The EMBO Journal vol.12 no.13 pp.4985-4992, 1993

Repression of adenovirus E1A enhancer activity by a novel zinc finger-containing DNA-binding protein related to the GLI-Kruppel protein

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Communicated by F.Blasi

We have previously shown that expression of the E1A oncogene is negatively regulated in rodent fibroblast cells by a nuclear factor (φAP3) that binds to the E1A promoter region just upstream of the canonical enhancer element. To understand how φAP3 can regulate E1A gene transcription by inactivation of the enhancer function, we have used an oligonucleotide probe containing a binding site for this protein to clone the mouse φAP3 gene. DNA sequence analysis of the 2.3 kb cDNA revealed the presence of six well-conserved zinc finger DNA-binding motifs, which were highly related to those found in the GLI-Kruppel family of human zinc finger proteins. Analysis of the tissue distribution of the φAP3 mRNA suggested that its expression was ubiquitous but at variable levels, most likely as a result of post-transcriptional regulation of mRNA stability. The φAP3 factor is a nuclear phosphoprotein; the extent of its phosphorylation is regulated during the cell cycle. Preferential binding of the hyperphosphorylated form of this protein to DNA was observed. Co-expression of the φAP3 cDNA and a luciferase reporter gene under the control of the E1A promoter/enhancer in several human cell lines resulted in repression of E1A enhancer activity. In contrast, when the φAP3 binding site upstream of the enhancer was mutated, no inhibition of enhancer function was observed. Based on these observations we conclude that we have cloned the cellular φAP3 gene, and that the DNA-binding activity of this protein is regulated during the cell cycle.

Key words: φAP3/negative regulation/transcription

Introduction

Eukaryotic enhancer sequences are among the most studied examples of a *cis*-acting regulatory elements and can modulate polymerase II transcription from both viral (Gruss *et al.*, 1981; Hearing and Shenk, 1983; Boshart *et al.*, 1985; Shaul *et al.*, 1985) and cellular genes (Neuberger, 1983; Fujita *et al.*, 1985; Hanahan, 1985). It is now generally accepted that the activities of many viral and tissue- or cell-specific enhancer elements are developmentally regulated or are active in specific cell types. As a consequence enhancer function can be either positively or negatively regulated. Several models have been proposed to explain the mechanism by which a cellular factor may negatively influence the rate of transcription initiation. The end result is often (but not exclusively) manifest by reduced occupancy of *cis*-regulatory elements by positively acting cellular transcription factors. Several viral and cellular genes, like the mouse albumin gene (Herbst *et al.*, 1989), the β-interferon gene (Goodbourn *et al.*, 1986), the *Drosophila hunchback* gene (Zuo *et al.*, 1991) and the adeno-associated P5 promoter (Shi *et al.*, 1991), have been shown to be negatively regulated as a consequence of the state of cellular differentiation, suggesting that this could represent a common mechanism by which tissue-specific gene expression is modulated.

Like many viral enhancers, the adenovirus E1A gene enhancer has been shown to be composed of multiple regulatory elements (Hearing and Shenk, 1983, 1986; Hardy and Shenk, 1988). Element I is present in two copies, at least one of which is required to maintain a high rate of E1A gene transcription. A cellular nuclear factor, EF-1A, has been identified that binds cooperatively to both copies of element I and to three enhancer-proximal sites (Bruder and Hearing, 1989, 1991). Element II is situated between the two elements I and modestly increases transcription from all promoters on the viral genome. The factor(s) that recognize these sequences have not yet been identified. A third region consists of two cellular E2F protein binding sites (Kovesdi *et al.*, 1986), although their contribution to the rate of E1A gene transcription during a virus infection is unclear. Additional sites of DNA-protein interactions have been mapped outside of the functional E1A enhancer domains, and are thought to be necessary for viral DNA replication and packaging (Barrett *et al.*, 1987; Hen *et al.*, 1983).

From previous studies in our laboratory, we observed that when compared with human cell types that support adenovirus DNA replication, the rate of viral E1A gene transcription was reduced by 5- to 10-fold in cell types that failed to support viral DNA replication (rodent fibroblast cells) or that were phenotypically undifferentiated (Herbst *et al.*, 1990a). We and others (Chinnadurai, 1992) have observed a negative effect of increased rates of E1A gene transcription on cellular transformation efficiencies of rodent cells. Since most of these rodent cell types are susceptible to transformation by adenovirus, we concluded that an early stage leading to establishment of the transformed cell phenotype is facilitated by an active mechanism which results in a reduced rate of viral E1A gene expression (Adami and Babiss, 1990).

Using several genetic approaches we identified a cellular protein (φAP3) that bound to a DNA sequence 5′ to the E1A enhancer region (between nucleotides −341 and −330 relative to the E1A gene capsite), which mediated repression of viral E1A gene expression through the inactivation of E1A enhancer function (Herbst *et al.*, 1990a). The DNA-binding activity of this factor was higher in cell types where we

observed this E1A enhancer repression phenotype, but interaction of the φAP3 protein with DNA did not preclude EF-1A or E2F protein−DNA interactions. Further support for this model was provided by the observation that deletion of the φAP3 binding site on the viral genome resulted in the restoration of enhancer-dependent E1A transcription in cells that maintained high φAP3 protein expression.

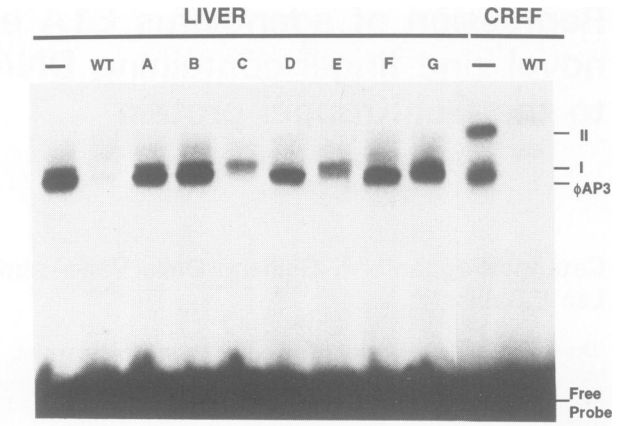
In an effort to determine the mechanism(s) that is responsible for φAP3-mediated inactivation of E1A enhancer function, we have cloned the cellular gene encoding this factor. DNA sequence analysis revealed the presence of six well-conserved zinc finger motifs which were highly related to the GLI-Kruppel family of human zinc finger proteins. Expression of the cloned φAP3 mouse liver cDNA in several human cell types resulted in the repression of E1A-enhancer dependent expression. In contrast, when the φAP3 DNA-binding site was mutated, over-expression of the mouse φAP3 protein did not lead to a reduction in E1A enhancer-dependent gene transcription. Based on these findings we believe we have isolated a cellular gene encoding a DNA-binding activity that functions to repress the activity of the viral E1A enhancer element. We propose several mechanisms to explain how this protein functions and what its role may be in regulating cell growth and development.

Results

Specificity of φAP3−DNA interactions upstream of the E1A enhancer element

A mutational analysis of the φAP3 DNA-binding site was performed to establish which bases were required for protein interaction and to identify a DNA sequence that had the greatest affinity for solely the φAP3 protein. This would provide us with a DNA probe which would be used to screen a mouse liver cDNA expression library. As shown in Figure 1, when we used a 32 bp oligonucleotide probe containing the φAP3 DNA-binding site [the minimal binding site is 5′-ATGTGGCAAAAG-3′ (Herbst *et al.*, 1990a)], a shifted complex (labeled φAP3) was observed using both mouse liver (heparin-fractionated) and cloned rat embryo fibroblast (CREF, Fisher *et al.*, 1972) cell (unfractionated) extracts. While φAP3 DNA-binding activities were comparable in this experiment, it should be noted that, as previously observed (Herbst *et al.*, 1990a), φAP3 DNA-binding activity was 5- to 7-fold greater for unfractionated CREF cell extracts than for unfractionated mouse liver extracts.

Formation of the φAP3−DNA complex was blocked by the addition of a 100-fold molar excess of the unlabeled WT oligo, but not by an oligonucleotide containing the C/EBP binding site which shares weak DNA homology (Figure 1, lanes marked WT and A respectively). An additional complex (complex I) was present in both extracts and most likely is an unrelated protein, as indicated by the competition pattern (see Figure 1). We have determined that this DNA-binding activity will not repress E1A transcription by replacing the WT φAP3 DNA-binding element with oligo C on a viral genome, and observing high E1A gene expression following CREF cell infection (R.S.Herbst and L.E.Babiss, data not shown). A third sequence-specific DNA−protein complex (complex II) was detected when either crude liver nuclear or CREF cell nuclear extracts were used in the shift gel assay. Since complex II could not be detected following heparin-fractionation of either extract, this



COMPETITOR	SEQUENCE	φAP3	I
WT	CGG ATG TGG CAA AAG TGC	+	+
A	AGG GTG TGG AAA GTC TCC	−	−
B	CGG ATc gGG CAA gcG TGC	−	−
C	CGG ATG TtG CAA cAG TGC	+	−
D	CGG ATG gGG CAA gAG TGC	−	+
E	CGG ATG gGG CAA cAG TGC	+	−
F	CGG ATc gGG CAA AAG TGC	−	+
G	CGG ATG TGG CAA gcG TGC	−	−

Fig. 1. Gel retardation analysis of mouse liver (heparin-fractionated) and CREF cell nuclear extracts. A ^{32}P-labeled oligonucleotide containing the φAP3 binding site (WT) was used as a substrate for binding reactions with each extract. The letter above each lane indicates the unlabeled competitor oligonucleotide used in each shift reaction. Bands corresponding to the φAP3 protein complex and to the protein complexes I and II are labeled. The sequences of the oligonucleotide competitors used in the reactions are shown. Competitor WT represents the oligonucleotide containing the binding site for φAP3 (underlined in the figure). Competitor A represents the oligonucleotide containing the C/EBP binding site. Competitors B−G represent oligonucleotides containing several point mutations (bold lower case in the figure) in the φAP3 binding site. The different competition profiles for the φAP3 protein complex and for protein complex I are indicated.

suggests that φAP3 protein can associate with another cellular protein(s) and this complex can recognize the same DNA sequence.

To identify which bases were required to stabilize the φAP3−DNA interaction and not complex I, we generated a series of oligonucleotides containing several transition or transversion mutations within the minimal φAP3 DNA-binding site. As shown in Figure 1, oligos with mutations of two bases (oligos C and E) blocked the formation of the φAP3−DNA complex, but did not block formation of complex I. In contrast, an A to G (competitor D) or G to C (competitor F) substitution blocked formation of complex I, but left φAP3 binding intact.

Isolation of a cDNA clone encoding φAP3

A mouse liver cDNA library, cloned in λgt11, was screened with a ^{32}P-labeled concatemerized form of oligo C. While it may have been preferable to screen a CREF cell cDNA library, the liver library was chosen since we could optimize the reaction conditions using a DNA probe that contained a C/EBP factor (liver-enriched) binding site. A single clone (mφAP3), was isolated after screening approximately one million plaques. The DNA-binding specificity of the fusion protein produced in this phage clone was first assessed by using the filter binding assay that formed the basis for the screening (Vinson *et al.*, 1988). Purified phages were

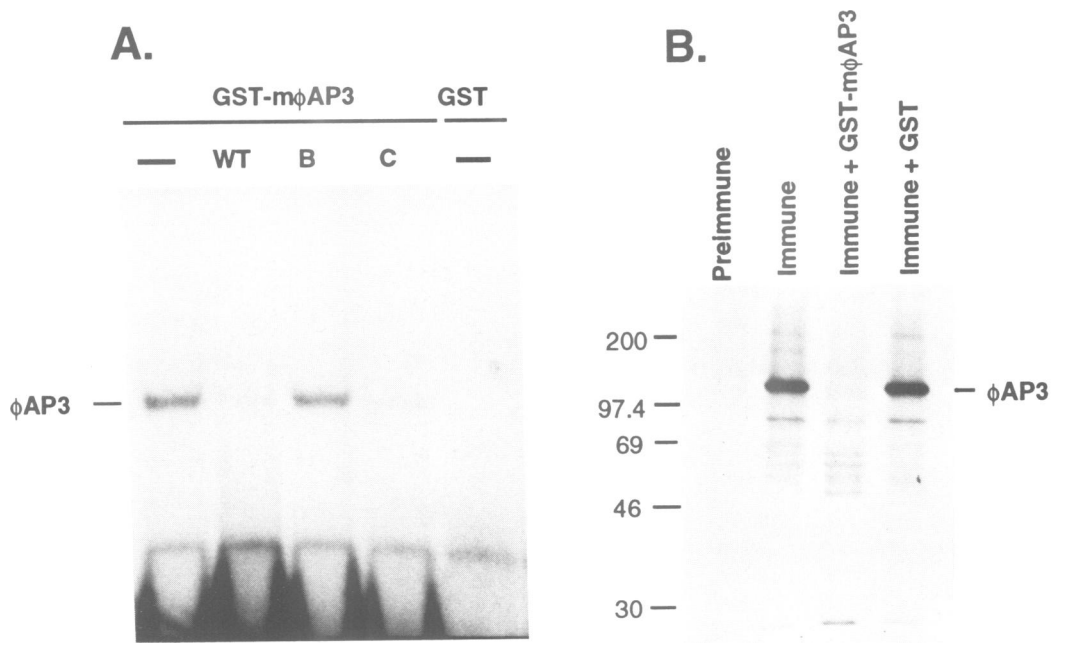


Fig. 2. (A) DNA-binding specificity of the *E.coli*-expressed GST−mφAP3 fusion protein. The DNA-binding activity of the purified GST−mφAP3 fusion protein (20 μg per lane) was tested in a gel mobility shift assay using the ^{32}P-labeled WT oligonucleotide. Purified GST alone was used as a control. Letters above the lanes indicate the unlabeled competitor oligonucleotides used in each reaction. The φAP3 protein complex is labeled. (B) φAP3 protein was immunoprecipitated from [^{35}S]methionine-labeled CREF cells using anti-GST−mφAP3 serum. Preimmune serum served as a control. Incubation of excess fusion protein GST−mφAP3 and GST in these reactions was used to compete for specific anti-φAP3 binding. The anti-φAP3 immunoprecipitate and the migration of molecular size markers are indicated.

transferred on to nitrocellulose filters, treated with guanidine-HCl and incubated with a ^{32}P-labeled concatenated oligo WT probe under the conditions used for library screening (Materials and methods). The mφAP3 phage fusion protein bound strongly to the concatenated oligo WT probe, but failed to bind the C/EBP binding site oligo and the oligo containing the mutated φAP3 binding site (oligo B) (data not shown).

DNA-binding specificity was studied in greater detail by expressing the mφAP3 protein on the pGEX-2T plasmid, where the cloned φAP3 protein was fused to the carboxyl-terminus of glutathione-S-transferase (GST), giving the GST−mφAP3 fusion protein. Bacterial extracts containing products of this clone were tested in band shift assays with WT oligo as a probe. Extracts from this clone gave a single band (Figure 2A), which was competed by a 100-fold molar excess of unlabeled WT oligo and oligo C competitor DNAs. In contrast, oligo B failed to compete in this assay, suggesting that we had in fact isolated and expressed the protein that is responsible for the φAP3 shifted complex, and not the protein(s) involved in complex I (see Figure 1).

Antibodies against the mφAP3 protein recognize the CREF cell φAP3 protein

To determine the relatedness of the endogenous and cloned φAP3 proteins, the purified fusion protein, GST−mφAP3, was used to generate a rabbit antiserum. The specificity of this serum was tested by immunoprecipitation using whole-cell lysates prepared from [^{35}S]methionine-labeled CREF cells. SDS−PAGE analysis of an anti-φAP3 immunoprecipitate revealed a predominant 100−110 kDa band, which was not recognized by the cognate preimmune serum (Figure 2B). Immunoprecipitation of the 100−110 kDa protein was competitively inhibited by excess GST−mφAP3 protein, but not by excess GST alone. This suggests that the p100−110 protein and the GST−mφAP3 fusion protein share common epitopes and are likely to be related. A similar pattern of endogenous φAP3 protein expression was observed when [^{35}S]methionine-labeled extracts from mouse and rat hepatoma cells were analyzed by immunoprecipitation (data not shown).

In addition to the p100−110 protein, a protein of 85 kDa was also observed following immunoprecipitation using the anti-φAP3 serum. While this may represent a p100−110 breakdown product (although it was not competed by excess GST−mφAP3 protein), it is possible that this protein interacts with φAP3 to produce the shifted complex II. While the p100−110 protein could be detected by Western blot analysis, the 85 kDa protein was not, again suggesting that the proteins are distinct.

The anti-mφAP3 serum was also used to determine the subcellular localization of the cloned and endogenous φAP3 proteins. Whether expressed endogenously or from a cytomegalovirus (CMV) plasmid expression vector, the φAP3 protein showed a very distinct nuclear staining pattern with exclusion of nucleoli (data not shown). Additional studies revealed that the intensity and subcellular localization of the φAP3 protein did not change as a function of the cell cycle.

Finally, we used the anti-mφAP3 serum to determine whether the φAP3 protein is phosphorylated. [^{35}S]methionine- or [^{32}P]orthophosphate-labeled CREF cell extracts were immunoprecipitated using the mφAP3 polyclonal serum. As shown in Figure 3, cells that were serum-starved (lane 5), cycling (lane 6) or serum-starved and induced to cycle by TPA (phorbol ester tumor-promoting agent) or adenovirus infection (induces cells to cycle; lanes 2, 3 and 4), all maintained similar amounts of [^{35}S]methionine-labeled φAP3 protein (Figure 3A). To control for variations in cell numbers during the labeling

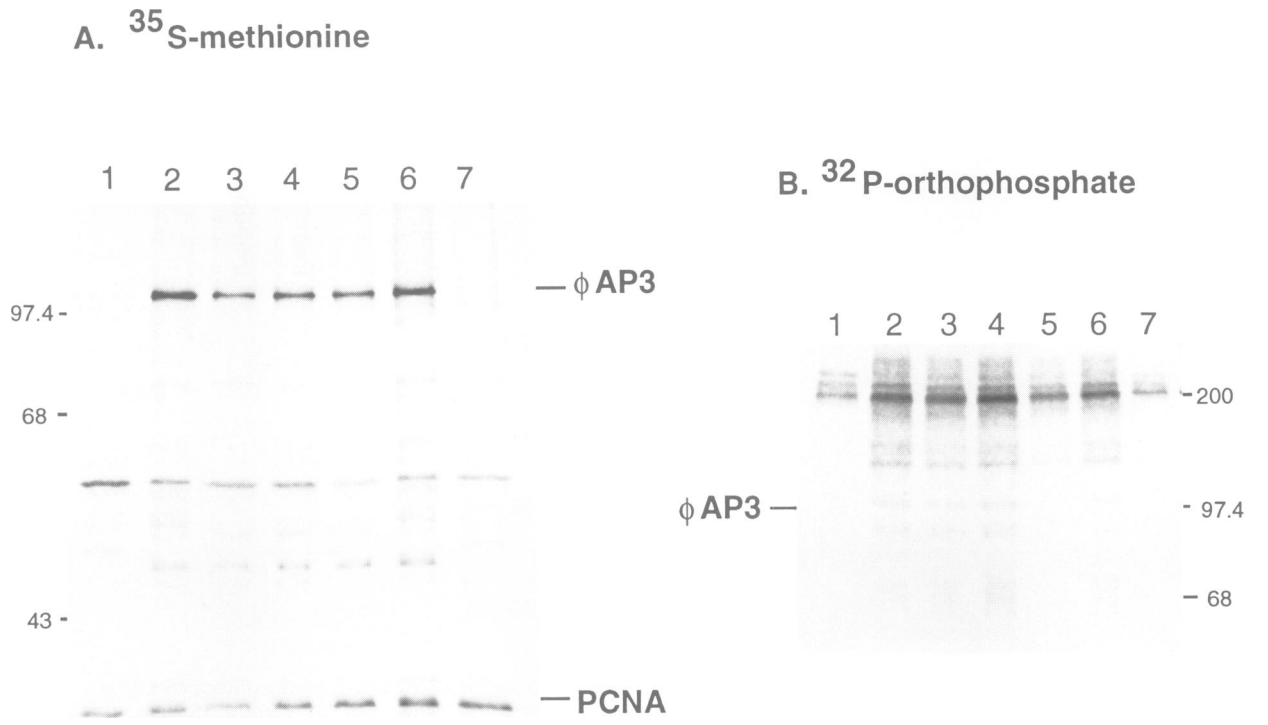


Fig. 3. Induction of φAP3 phosphorylation following treatment with TPA or infection by adenovirus of CREF cells. (**A**) φAP3 protein was immunoprecipitated from [^{35}S]methionine-labeled CREF cells, using anti-mφAP3 serum (lanes 2–6). Preimmune serum (lanes 1 and 7) was used as a control. CREF cells were serum-starved for 48 h (lanes 1–5), at which time serum (lane 2), TPA (100 ng/ml for 6 h, lane 4), or Ad5Wt (20 p.f.u./cell for 12 h, lane 3) was added to cells, and the cells were labeled for 4 h. CREF cells maintained in 10% serum throughout the study are shown in lane 6. (**B**) CREF cell were labeled in phosphate-free DMEM containing [^{32}P]orthophosphate (400 μCi/ml, for 2 h) and immunoprecipitated with anti-mφAP3 serum (lanes 2–6). Preimmune serum (lanes 1 and 7) was used as a control. The lane designations are as described for panel A. The anti-φAP3 immunoprecipitate and the anti-phospho-φAP3 immunoprecipitate are indicated.

period and extract preparation, we co-immunoprecipitated the rodent PCNA protein. Expression of this protein was found to be similar for all the cell incubation conditions. Cycling cells as well as serum-starved cells treated with TPA or adenovirus, expressed a p100–110 protein activity that was highly phosphorylated (Figure 3B, lanes 2–4 and 6). In contrast, growth-arrested cells maintained a high basal level of the protein, but very little existed in a phosphorylated state (lane 5). Additional bands that are present only in the immune serum-treated samples probably represent φAP3 protein breakdown products. We have previously shown that when serum-starved CREF cells are exposed to TPA, φAP3 DNA-binding activity is enhanced by 5-fold (Herbst *et al.*, 1990a). Therefore, DNA-binding activity is apparently determined by the phosphorylation state.

The φAP3 protein contains six zinc fingers that are highly related to the GLI-Kruppel family of genes

DNA sequence analysis revealed that the mφAP3 clone was 2321 bases in length and contained a single open reading frame that initiated with an ATG codon at nucleotide position 286 and terminated at nucleotide 2157 (asterisk in Figure 4). By primer extension analysis using both CREF cell and mouse hepatocyte mRNA, and by DNA sequence analysis of several CREF cell-derived φAP3 cDNA clones, we conclude that the mφAP3 cDNA clone lacked ~100–200 bases of 5′ untranslated sequence, while the 3′ end was complete (data not shown). The most obvious structural characteristic of the φAP3 protein was the six putative zinc fingers, two of which were clustered at the amino-terminus. The other four were located between nucleotides 1113 and 1512. The φAP3 zinc finger motifs were found to be related to those of GLI-Kruppel family genes (Ruppert *et al.*, 1988). All six belong to the Kruppel subgroup (C-X_2-C-X_{12}-H-X_3-H) and four of them show the conserved intrafinger Phe and Leu. DNA and protein searches in the GenBank and EMBL databases produced no extensive homologies with other genes, with the exception of the zinc finger domains, indicating that φAP3 is a unique protein. Southern hybridization analysis using genomic DNA from rodent cell types revealed that this gene is most probably not a member of a multigene family of zinc finger-containing proteins (data not shown).

Analysis of mRNAs from a wide variety of mouse tissues and rodent CREF cells (the latter was probed with a CREF cell-derived φAP3 cDNA clone, data not shown) revealed a single mRNA species that was ~2.5 kb in size (Figure 5). Expression of this mRNA was ubiquitous, although φAP3 mRNA levels varied widely among the different tissue types. When analyzed at the level of transcriptional initiation by nuclear run-on analysis, all cell types showed low but equal rates of transcription (data not shown). Based on these observations we conclude that φAP3 mRNA levels among the different tissue types is most probably regulated post-transcriptionally.

Exogenous transient expression of the mφAP3 cDNA in human 293 and WI38 cells blocks E1A enhancer function

To determine whether the cloned mφAP3 protein could function to suppress E1A enhancer-dependent transcription, transient transfection experiments were performed. The

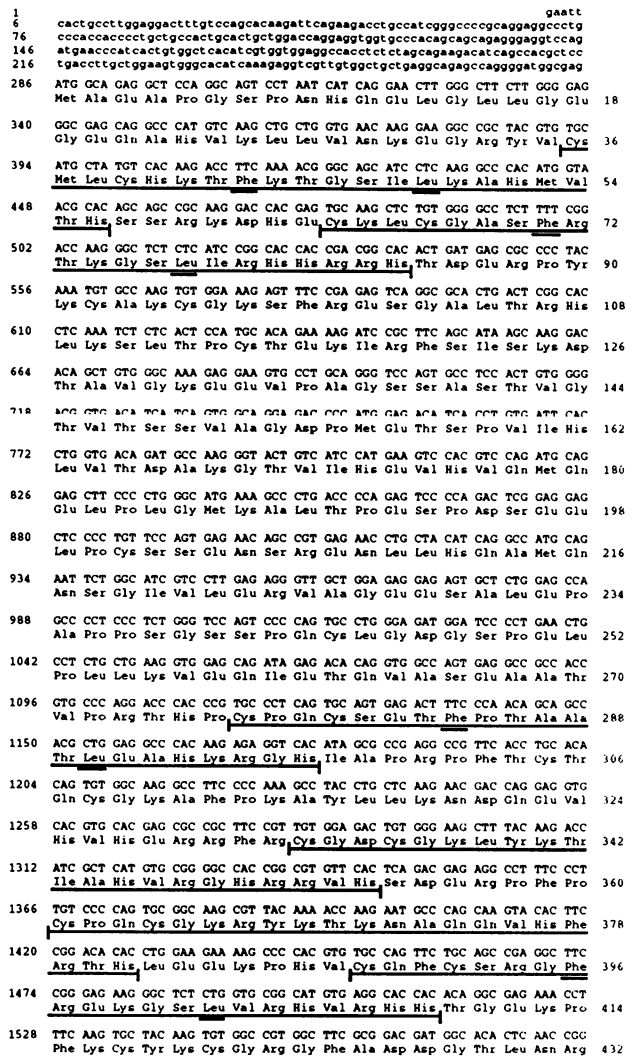


Fig. 4. Nucleotide and deduced protein sequence of the mϕAP3 cDNA. The cDNA sequence of ϕAP3 and the predicted protein sequence are shown. Peptide sequence numbering starts at the putative translation initiation codon ATG (nucleotide 286). The stop codon is denoted by asterisks. The zinc finger sequences are underlined. The conserved intrafinger Phe and Leu are doubly underlined, and the potential polyadenylation signal sequence located in the 3′ untranslated region is noted.

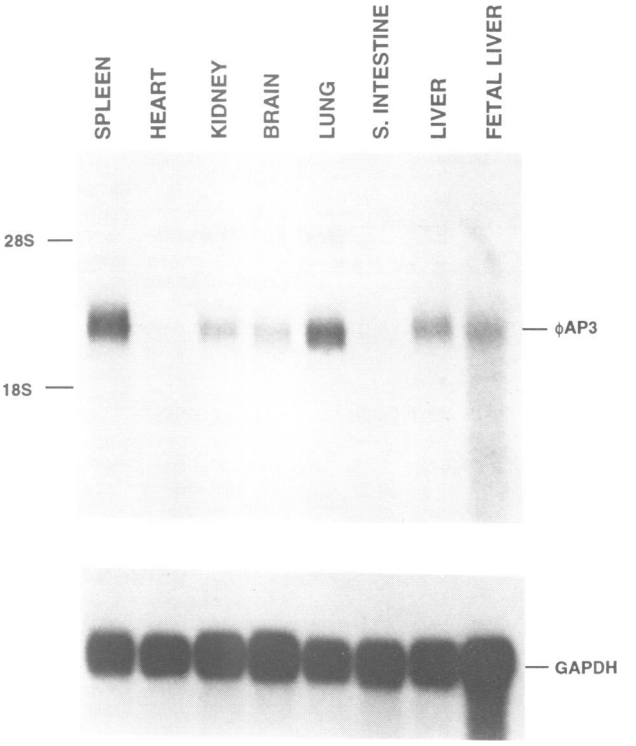
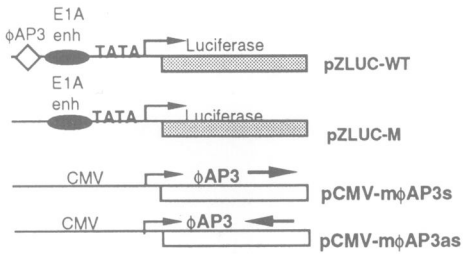


Fig. 5. Analysis of tissue distribution of the ϕAP3 mRNA. Poly(A)$^+$ RNA (5 μg per lane) from the different tissues shown above each lane were analyzed by Northern hybridization, using the full-length ^{32}P-labeled mϕAP3 cDNA and GAPDH cDNA probes. The bands corresponding to the 18S and 28S ribosomal RNA and to the ϕAP3 mRNA are indicated.

mϕAP3 cDNA was cloned in the sense and antisense orientation downstream of the CMV promoter within the expression plasmid vector pCMV (Figure 6A). As a reporter, we used pZLUC, in which the luciferase gene was placed under the control of the E1A promoter including the enhancer and the ϕAP3 binding site (pZLUC-WT). As controls, we used the pCMV vector alone and a modified reporter plasmid, pZLUC-M, which contained four point mutations within the ϕAP3 DNA-binding sequence. CREF cells were not used in these studies since they were found to be refractory to plasmid DNA transfection. 293 (human embryo kidney) and WI38 (human fibroblast) cells were chosen since the former have a high transfection efficiency owing to constitutive viral E1B gene expression, and endogenous ϕAP3 protein expression was low as judged by Western blotting (Figure 6C). In contrast, human WI38 cells expressed ϕAP3 protein at levels comparable to that of CREF cells.

When the CMV-based plasmids were independently transfected into 293 or WI38 cells, only the plasmid construct containing the ϕAP3 cDNA in the sense orientation resulted in expression of the mϕAP3 protein, as detected by Western blot analysis (Figure 6C). Under these experimental conditions we would predict that E1A enhancer-dependent expression would be repressed. When the antisense version of the mϕAP3 cDNA was expressed in these cells, endogenously expressed ϕAP3 protein could not be detected. Under these experimental conditions and in situations where the ϕAP3 DNA-binding site was mutated (pZLUC-M) we would predict a higher rate of luciferase gene transcription from the E1A promoter, when compared with cells transfected with the pCMV vector alone.

A.



B.

ϕAP3 mediated suppression of the adenovirus E1A enhancer

Plasmids	Cell Line	Relative Luciferase Activity
pZLUC-WT + pCMV-mϕAP3s	293	0.17 ± .06
pZLUC-WT + pCMV-mϕAP3as	293	2.6 ± .06
pZLUC-WT + pCMV	293	1.15 ± .08
pZLUC-WT	293	1.0 ± .08
pZLUC-M	293	2.0 ± .08
pZLUC-M + pCMV-mϕAP3s	293	2.5 ± .1
pZLUC-WT + pCMV-mϕAP3s	WI38	0.2 ± .047
pZLUC-WT	WI38	1.0 ± .015

C.

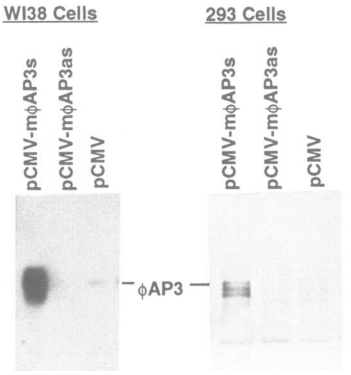


Fig. 6. Down-regulation of E1a enhancer activity by the mϕAP3 protein. **(A)** Plasmid constructs used in the transfection experiments. The E1a promoter which includes the enhancer and the ϕAP3 binding site, was subcloned upstream of the luciferase reporter gene (pZLUC-WT). pZLUC-M represents the same construct, but it contains four point mutations in the ϕAP3 binding site so the ϕAP3 factor no longer binds to it. The ϕAP3 cDNA was placed under the control of the cytomegalovirus promoter in the sense (pCMV-mϕAP3s) and antisense orientations (pCMV-mϕAP3as). **(B)** The constructs shown were transfected alone or in combination into 293 or WI38 cells. Following 48 h of incubation, the cell extracts were used to determine relative luciferase activity. The table summarizes the relative luciferase activity of each transfection and is the average of at least three experiments. **(C)** Western blot analysis of endogenous and mϕAP3 gene expression following DNA transfection of 293 and WI38 cells. Following 48 h of incubation post-transfection, total cell extracts were prepared, and 10 μl of each extract was electrophoresed on a 10% SDS−polyacrylamide gel. The proteins were blotted on to nitrocellulose and incubated with anti-mϕAP3 serum. Protein bands were visualized following the ECL protocol (Amersham). The different transfected constructs used are indicated above each lane and the position of the ϕAP3 factor is shown.

As shown in Figure 6B, over-expression of the mϕAP3 protein from the pCMV-mϕAP3 vector in both WI38 and 293 cells resulted in a 5-fold decrease in luciferase protein expression, while the control pCMV had no effect. When antisense mϕAP3 RNA was expressed in 293 cells, we observed a 2.5-fold increase in luciferase activity, suggesting that inhibition of endogenous ϕAP3 protein expression by antisense RNA in this cell line leads to up-regulated E1A enhancer activity. However, over-expression of the antisense mϕAP3 cDNA in WI38 cells during the time required to detect high luciferase activity was cytotoxic. This could be explained by the specific down-regulation of endogenous ϕAP3 expression or expression of a cytotoxic protein from the antisense RNA. However, sequence and codon usage analysis of the antisense RNA revealed no functional capsites or open reading frames of significant length. These results indicate that the mϕAP3 and human ϕAP3 proteins function in a similar manner to regulate negatively the enhancer-dependent E1A transcription.

To confirm that the mϕAP3 was able to exert its effects by binding to the cognate DNA sequence upstream of the E1A enhancer, 293 cells were co-transfected with the CMV constructs and the pZLUC-M plasmid. As shown in Figure 6B, mutating the ϕAP3 DNA-binding site led to a 2-fold increase in luciferase gene expression, regardless of which pCMV vector was used in the co-transfection assay. Therefore, when the ϕAP3 protein−DNA binding site is mutated, neither the mϕAP3 or human ϕAP3 proteins could exert their effects.

Discussion

In this paper we have begun to explore the mechanism by which viral E1A gene enhancer activity is repressed by the cellular ϕAP3 protein. To achieve this goal we have cloned a mouse liver cDNA that encodes a protein that can bind to the ϕAP3 DNA sequence. Using several experimental approaches, we demonstrated that the cloned ϕAP3 protein had all of the biological attributes of the ϕAP3 protein expressed by CREF cells. Like many cellular proteins that have been found to function by inhibiting the rate of initiation of pol II transcription, the mϕAP3 protein was shown to contain six zinc finger motifs, which were related to the GLI-Kruppel proteins. Interestingly, like the zinc finger-containing PRDII-BF1 protein described by Fan and Maniatis (1990), the mϕAP3 protein contained two widely separated zinc finger motifs. We have now expressed the two zinc finger-containing domains of the mϕAP3 protein in *Escherichia coli* (nucleotides 0−933 and 933−2321, see Figure 4) and have found that both are capable of recognizing the consensus ϕAP3 DNA sequence (unpublished observation). The cloning of the gene encoding

the φAP3 factor represents the first step towards our understanding of the molecular mechanism(s) that contribute to E1A transcription regulation.

While much attention has been focused on understanding how cellular transcription factors activate and/or stimulate viral and cellular gene transcription, increasing evidence suggests that the regulation of many genes is the result of a balance between positive and negative regulatory proteins. Negative regulatory proteins can function to block completely the expression of specific genes in specialized cells at distinct developmental stages, to fine tune expression in response to an extracellular stimulus, or—as is probably the case for the φAP3 protein—they can regulate the rate of transcriptional initiation in specific cell types (Herbst *et al.*, 1990a). Analyses of the mechanisms of action of the known negative regulatory proteins have thus far revealed three distinct functional classes [for a review see Levine and Manley (1989) and Renkawitz (1990)]. These include (i) proteins which compete with positively acting DNA-binding proteins for the same DNA-binding site, (ii) proteins that can either physically interact with a positive regulatory protein or bind to a different DNA sequence, and block transcriptional activation (quenching and squelching) and (iii) proteins that recognize a DNA element which can function in an orientation- and enhancer-independent manner to block (silence) the formation of an active transcription complex. In this case the silencer could function in a promoter-independent manner, much as we have proposed for the NLS-1 protein which regulates mouse albumin gene expression (Herbst *et al.*, 1990b).

While we have demonstrated that E1A gene expression can be repressed by the cloned mφAP3 protein, it should be noted that the E1A proteins (in particular the 243R protein) have been shown to repress the expression of enhancer-dependent genes. Therefore, does E1A expression lead to deregulated cellular φAP3 gene expression? We have tested this idea and have found that expression of either E1A gene protein in cell types that are permissive (HeLa or 293) or non-permissive (CREF or NIH 3T3) for adenovirus DNA replication did not affect cellular φAP3 transcription rates, mRNA levels or DNA-binding activity (Herbst *et al.*, 1990a and unpublished observations).

Ruppert *et al.* (1990) have described the transformation of rat embryo fibroblast cells by a cooperative effect of adenovirus E1A and the human GLI genes. Like φAP3, GLI is a nuclear phosphoprotein containing five zinc fingers. Interestingly, when E1A expression was measured in several co-transformed cell lines, it was lower than that found in parental cells expressing E1A alone. While φAP3 and GLI do not share the same DNA-binding site sequences, this observation supports our contention that like φAP3, GLI may repress E1A expression. This may explain the mechanism that allows GLI and E1A to cooperate to elicit a transformed cell state.

Antibodies raised against *E.coli*-expressed GST−mφAP3 reacted with a nuclear phosphoprotein expressed in CREF cells (Figure 3). By immunoprecipitation, the anti-mφAP3 serum recognized a set of proteins, which were ~110−100 kDa in size. The multitude of bands probably reflects post-translational modification (e.g. phosphorylation) of a single protein species. Interestingly, the extent of φAP3 protein phosphorylation varied during the cell cycle. Actively dividing cells expressed the hyperphosphorylated φAP3 protein and had enhanced φAP3 DNA-binding activity. In contrast non-dividing quiescent cells expressed a hypophosphorylated φAP3 protein that had less DNA avidity. These observations suggest that the cellular genes that are targets for negative regulation by the φAP3 protein may encode proteins which function to inhibit cells from cycling. Such genes could include those that function to maintain a terminal state of cellular differentiation. As indirect support for this hypothesis, we have observed that over-expression of the mφAP3 cDNA in the antisense orientation led to WI38 cell death (Figure 6).

An understanding of the biological significance of down-regulation of E1A gene expression in fetal rodent cell types may help to explain why adenovirus can transform these cell types. However, these cells are not generally targets for adenovirus infection in nature. Upper respiratory tract infections caused by adenovirus in humans can lead to latent infections of cells in the lymphatic system (including B cells and adenoidal tissues). Since adenovirus fails to replicate efficiently in these cell types, it would be interesting to measure the expression and percentage of hyperphosphorylated φAP3 protein that was present in these cells. Based on our model we would predict that like CREF cells, these cells would possess elevated φAP3 DNA-binding activities. Such a study would also lead to the identification of those cellular genes whose rate of transcription is regulated by the φAP3 protein.

Materials and methods

Gel mobility shift analysis

Nuclear extracts were prepared from CREF cells and mouse liver hepatocytes according to the protocols of Dignam *et al.* (1983) and Herbst *et al.* (1990b), and gel retardation analysis was performed as previously described (Herbst *et al.*, 1990a). End-labeled double-stranded oligonucleotides containing a wild-type or mutated φAP3 binding site were incubated with 1−10 μg of nuclear protein, 4 μg of poly(dI−dC) (Pharmacia) and 0.5 μg of PGEM1 plasmid DNA, and incubated for 20 min at room temperature in 20 μl of binding buffer containing 40 mM KCl, 20 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol and 4% Ficoll (Pharmacia Fine Chemicals). Protein−DNA complexes were separated from free probe on a 6% non-denaturing polyacrylamide gel.

Library screening

A λgt11 mouse liver cDNA library was used to screen for the φAP3 cDNA. 1×10^6 p.f.u. of phage were plated with *E.coli* Y1090 on 150 mm plates and grown at 42°C for 4 h. The plates were overlaid with nitrocellulose filters saturated with 10 mM IPTG, for 6 h at 37°C. The filters were air-dried and processed for binding following the protocol described by Vinson *et al.* (1988). The probe was generated by concatenating the double-stranded oligo WT (Figure 1) and labeled using DNA polymerase (Klenow) and [^{32}P]dNTPs. The filters were then washed three times in binding buffer with 0.1% Triton X-100, air-dried and exposed for autoradiography.

mφAP3 protein expression, isolation and analysis

The pGST−mφAP3 plasmid contains the full-length mφAP3 cDNA, fused to the carboxyl-terminus of the GST protein. It was constructed by insertion of the partial *Eco*RI digestion product from mφAP3 λ phage DNA into *Eco*RI-linearized pGEX-2T plasmid (Smith and Johnson, 1988). Expression and isolation of the GST−mφAP3 fusion protein expressed from this plasmid vector were performed as described by Smith and Johnson (1988). The eluted purified protein, with 30% glycerol added, was stored at −70°C .

The GST−mφAP3 fusion protein was purified by preparative SDS−PAGE. The GST−mφAP3 fusion protein was visualized by briefly staining the gel with 0.05% Coomassie brilliant blue R-250 prepared in water and then extensive destaining in water. The gel slice was minced and passed through a 21 gauge needle several times. Two New Zealand White rabbits were injected with this material every 2 weeks. Preimmune sera were obtained prior to the first injections.

Endogenous and exogenously expressed mφAP3 protein was analyzed by immunoprecipitation of [^{35}S]methionine- or [^{32}P]orthophosphate-labeled crude cell lysates. Briefly, 10^6 CREF cell were incubated for 4 h at 37°C

in DMEM minus methionine, and containing 250 μCi per ml of [^{35}S]methionine or 400 μCi per ml of [^{32}P]orthophosphate. Prior to labeling, in some experiments, CREF cells were serum-starved for 48 h (0.2% serum) prior to incubation with TPA (100 ng per ml for 6 h) or adenovirus type 5 (20 p.f.u./cell for 12 h), and metabolic labeling. The cells were washed with ice-cold PBS and harvested by the addition of ice-cold RIPA buffer [10 mM Tris−HCl (pH 7.5), 0.15 M NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 mM EDTA and 10 mM KCl]. The lysates were vortexed for 30 s and cleared by centrifugation for 10 min at 4°C. The supernatant was incubated with preimmune serum (10 μl) for 60 min at 4°C, then protein A/G agarose (15 μl, Oncogene Science) was added for 60 min. Following centrifugation, the supernatant was incubated with 10 μl of anti-GST−mφAP3 serum for 16 h at 4°C with agitation. Antibody−antigen complexes were recovered using protein A−Sepharose beads and washed four times in ice-cold RIPA buffer and twice in ice-cold RIPA buffer containing 1 M NaCl. The bound proteins were eluted in SDS gel loading buffer and the proteins were resolved on a 10% polyacrylamide−SDS gel and visualized by autoradiography.

Cell transfection and luciferase assays

The pCMV-mφAP3 plasmid contains the entire cDNA insert from a mφAP3 phage clone under the control of the CMV promoter. It was obtained by partial digestion of phage DNA with *Eco*RI. Two *Not*I polylinkers were ligated to the insert and the cDNA insert was subcloned into *Not*I-linearized pCVM plasmid (MacGregor *et al.*, 1989). The E1A promoter, enhancer and φAP3 protein binding regions (extending from nucleotide −599 to +41 relative to the E1A gene capsite) were subcloned into the *Bam*HI site upstream of the luciferase gene in the pZLUC plasmid (Maxwell *et al.*, 1989). Plasmid DNA transfections were performed as follows. 5×10^5 293 or WI38 cells were transfected with various combinations of plasmid DNA expression vectors and reporter gene constructs, by calcium phosphate precipitation (Graham and van der Eb, 1973). Forty-eight hours following transfection, the cells were harvested in 200 μl of lysis buffer [25 mM Tris−phosphate (pH 7.8), 2 mM DTT, 2 mM EDTA, 10% glycerol and 1% Triton X-100]. 10 μl of the cell extracts were used to determine the luciferase activities as previously described (Brasier *et al.* 1989).

RNA analysis

Cytoplasmic RNA was isolated from mouse tissues as previously described (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was separated using oligo(dT)−cellulose (Sambrook *et al.*, 1989). The φAP3 mRNA levels of the different tissues were compared by Northern hybridization analysis. 5 μg of poly(A)$^+$ RNA was loaded into each lane of a 1.0% agarose−formaldehyde gel and, following electrophoresis, the RNA was blotted on to a Hybond-N filter (Amersham). The filter was prehybridized and subsequently incubated with a ^{32}P-labeled randomly primed cDNA probe (mφAP3 cDNA or human GAPDH cDNA) in 6 × SSC, 10 × Denhardt's, 0.5% SDS, 2 mM EDTA, 50 mM sodium phosphate pH 7.2 and 100 μg/ml of salmon sperm DNA, for 16 h at 65°C. Following hybridization, the filters were washed to a final stringency of 0.5 × SSC, 0.2% SDS at 65°C and exposed to autoradiography film.

Acknowledgements

The authors wish to thank Dr Jeff Hanvey, Dr Suzanne Neill, Dr Guy Adami and Nancy Peffer for critically reading this manuscript, Dr Jovan Mirkovitch for his assistance with the Southern hybridization studies, and Dr Ulrich Nielsch. C.F. was supported by the Italian Ph.D. program (University of Piacenza) and the Associazione Italiana Riceerca Cancro. This work was aided by a grant from the American Cancer Society (JFRA-235) and by Public Health service grant CA48707-10 from the National Institutes of Health to L.E.B.

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Received on March 11, 1993; revised on August 26, 1993