A DNA Element That Regulates Expression of an Endogenous Retrovirus during F9 Cell Differentiation Is E1A Dependent

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The retinoic acid-induced differentiation of F9 cells into parietal endoderm-like cells activates transcription of the endogenous mouse retrovirus, the intracisternal A-particle (IAP). To investigate the elements that control IAP gene differentiation-specific expression, we used methylation interference, Southwestern (DNA-protein), and transient-transfection assays and identified the IAP-proximal enhancer (IPE) element that directs differentiation-specific expression. We find that the IPE is inactive in undifferentiated F9 cells and active in differentiated parietal endoderm-like PYS-2 cells. Three proteins of 40, 60, and 68 kDa bind to the sequence GAGTGAC located between nucleotides -53 and -47 within the IPE. The 40- and 68-kDa proteins from both the undifferentiated and differentiated cells exhibit similar DNA-binding activities. However, the 60-kDa protein from differentiated cells has greater binding activity than that from undifferentiated cells, suggesting a role for this protein in F9 differentiation-specific expression of the IAP gene. The IAP gene is negatively regulated by the adenovirus E1A proteins, and the E1A sequence responsible for repression is located at the N terminus, between amino acids 2 and 67. The DNA sequence that is the target of E1A repression also maps to the IPE element. Colocalization of the differentiation-specific and E1A-sensitive elements to the same protein-binding site within the IPE suggests that the E1A-like activity functions in F9 cells to repress IAP gene expression. Activation of the IAP gene may result when the E1A-like activity is lost or inactivated during F9 cell differentiation, followed by binding of the 60-kDa positive regulatory protein to the enhancer element.

The use of the embryonal carcinoma cell line F9 has greatly aided analyses of the molecular mechanisms controlling cellular differentiation during mammalian embryogenesis. F9 is a nullipotent teratocarcinoma-derived cell line that resembles early embryonic cells in its antigenic and biochemical properties and in its ability to differentiate into parietal endoderm (PE) (41). Treatment of F9 cells with retinoic acid plus dibutyryl cyclic AMP induces differentiation into PE-like cells, with a corresponding change in the pattern of gene expression. Numerous cellular and viral genes are transcriptionally activated upon differentiation of F9 cells through the appearance of positive-acting, stage-specific transcription factors or loss of negative-acting repressors (8, 10, 24, 27, 37, 45, 52). The polyomavirus enhancer, for example, which is inactive in undifferentiated F9 cells and active in differentiated F9 cells, is regulated by multiple stage-specific transcription factors, including PEA1, PEA2, and PEA3 (32, 45, 50). PEA1, a transcriptional activator homologous to transcription factor AP-1, is induced upon F9 differentiation, while PEA2 and PEA3, labile repressors present in F9 cells, are lost upon differentiation.

The restricted expression of the differentiation-specific genes in F9 cells is believed to be due in part to a cellular activity similar to that of the adenovirus E1A gene. Undifferentiated but not differentiated F9 cells complement an adenovirus E1A deletion mutant with respect to activation of adenovirus early genes (14). Additional evidence that this

activity is functionally similar to that of E1A comes from the observations that (i) many genes that are repressed in undifferentiated F9 cells are also repressed by E1A and (ii) this repression maps to enhancers of some genes that are repressed in undifferentiated F9 cells (10, 11, 21, 27, 33, 36, 43, 52). Although colocalization of DNA elements responsible for F9 cell-specific repression and E1A-mediated repression has not been established, the findings suggest that adenovirus E1A and the E1A-like activity in undifferentiated F9 cells may repress differentiation-specific gene expression by a common mechanism.

We have studied the regulation of a gene that encodes the endogenous retrovirus intracisternal A-particle (IAP). IAP genes are reiterated throughout the mouse genome and contain many features characteristic of retroviruses, including long terminal repeats (LTRs) that control IAP transcription. IAPs exhibit restricted expression in F9 cells and are transcriptionally activated upon differentiation into PE-like cells (13). Previously, we identified a DNA element (nucleotides [nt] -180 to -173), located within an IAP upstream enhancer (IUE), that is active in both undifferentiated and differentiated cells but whose activity is inhibited by CpG methylation (18). We also found, by 5' deletion analysis of the IAP LTR (18), that all the constructs bearing sequence from nt -307 to +78 had seven- to eightfold-greater transcriptional activity in the PE-like teratocarcinoma-derived PYS-2 cells (and in F9 cells treated with retinoic acid plus dibutyryl cyclic AMP) than in F9 cells, including the 3'-most deletion construct containing the sequences from nt -67 to +78 (see Fig. 1 for diagrammatic representation of the IAP LTR). This observation indicated the presence of at least one DNA element within nt -67 to +78 that directs differentiation-specific expression. In this report, we show for the first time that an enhancer element (nt -53 to -47) located

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FIG. 1. Transcriptional activity of IAP LTR elements. CAT constructs containing either the IAP -161/-130 oligonucleotide (B), the IAP -134/-73 fragment (C), or the IAP -77/-49 oligonucleotide (D) cloned upstream of the TK promoter in different orientations and copy numbers (arrows indicate orientation of the elements) were transfected into F9 and PYS-2 cells. CAT activity is expressed relative to the activity of the TK promoter driving CAT expression (A, pBLCAT2). The numbers of independent transfection experiments with each construct are shown in parentheses. The relative error between independent experiments varied from 4 to 17%. (E) Schematic representation of the IAP elements tested for transcriptional activity. The beginning of the LTR is shown. Boxed sequences represent transcriptional elements, most of which were identified on the basis of homology to known transcriptional elements, including an SP1 binding site (SP1) (5), a glucocorticoid response element (GRE) (34), a simian virus 40 core enhancer sequence (CORE) (48), a cyclic AMP response element or the homologous ATF binding site (CRE) (23, 28), a nuclear factor 1 or the homologous CCAAT transcription factor binding site (NF1/CTF/CAAT) (15), an E4TF1 binding site (E4TF1) (46), and a nonconsensus TATA box (GAGA). Also boxed are two enhancer elements identified on the basis of function: the transcriptional enhancer Enh2, which binds protein factor EBP80 (ENH2) (6), and the IUE, which binds protein factor IUEB (18).

proximal to the IAP basal promoter is repressed by endogenous factors in undifferentiated F9 cells and by E1A in differentiated PE-like PYS-2 cells. Colocalization of the differentiation-specific and E1A-sensitive elements suggests that the E1A-like activity present in undifferentiated F9 cells directs differentiation-specific expression from the IAP enhancer located proximal to the IAP basal promoter.

MATERIALS AND METHODS

Oligonucleotides. The following synthetic double-stranded oligonucleotides were used:

-161/-130 GATCCGTTCTTAAGTGGTAAACAAATAATCTGCGCATG
GCAAGATTACCAATTTGTTATTAGACGGGTACCTAG
-77/-49 GATCCTCGCCGATGGCTGCAGCCAAATCAGGAGTG
GAGCCGGTACCGACGTCGGTTAGTCCCTCACCTAG
-62/-18 GATCCCCAATCAGGGAGTGACAGTCCGAGGCGAAGAGATGCTCCTTAAG
GGGTTAGTCCCTCACTGTGCAGGCTCCGCTTCTCTTACGAGGAATTCCTAG
-59/-43 GATCATCAGGGAGTGACAGCT
TAGTCCCTCACTGTGCACTAG
-59/-43 mutant GATCATCAGGtctGTGA-ACGT
(mt) 1 TAGTCCgagCACT-TGCACTAG
-59/-43 mutant GATCATCAGGgaatttACGT
(mt) 2 TAGTCCCTttaaTGCACTAG
Intron GGACATCCTTTTCTCTCTCCGTACGAGATCTCTGAGTGG
CCTGTAGGAAAAAGAGAGGAGTCTGTAGAGACTCACC

Plasmid constructs. The IAP nt -134 to -73 fragment was cloned upstream of the herpesvirus thymidine kinase (TK) promoter by digesting pIAP.1 (13) with *FspI* and *HaeIII*, adding *BamHI* linkers, and cloning into the *BamHI* site of pBLCAT2 (a construct with the TK promoter from nt -105 to +51 driving chloramphenicol acetyltransferase [CAT] gene expression in a pUC18 background [26]). The -161/-130, -77/-49, and wild-type and mutant -59/-43 oligonucleotides, all of which contain *BamHI* or *BglII* ends, were cloned into the *BamHI* site of pBLCAT2. The IAP nt -245 to -73 fragment was obtained by digesting pIAP.1 (13) with *HaeIII* and inserted into the *BamHI* site of pBLCAT2 upstream of the TK promoter.

The nt -67 to +78 region driving CAT expression (18) was subcloned into the polyclonal region of pGEM3Z (Promega). The polyomavirus mutant enhancer PyF101 (9) (generously provided by E. Linney, Duke University, Durham, N.C.) was then cloned into the *EcoRI* site upstream of nt -67 to +78 by removing the PyF101 cassette with *PstI* and *EcoRI* and using *PstI* and *EcoRI* adaptors to convert the ends to *EcoRI* ends. Both pBLCAT2 and pBLCAT3 were gifts of D. Hall (Thomas Jefferson University, Philadelphia, Pa.), while the adenovirus E3 promoter expression vector pKCAT23 (47) was provided by R. Ricciardi (The Wistar Institute, Philadelphia, Pa.), and the adenovirus type 5 genomic E1A constructs pE1A.WT and pE1A.928 (30) were generously provided by E. Moran (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). The E1A-expressing vectors containing 12S and 13S cDNAs driven by the cytomegalovirus immediate-early promoter (2) and the deletion and point mutants were generously provided by J. R. Nevins (Duke University Medical Center, Durham, N.C.). The copy number and orientation of the various constructs were determined by restriction enzyme analysis or by nucleotide sequence analysis by the dideoxy chain termination method (Sequenase; United States Biochemical).

Cell culture, transfections, and growth hormone and CAT assays. F9 and PYS-2 cells in 60-mm plates were cotransfected with 10 μ g of CAT plasmid DNA, 4 μ g of pTKGH control plasmid, and 1 μ g of pUC18 by the standard calcium phosphate precipitation technique as described before (18). E1A cotransfections were performed in a similar manner but with only 0.6 μ g of pUC18 and 0.4 μ g of E1A expression plasmids. The levels of growth hormone were used to determine relative transfection efficiency within a cell type and to adjust the amount of cell extract used for the individual CAT assays. CAT activity was assayed by thin-layer chromatography as described before (18). Relative CAT activity was calculated by subtracting the activity of pBLCAT3 (background control) and then normalizing to pBLCAT2 activity.

Nuclear extracts and band shift and methylation interference assays. Nuclear extracts were prepared as described

before (18). The protease inhibitors phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (0.5 mg/ml), and pepstatin (0.7 mg/ml) were added at all steps of extract preparation (all were from Boehringer Mannheim). Protein concentrations were determined by a dye-binding assay (Bio-Rad). Band shift assays were initiated by incubating 5 μ g of nuclear extract with 0.5 μ g of double-stranded poly(dI-dC) (Pharmacia) for 20 min at room temperature. Probe was then added (50,000 cpm or approximately 0.2 ng), and reaction mixtures were incubated continuously for 20 min at 30°C. Probes were prepared by end labeling oligonucleotides before annealing with T4 polynucleotide kinase (Boehringer Mannheim) and [γ - 32 P]ATP by conventional procedures. DNA-protein complexes were resolved on 5% polyacrylamide gels run at 120 V for 120 min. The methylation interference assay was that described previously (18). Briefly, the end-labeled -62/-18 oligonucleotide was treated with dimethyl sulfate and used for binding. Binding reaction mixes were scaled up 30-fold, and after gel electrophoresis, free and bound probes were isolated, cleaved by piperidine, and run on a 12% polyacrylamide sequencing gel.

Southwestern (DNA-protein) analysis. Proteins (250 μ g) from nuclear extracts were resolved by electrophoresis in sodium dodecyl sulfate-9% polyacrylamide gels and transferred to nitrocellulose by overnight electrophoresis at 30 V, 4°C, in a buffer containing 25 mM Tris, 192 mM glycine and 15% (vol/vol) methanol, pH 8.3. Proteins on the nitrocellulose were then denatured with 6 M guanidine hydrochloride, renatured, and probed as described before (38). The probe consisted of a -59/-43 oligonucleotide end labeled with [γ - 32 P]ATP and ligated to form random concatamers.

RESULTS

Identification of a differentiation-specific element. We examined DNA elements that mediate a higher level of transcriptional activity in differentiated PE-like PYS-2 cells than in undifferentiated F9 cells. We reported previously that the IUE located between nt -186 and -167 within the IAP LTR is active in both undifferentiated and differentiated cells (18). The present study focuses on the sequence downstream of the nt -186 to -167 region. Transfection assays with plasmids containing an oligonucleotide from nt -161 to -130, cloned in different orientations and copy numbers upstream of the TK promoter, revealed a slight stimulation of transcription in both F9 and PYS-2 cells (Fig. 1A). By contrast, a DNA fragment from nt -134 to -73 cloned upstream of the TK promoter enhanced transcription in both F9 and PYS-2 cells independent of orientation and with increasing activity after multimerization (Fig. 1B). An oligonucleotide spanning the sequences from nt -77 to -49 cloned upstream of the TK promoter in different orientations and copy numbers also exhibited strong enhancer activity irrespective of cell type (Fig. 1C). These results indicate the absence of a differentiation-specific element upstream of nt -49 within the IAP LTR.

The nt -67 to +78 region was previously shown to direct differential expression of the reporter CAT gene, resulting in higher transcriptional activity in differentiated cells than in undifferentiated cells. Thus, an oligonucleotide spanning from nt -62 to -18 was used to look for elements that might be responsible for the differentiation-specific expression. Figure 2A shows that the sequence from nt -62 to -18 binds to a protein factor(s) present in both F9 and PYS-2 nuclear extracts to yield a DNA-protein complex, with the PYS-2 complex slightly more abundant than the F9 complex. The

FIG. 2. Localization of the IPE element (nt -53 to -47) by band shift (A) and methylation interference (B) analysis with PYS-2 and F9 nuclear extracts. (A) 32 P-labeled -62/-18 oligonucleotide was incubated with 6 μ g of either PYS-2 (lanes 1 to 3) or F9 (lanes 4 to 6) nuclear extracts, and a 20-fold molar excess of unlabeled -62/-18 (lanes 2 and 5) and irrelevant oligonucleotide (intron; lanes 3 and 6) competitor was added. (B) The piperidine cleavage patterns for both noncoding (lanes 1 to 3) and coding (lanes 4 to 6) strands. The probe not incubated with extract (G, lanes 1 and 4), and the free (F, lanes 2 and 5) and bound (B, lanes 3 and 6) forms of the probe are shown. The methylated G residues, from nt -53 to -47, that interfered with DNA-protein interaction are indicated.

unlabeled nt -62 to -18 fragment abolished DNA-protein interaction, but an unrelated DNA fragment had no effect. Thus, one or more factors bind specifically within the nt -62 to -18 region. Analysis of the dimethyl sulfate-methylated DNA-protein complex (Fig. 2B) showed that methylation of G residues at nt -49, -51, and -53 on the noncoding strand and nt -47 on the coding strand interfered with the DNA-protein interaction. Therefore, the factor-binding site, GAGTGAC, is located between nt -53 and -47.

Transfection assays with a plasmid containing five copies of the oligonucleotide (-59/-43) encompassing the protein-binding site cloned upstream of the TK promoter revealed the stimulation of expression from the TK promoter in differentiated PYS-2 cells but not in undifferentiated F9 cells

FIG. 3. Characterization of the IPE (nt -59 to -43) element. (A) Transient-transfection assays, indicating higher transcriptional activity in PYS-2 than in F9 cells from the reporter plasmid containing the nt -59 to -43 enhancer linked to the TK promoter. The mutant enhancers failed to promote such activity. The reporter plasmid used in the transient transfection is indicated above each lane. Levels of CAT activity, expressed as percentages of [14 C]chloramphenicol converted to acetylated products, are averages of three independent experiments and are indicated below the lanes. The CAT activity relative to the activity obtained with transfections of the TK promoter alone (pBLCAT2) is also shown. (B) Band shift analysis showing the specific binding of the IPE element to a transcription factor(s). The oligonucleotide competitors (5 ng, 50-fold molar excess to the -62/-18 probe) are indicated above each lane. The intron has sequence unrelated to nt -62 to -18. Mutations within the protein-binding site abolished the ability of the IPE element within nt -62 to -18 to bind to a factor.

(Fig. 3A, compare lanes 4 to 8). Thus, the nt -59 to -43 region functions as an IAP differentiation-specific enhancer and is hereafter designated the IAP-proximal enhancer (IPE).

To verify that the binding of a protein to the nt -53 to -47 element is responsible for the differentiation-specific expres-

FIG. 4. Southwestern analysis of nuclear proteins from PYS-2 and F9 cells. The sources of the proteins are indicated above the lanes. Proteins probed with the wild-type -59/-43 probe (lanes 1 to 4) and with mutant 2 -59/-43 probe (lane 5) are shown. Autoradiograms of lanes 1 and 2 were underexposed to illustrate the greater binding activity of the 60-kDa protein from PYS-2 cells than that from F9 cells and the presence of 68-kDa protein in both F9 and PYS-2 cells. Lanes 3 and 4 were overexposed to show the presence of 60-kDa protein-binding activity in F9 cells and also of 40-kDa proteins in F9 as well as PYS-2 cells.

sion of the IPE, oligonucleotides containing mutations covering the binding site were analyzed. In band shift assays, the wild-type -59/-43 oligonucleotide competed for protein binding to the labeled -62/-18 oligonucleotide, whereas the mutant oligonucleotides failed to compete, as is the case with an irrelevant sequence (Fig. 3B). Transfection assays showed that the mutations diminished the enhancer activity in differentiated PYS-2 cells almost to basal levels (Fig. 3A). Thus, mutations abolished the ability of the nt -53 to -47 element to bind to a specific protein factor(s) and elicit differentiation-specific expression.

Protein factors binding to the IPE element. Southwestern analyses were carried out to characterize the factors that bind to the IPE element. The concatenated -59/-43 probe bound to four polypeptides of 31, 40, 60, and 68 kDa (Fig. 4). The 31-kDa protein probably represents a nonspecific protein, since mt 2, with a mutation within the nt -59 to -43 region, also bound to this protein. The 40- and 68-kDa proteins from both undifferentiated F9 and differentiated PYS-2 cells had similar DNA-binding activities. However, the 60-kDa protein from PYS-2 cells had much greater

FIG. 5. Schematic representation of the two E1A proteins 243R and 289R (12S and 13S products) resulting from differential splicing. Three CRs shared by adenoviruses are indicated. The two proteins have identical amino- and carboxyl-terminal sequences, with CR3 unique to the 289R protein. Also shown are the two mutants with amino acids 2 to 36 and 38 to 67 deleted from the 243R proteins and a point mutation at amino acid 124 in the 243R protein.

FIG. 6. Effect of adenovirus E1A on IAP expression. PYS-2 cells were cotransfected with either pUC18, pE1A.WT, or pE1A.928 and the IAP CAT deletion constructs $-307/+78$ (lanes 1, 2, and 3, respectively), $-157/+78$ (lanes 4, 5, and 6, respectively), $-134/+78$ (lanes 7, 8, and 9, respectively), and $-67/+78$ (lanes 10, 11, and 12, respectively) or the adenovirus E3 CAT construct pKCAT23 (lanes 13, 14, and 15, respectively). Five independent transfection experiments yielded similar results.

DNA-binding activity than that from F9 cells, suggesting a role for this protein in differentiation-specific expression.

Identification of an E1A-sensitive element. Because several genes that are not transcribed in the undifferentiated F9 cells are also repressed by the adenovirus E1A gene, we analyzed

the effect of E1A on IAP expression. We cotransfected PYS-2 cells with the 5' IAP LTR deletion constructs (18) and a wild-type genomic construct (pE1A.WT) that expresses two E1A proteins 243 and 289 amino acids in length (243R and 289R, respectively). The two proteins are products of

FIG. 7. Mapping of the E1A-responsive element to the nt -67 to $+78$ region. (A) CAT activity of constructs containing either the IAP nt -307 to $+78$ (18) cotransfected with pUC18 (lane 1) and pE1A.WT (lane 2), the TK promoter alone (lane 3), or the IAP nt -245 to -73 enhancer fragment cloned upstream of the TK promoter cotransfected with pUC18 (lane 4) and pE1A.WT (lane 5) in PYS-2 cells. Four independent transfection experiments yielded similar results. (B) CAT activity of constructs containing either the IAP nt -67 to $+78$ (lane 1), the PyF101 enhancer cloned upstream of the IAP nt -67 to $+78$ region cotransfected with pUC18 (lane 2), and pE1A.WT (lane 3), the TK promoter alone (lane 4), or the PyF101 cloned upstream of the TK promoter cotransfected with pUC18 (lane 5) and pE1A.WT (lane 6) in PYS-2 cells. Three independent transfection experiments yielded similar results. (C) Schematic representation of CAT constructs containing either the IAP $-307/+78$ full-length deletion construct (drawing 1), the IAP $-245/-73$ enhancer fragment cloned upstream of the TK promoter (drawing 2), or the PyF101 enhancer cloned upstream of the IAP nt -67 to $+78$ region (drawing 3) or the TK promoter (drawing 4).

FIG. 8. Localization of the E1A-sensitive element to the IPE element. CAT activity of corresponding constructs cotransfected without or with pE1A.WT in PYS-2 or F9 cells. CAT activity, expressed as percentage of total chloramphenicol converted to acetylated derivatives, represents averages of three independent experiments and is shown below the lanes. CAT activity relative to the activity obtained from transfections with the TK promoter alone is also shown.

the 12S and 13S mRNAs which result from differential splicing of the primary E1A transcript. These two proteins have identical amino and carboxyl termini and differ in their primary sequences only by the presence of 46 amino acids that are unique to the 289R protein (4) (Fig. 5). pE1A.WT repressed expression of all of the IAP LTR deletion constructs (Fig. 6) and reduced CAT activity by 5- to 12-fold. The mutant E1A genomic construct pE1A.928 also repressed IAP expression. This construct expresses 12S and 13S products mutated at nt 928 (30) within a region (domain 2) that is common to all adenoviruses and important for the transformation and transcription repression activities of E1A (29). These results indicate that the mutated region is not essential for repression of the IAP enhancer activity in differentiated PYS-2 cells.

As a control for nonspecific inhibition by the E1A constructs, PYS-2 cells were cotransfected with the E1A constructs and pKCAT23, a construct in which the E1A-inducible adenovirus E3 promoter directs CAT gene expression (47). pKCAT23 expression was activated by cotransfection with either pE1A.WT or pE1A.928 (Fig. 6, lanes 13 to 15), indicating that repression of the IAP LTR by E1A is a specific effect. These experiments establish the existence of an E1A-sensitive regulatory element within the region between nt -67 and +78. This result was further confirmed by the observation that the IAP -245/-73 enhancer and the mutant polyomavirus enhancer (PyF101) (24), while not repressed by E1A when juxtaposed to the TK promoter, were highly repressed by E1A when joined to the IAP nt -67 to +78 region (Fig. 7A and B, respectively).

To determine whether the IPE (nt -59 to -43) is the site of E1A repression, pE1A.WT was cotransfected with the CAT plasmids containing the IPE linked to the TK promoter. Figure 8 shows that the E1A proteins repressed the IPE activity in PYS-2 cells and, as expected, did not affect the negatively controlled IPE activity in F9 cells.

As a step toward understanding the mechanism of E1A-mediated repression, we have determined the E1A sequences that are essential for repression. Three E1A domains are conserved among adenoviruses (Fig. 5). Conserved regions 1 (CR1, amino acids 41 to 79) and 2 (CR2, amino acids 121 to 139) are common to both the 289R and 243R proteins and appear to be required for both transcription repression (4) and oncogenic transformation, but not for

trans activation. Region 3 (CR3, amino acids 140 to 189) is specific to the 289R protein and is sufficient for E1A-mediated transcriptional activation (4). Our results indicate that both the 243R and 289R proteins, expressed from vectors containing 12S and 13S E1A cDNAs, respectively, repressed the IPE activity in PYS-2 cells (Fig. 9A), indicating that CR3 is not responsible for the repression. Nor is CR2 responsible for the repression, since a point mutation in amino acid residue 124 (nt 928) located within CR2 of the 12S cDNA did not affect the repression. Deletion of amino acids 2 to 36 from the N-terminal region and of amino acids 38 to 67 from CR1 alleviated the repression, although the deletion mutants encode stable proteins (Fig. 9B). Thus, the region within the N terminus and CR1 is essential to repression of the IPE. These results also show that it is E1A itself which represses transcription.

DISCUSSION

We showed previously that F9 cell differentiation-specific expression of the IAP gene, which occurs in differentiated PE-like PYS-2 cells but not in undifferentiated F9 cells, is likely mediated by CpG methylation of a developmentally neutral IUE element located between nt -180 and -173 relative to the transcription start site (18). In the present study, we demonstrate that an enhancer (IPE) element, GAGTGAC, located between nt -53 and -47 proximal to the basal promoter of the IAP gene, is also active in differentiated PYS-2 cells but not in undifferentiated F9 cells. Furthermore, the transcription-enhancing activity of the IPE element is repressed by E1A.

Three proteins, 40, 60, and 68 kDa in size, bind to the element. While the 40- and 68-kDa proteins from both undifferentiated and differentiated cells bind equally well to the IPE element, the 60-kDa protein from differentiated cells binds with greater efficiency than that from undifferentiated cells, resulting in a complex that is more abundant in differentiated cells than in undifferentiated cells. Thus, binding of the 60-kDa protein, which is either more abundant or more active in differentiated cells, is essential for active expression of the IPE. Our observation that the enhancers located upstream of the IPE do not contain a differentiation-specific element further suggests that restricted expression elicited by poor binding of the 60-kDa protein to the

FIG. 9. Identification of the N-terminal region of E1A as being responsible for *trans* repression in PYS-2 cells. **(A)** CAT activity of corresponding constructs cotransfected with expressing plasmids that contain E1A 12S or 13S cDNA or mutant cDNAs linked to the cytomegalovirus immediate-early promoter (see also Fig. 5). Deletion of amino acids 2 to 36 (Δ 2-36) and deletion of amino acids 38 to 67 (Δ 38-67) in the 12S cDNA. Construct 124 (928) mt has a point mutation at amino acid 124 (nt 928) in the 12S cDNA. CAT activity values expressed relative to the amount determined for the TK promoter alone and expressed relative to the activity from the TK promoters cotransfected by electroporation with plasmids expressing E1A or E1A mutants are from three different experiments. **(B)** Control experiments showing expression of E1A or E1A deletion mutants. PYS-2 cells were transfected with TKCAT (**a**), pE1A.WT (**b**), the Δ 2-36 mutant (**c**), and the Δ 38-67 mutant (**d**). Magnification, $\times 380$. Immunohistochemistry was performed by using a Vectastain ABC kit (Vector) with the M73 monoclonal antibody (kindly provided by E. Harlow [Massachusetts General Hospital Cancer Center, Charlestown, Mass.]).

IPE element in F9 cells is a dominant effect. That is, binding of the 60-kDa protein to the IPE may be essential for the active expression of the IAP gene. Analysis of the effect of mutations within this element on the transcriptional activity of the full-length IAP LTR should serve to clarify this question.

Although our results show that the 40-, 60-, and 68-kDa proteins bind independently to the IPE element, the binding sites must be very close or overlapping to yield the F9 and PYS-2 complexes with virtually identical mobilities and footprints in band shift and methylation interference assays, respectively. The identities of these IPE-binding proteins remain unknown. The IPE element, GAGTGAC, bears some resemblance to the E4TF1 and E4TF3 binding site (44, 46) and to the AP-1 (TGACTCA) and the CREB/ATF binding sites (TGACGTCA) (16). Although both AP-1 and ATF are associated with differentiation-specific and/or E1A-responsive gene expression (25, 31, 42, 45), and the sizes of the 40- and 60-kDa proteins resemble those of Jun and Fos, components of AP-1, respectively, our preliminary results indicate that the two proteins are not Jun or Fos.

We have shown that the transcriptional activity of the IPE is repressed by the N-terminal region of E1A that includes CR1. Since differentiated cell-specific expression of the IPE is controlled by the positive regulatory 40-, 60-, and 68-kDa proteins interacting with the enhancer, colocalization of the element associated with differentiation-specific expression and the element responsible for E1A-mediated repression indicates that the activities of the positive regulatory factors are altered by E1A expression. We speculate that the negative control mechanism(s) utilized by E1A may apply to the control of the IPE in F9 cells. In fact, the presence of E1A-like activity in undifferentiated F9 cells (14) has been suggested as a cause of the inability of the cells to support expression of many viral as well as cellular enhancers. It has been shown that an insulin enhancer under a negative control in non-insulin-producing cells is also the target for repression by the N-terminal region that includes the CR1 of E1A (39, 40, 49). Thus, the negative control mechanism mediated by E1A-like activity may not be unique to F9 cells but may instead represent a general mechanism that is operative in many viral and cellular genes in their nonexpressing cells.

The mechanism by which E1A controls transcriptional repression is not known. E1A functions not only as a *trans* repressor but also as a *trans* activator. The mechanism of activation by E1A has been investigated intensively. E1A does not bind to a specific DNA sequence (7), but instead stimulates transcription from many different promoters. Several studies have indicated that E1A regulates *trans* activation through three mechanisms. First, it increases transcription by directly binding to cellular transcription factors such as TFIID, ATF-2, and Oct 4, which in turn bind to elements found in various promoters (12, 20, 25, 35) and allow interaction of the E1A activating domain with the basic transcriptional machinery (22). Second, E1A *trans* activates by binding to cellular proteins such as the retinoblastoma gene product to dissociate transcriptionally inactive complexes such as E2F-RB, allowing E2F to become transcriptionally active (3). Lastly, E1A activates transcription by phosphorylation of preexisting transcription factors such as CREB and E2F, possibly through modification of a cellular kinase or phosphatase (1, 20, 51). Conceivably, similar E1A-protein interactions or post-transcription-

al modifications are involved in E1A-directed *trans* repression.

The permissiveness for growth of certain viruses during early mouse embryogenesis and during embryonal carcinoma cell differentiation (reviewed in reference 17) suggests the presence of an E1A-like activity in early embryos but not in differentiated cells. Given the ability of E1A to act as either a repressor or an activator, changes in the levels of the E1A-like activity could modulate several genes simultaneously upon differentiation of the pluripotent cells. In addition, since E1A immortalizes cells by stimulating DNA synthesis in quiescent cells, the E1A-like activity in early embryonic cells or embryonal carcinoma cells may help to promote rapid proliferation during early embryogenesis. Since the activity of the IPE-binding proteins is regulated by E1A, one of these proteins may be a candidate factor for exerting the E1A-like activity and provide a useful means to isolate the E1A-like activity and study its role in gene regulation during early embryogenesis.

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