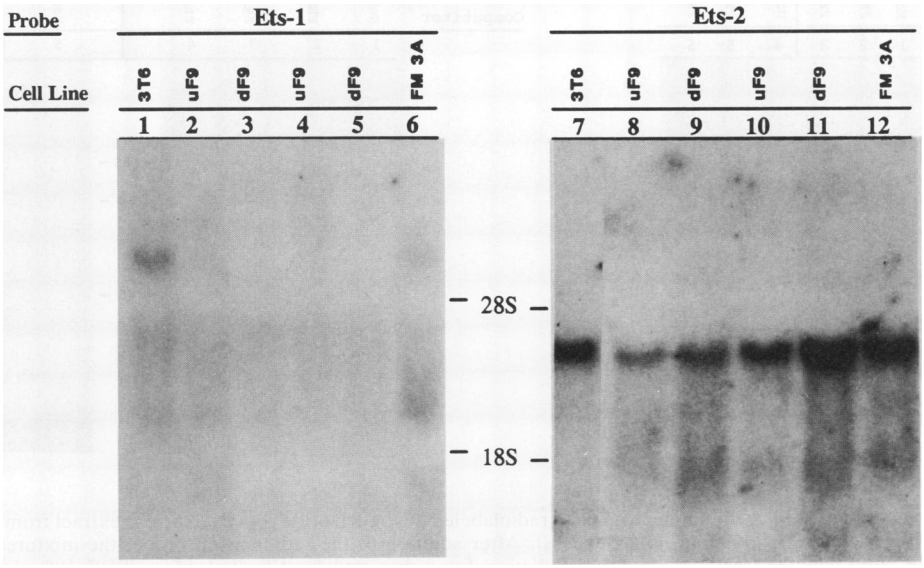

FIG. 5. Expression of c-*ets*-1 (5.3 kb) and c-*ets*-2 (3.5 kb) mRNAs in uF9 and dF9 cells. Total RNA was isolated from cultured cells and fractionated on denaturing agarose gels. The RNAs were transferred to nitrocellulose and hybridized to c-*ets*-1- or c-*ets*-2-specific random-primed DNA probes. The positions of the 28S and 18S rRNAs are indicated.

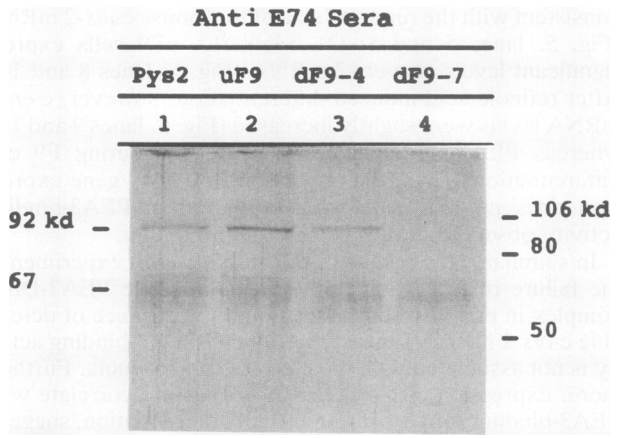

FIG. 6. Immunoblot analysis of E74-related proteins expressed in differentiating F9 cells. Nuclear extracts (50 μg) from PYS-2 cells (lane 1), uF9 cells (lane 2), and dF9 cells treated with retinoic acid for 4 days (dF9-4, lane 3) or 7 days (dF9-7, lane 4) were fractionated on an SDS–10% polyacrylamide gel, the proteins were transferred to a nitrocellulose filter, and the filter was probed with a 1:1,000 dilution of an E74A-specific antiserum as described in Materials and Methods.

92-kDa species was decreased in dF9 cells treated for 4 days with retinoic acid (Fig. 6, lane 3) and was undetectable in dF9 cells after 7 days of treatment (Fig. 6, lane 4). PYS-2 cells contained levels of the 92-kDa species similar to those observed in dF9 cells after 4 days of retinoic acid treatment (Fig. 6, lane 1). These results are very similar to those observed for PEA3-binding activity in mobility shift assays (compare Fig. 2 and 6), suggesting that this 92-kDa E74-related protein may be involved in PEA3-binding activity.

The E74A peptide to which the anti-E74A serum was raised contains a portion of the highly conserved Ets domain. Therefore, it is possible that this antiserum might recognize Ets-related proteins other than E74, although most, such as c-*ets*-1, c-*ets*-2, c-*erg*, c-*elk*, GABP, and PU.1, are consistently smaller (29 to 51 kDa) than E74 (87 to 95 kDa). The addition of anti-E74 serum to mobility shift assays only slightly inhibited formation of the PEA3 complex in mobility shift assays (35). This might occur because the epitopes recognized by the E74 antiserum are not accessible in the native protein or in the DNA-protein complex, or alternatively, multiple activities may be present in the PEA3 complex detected in mobility shift assays, only one of which is inhibited by the anti E74 serum.

The PEA3 motif associates specifically with a 91-kDa protein in uF9 cell nuclear extracts. As shown above, PEA3-binding activity during differentiation of F9 cells correlates with expression of a 92-kDa E74A-related protein. To determine whether a protein of this size binds to the PEA3 motif, we used UV cross-linking to specifically label proteins bound to radiolabeled PEA3 (Fig. 7A and B) or Ets-1 (Fig. 7B) oligonucleotide probes. After UV cross-linking of the PEA3 probe to factors present in uF9 cell nuclear extract, a strong band with an apparent molecular mass of 97 kDa (p97) and a more weakly labeled band of 104 kDa (p104) were labeled (Fig. 7A, lane 1). Labeling of these proteins was greatly reduced by competition with excess unlabeled PEA3 oligonucleotide (Fig. 7A, lane 3) but not by excess PEA3mut oligonucleotide (Fig. 7A, lane 2), confirming that cross-linking of these proteins was specific for the PEA3 motif. Nuclear extracts from dF9 cells contained substantially

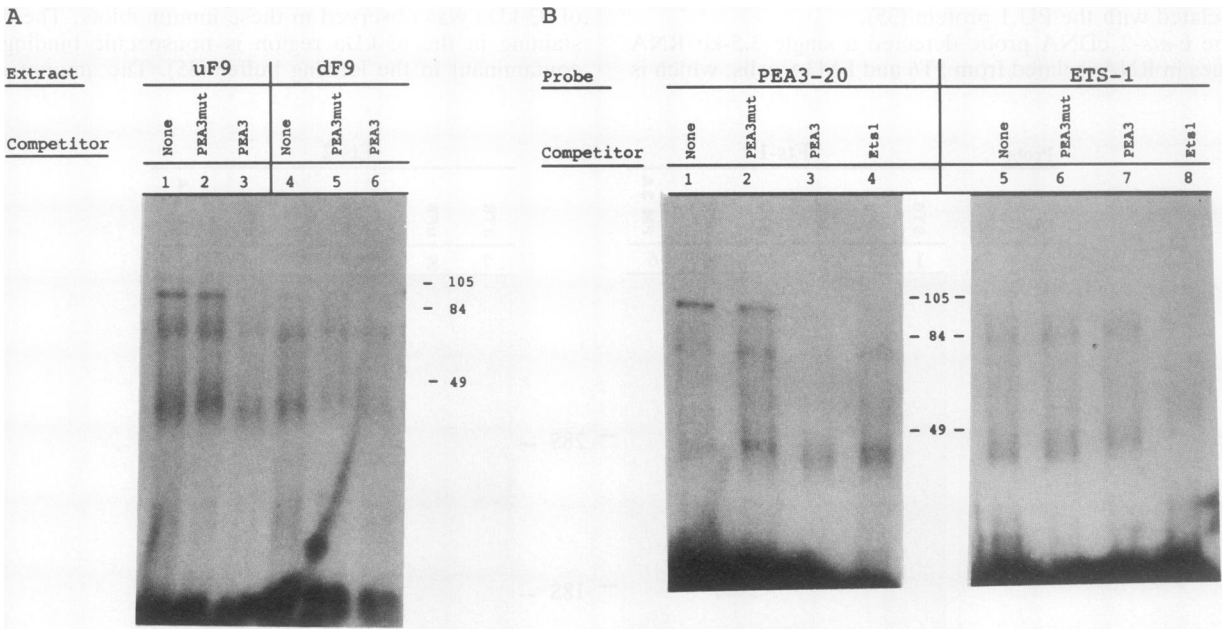

FIG. 7. (A) UV cross-linking of PEA3-binding proteins to radiolabeled oligonucleotide probes. Nuclear extract from uF9 and dF9 cells was preincubated with competitor DNAs (500-fold molar excess). After addition of the radiolabeled probe, the mixture was incubated for an additional 10 min and subsequently treated with UV irradiation for 8 min and fractionated on an SDS–10% polyacrylamide gel. (B) Comparison of PEA3- and Ets-1-binding proteins by UV cross-linking. Nuclear extract from uF9 cells was incubated with the PEA3 or Ets-1 probe and treated with UV irradiation in the presence or absence of the indicated competitor oligonucleotides (500-fold molar excess) as described for panel A.

reduced levels of p97 and p104, similar to the reduction of PEA3-binding activity observed in mobility shift assays (Fig. 7, lanes 4 to 6).

Previous studies have shown that covalently bound double-stranded or single-stranded DNA fragments increase the apparent molecular masses of proteins in SDS–polyacrylamide gels by an amount equal to the molecular mass of the DNA fragment (28, 45, 63). The short duration of UV treatment used in these experiments likely cross-linked only a fraction of the PEA3 DNA-protein complexes, and the majority of the labeled proteins may have been cross-linked to only one strand. This is consistent with p97 and p104 being the products of cross-linking of a single protein to one strand or to both strands of the PEA3 oligonucleotide probe, respectively. Factor binding predominantly to one strand of a target sequence has been reported for other transcription factors, including the Ets-related factor PU.1 (29). Therefore, by subtracting the molecular mass of one strand (6.5 kDa) from the apparent molecular mass of p97, or the molecular mass of two strands (13 kDa) from the apparent molecular mass of p104, we estimate the actual molecular mass of the PEA3-specific p97 and p104 cross-linked proteins in F9 cells to be 91 kDa (PEA3-91). These results are consistent with the notion that PEA3-91 and the 92-kDa E74-related protein are one and the same.

UV cross-linking to the Ets-1 oligonucleotide resulted in a labeled protein(s) which migrated as a broadly diffuse band with an apparent molecular mass of 83 kDa (Fig. 7B, lanes 5 to 8). Labeling of this protein was greatly diminished by competition with excess Ets-1 oligonucleotide (Fig. 7B, lane 8) but not by competition with excess PEA3mut or PEA3 oligonucleotide (Fig. 7B, lanes 6 and 7). UV cross-linking to the Ets-1 oligonucleotide did not label p97 even though cross-linking of p97 to the PEA3 oligonucleotide was inhibited by excess Ets-1 oligonucleotide (Fig. 7B, lanes 3 and 4). These results suggest that while the Ets-1 oligonucleotide is weakly bound by PEA3-91, under the conditions used during UV treatment, the PEA3-91 protein is not cross-linked to the Ets-1 oligonucleotide. Assuming that T residues are the primary target of UV irradiation in these studies, this result may be explained by the unequal distribution of T residues in the Ets-1-binding motif compared with that in the PEA3 motif (28). We can further conclude that PEA3-91 is one of several proteins present in uF9 cells that bind to the PEA3 motif. Purification and characterization of PEA3-binding proteins from uF9 cells should elucidate the roles of these proteins during differentiation and in transcriptional regulation.

DISCUSSION

uF9 cells express high levels of PEA3-91-binding activity, which is dramatically reduced during retinoic acid-induced differentiation to levels similar to those observed in the PYS-2 parietal endoderm cell line (21). We have detected by UV cross-linking a 91-kDa PEA3-binding protein whose expression is regulated in a manner identical to that of PEA3-binding activity and which is one of several proteins expressed in uF9 cells which bind to the PEA3 motif. We have also demonstrated that a portion of the c-*ets*-1 binding site that is important in the activation of the MSV LTR is bound by PEA3-91 (complex II) and by a second activity (complex I) present in uF9 nuclear extracts.

The identity of the factor(s) associated with complex I is not clear. Since complex I levels decreased during differentiation, as for PEA3 and complex II, complex I does not correlate with c-*ets*-2 expression, which changes little during differentiation. It is possible that complex I is the result of c-*erg*, c-*elk*, or a previously unknown factor binding to the Ets-1D motif. However, since the c-*ets*-2 protein is known to be subject to rapid turnover and is stabilized by phosphorylation (15), we cannot rule out the possibility that the c-*ets*-2 protein forms complex I but is underphosphorylated and short-lived in dF9 cells and cannot be detected in crude nuclear extracts from these cells.

Since the anti-Ets-A serum did not recognize F9 cell PEA3-binding activity in mobility shift assays, nor did it detect a 91-kDa protein in immunoblot assays (35), we conclude that PEA3-91 (complex II) is not a modified form of the c-*ets*-1 or c-*ets*-2 gene product. PEA3-91 also appears to be different from an *ets*-related gene recently identified in FM3A cells which encodes a 66-kDa PEA3-binding protein (25). Purification of these activities and cloning of their corresponding cDNAs should clarify the identities of these factors and their importance during development.

Our data indicate a correlation between PEA3-91 and an *ets*-related gene product whose homolog has not previously been detected in mammalian cells, the *Drosophila E74A* gene (6, 53, 54, 56). This is an intriguing possibility since the *Drosophila* E74A protein has a molecular mass of 87 kDa, similar to that of PEA3-91, and because a protein of similar size (92 kDa) was identified in uF9 cell nuclear extracts with an E74A-specific antiserum. That the E74A-related protein detected in immunoblots was down-regulated in a manner identical to PEA3-91 suggests that they are the same protein and is consistent with the notion that PEA3-91 is a murine homolog of the *Drosophila E74A* gene product. The *E74* gene products are important in triggering the events leading to the onset of larval metamorphosis, and their ability to specifically bind to DNA suggests that they are transcription factors (6, 53, 54, 56). The regulation of PEA3-91 during F9 cell differentiation suggests that it also has important roles in the regulation of early events in mammalian embryonic development, some of which may be homologous to E74A functions.

We have recently demonstrated that PEA3-binding proteins function synergistically with PEA1-binding proteins to form a TATA-independent initiator complex that is required for polyomavirus late transcription (64). Many developmentally regulated genes contain one or more PEA3 motifs at or near their initiation start sites, including several with TATA-independent promoters (10, 23, 33, 37, 39, 43, 58). Since the PEA3 motif has been shown to be important for the transcription of some of these promoters, it is likely that proteins such as PEA3-91 are important in the transcriptional regulation of these TATA-independent genes.

Expression of PEA3-91 and other PEA3-binding proteins may have multiple effects on transcriptional regulation of promoters with PEA3 motifs. The PEA3-binding site in the polyomavirus enhancer is important for enhancer function (at least in 3T6 cells) (36) and is required for function of the viral late initiator element, yet down-regulation of PEA3-binding activity correlates with induction of polyomavirus gene expression in dF9 cells. One possible explanation for these observations is that one or more PEA3 binding proteins repress polyomavirus transcription either directly or indirectly by interacting with PEA2-binding proteins, which are known to repress the polyomavirus enhancer in uF9 cells (12, 17, 46). Following differentiation, lower levels of negatively acting PEA3-binding proteins may result in a functional polyomavirus enhancer. Similarly, the c-*jun* and c-*fos* promoters contain PEA3 motifs, including a region of the

c-*fos* promoter that is associated with negative transcriptional regulation (47). These genes may be expressed at low levels in uF9 cells because of repression by PEA3-91 or other PEA3-binding proteins, which results in low levels of PEA1-binding activity and contributes to reduced polyomavirus enhancer activity (26, 47).

In summary, we have identified a 91-kDa protein which is the predominant PEA3-binding activity in uF9 cells. PEA3-91 is down-regulated during F9 cell differentiation and may be related to the *Drosophila* E74A protein. Additional experiments are under way to clone the PEA3-91 gene, to analyze how PEA3-91 functions in TATA-dependent and TATA-independent transcription, and to determine under what conditions it may repress transcription.

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