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Two different factors bind to the α-domain of the polyoma virus enhancer, one of which also interacts with the SV40 and c-*fos* enhancers

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Two nuclear factors from mouse 3T6 cells bind to a 22-bp segment constituting the α-domain of the polyoma virus enhancer. Binding of each factor can be competed out selectively by the appropriate double-stranded oligonucleotide, indicating that this binding is not strictly cooperative. Sequence homology between the two binding sites and the similar size of the protected regions may indicate that both factors, PEA1 and PEA2, are closely related. The binding site of PEA1 is centered on a sequence showing strong homology to the SV40 enhancer, the binding site of PEA2 is located immediately adjacent to it and shows a strong homology to the c-*fos* enhancer. Surprisingly, both SV40 and c-*fos* enhancers interact with PEA1, probably due to the presence of an extra base pair relative to c-*fos* in the PEA2 site. Factor PEA1 is probably identical to the recently described activator protein 1 (AP1).

Key words: nuclear factor/polyoma virus enhancer/α-domain

Introduction

Functionally defined as DNA sequences activating promoters in a distance- and orientation-independent manner, enhancers probably form large nucleoprotein complexes involved, in a still unknown way, in transcription of the eukaryotic genome (Gruss, 1984; Wildeman *et al.*, 1986; Piette *et al.*, 1986; Echols, 1986). The DNA sequence of many enhancers is organized in repeated motifs, some of them shared by other enhancers (Serfling *et al.*, 1985). They contain probably redundant information. Indeed, the SV40 enhancer has been divided into independent domains that can replace each other (Herr and Clarke, 1986). The polyoma enhancer was localized in a 246-bp fragment extending from the *Bcl*I site to the origin proximal *Pvu*II site (Tyndall *et al.*, 1981; de Villiers and Schaffner, 1981). This fragment could be divided further into two independent enhancers by Herbomel *et al.* (1984), each one consisting of core and auxiliary sequences. Altogether the work of several groups shows that this region of polyoma may be divided into four subelements δ, α, γ and β (Veldman *et al.*, 1985; Hassel *et al.*, 1986; see Figure 1). Veldman *et al.* showed that several repeats of a 22-bp element containing the α-domain or A core can restore the full activity of the enhancer. This suggests that all necessary information to provide enhancer function is already contained in such a small domain: this information has only to be amplified to provide the levels of activity equivalent to those reached by the intact enhancer. It is now clear that enhancer sequences are the target of specific cellular proteins (e.g. Ephrussi *et al.*, 1985; Piette *et al.*, 1985; Singh *et al.*, 1986) and that the same protein may be involved in the regulation of different enhancers as suggested by *in vivo* and *in vitro* competition experiments (Schöler and Gruss, 1984, 1985; Sassone-Corsi *et al.*, 1985; Schöler *et al.*, 1986). The exact interrelationship between the modular organization of enhancers and the various cellular factors is however still a matter of speculation.

We show here that the 22-bp α-domain of the polyoma enhancer is the target of at least two different cellular proteins interacting with two adjacent and closely related sequences. One of these proteins also interacts with the viral SV40 enhancer and the cellular c-*fos* enhancer, suggesting it has a role in common regulatory facets of the polyoma, SV40 and c-*fos* enhancers.

Results

Specific interaction of cellular factors with the α-domain of the polyoma virus enhancer

Gel retardation and filter binding experiments have revealed the specific interaction of cellular factors with the *Pvu*II-4 fragment containing the β- and γ-domains of the polyoma enhancer (Piette *et al.*, 1985; Fujimura, 1986; Böhnlein and Gruss, 1986). The combination of DNase I footprinting and chemical interference or protection experiments coupled with gel retardation assays permitted us to analyse in detail the interaction of the β-γ-domains with a mouse nuclear factor, PEB1 (Piette and Yaniv, 1986). However, no stable complex could be detected by such approaches with the *Bcl*I−*Pvu*II fragment containing the α- and δ-domains of the enhancer. We therefore turned to another approach, a modified DNase I footprinting procedure not involving the isolation of the complex. In addition, use of poly(dI−dC) as carrier instead of sonicated herring sperm DNA gave an important improvement of the footprints (Singh *et al.*, 1986). A footprint of the large 246-bp *Bcl*I−*Pvu*II fragment covering the entire enhancer region by a nuclear extract of 3T6 cells is given in Figure 2a. Clear protection or alteration of the DNase I digestion pattern is observed approximately between nucleotides 5100 and 5200 covering, respectively, the α-, γ- and β-domains of the enhancer. The protection of the α-domain is almost complete whereas the β-γ-domains are only partially protected in direct footprinting experiments. Hypersensitive sites are present on the late site of the α-domain and on the early site of the β-domain at positions similar to those mapped *in vivo* (Herbomel *et al.*, 1981; Bryan and Folk, 1986; Caruso *et al.*, 1986). Variations in the intensity of these hypersensitive sites in different experiments may be due to variations in the extent of the DNase

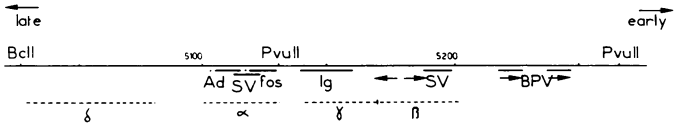

Fig. 1. The polyoma virus enhancer. The *Bcl*I−*Pvu*II fragment containing the enhancer region is shown. Homologies with other enhancers are indicated, as are palindromes or repeated sequences. The functional domains of the enhancer are symbolized by Greek letters. See text for further details.

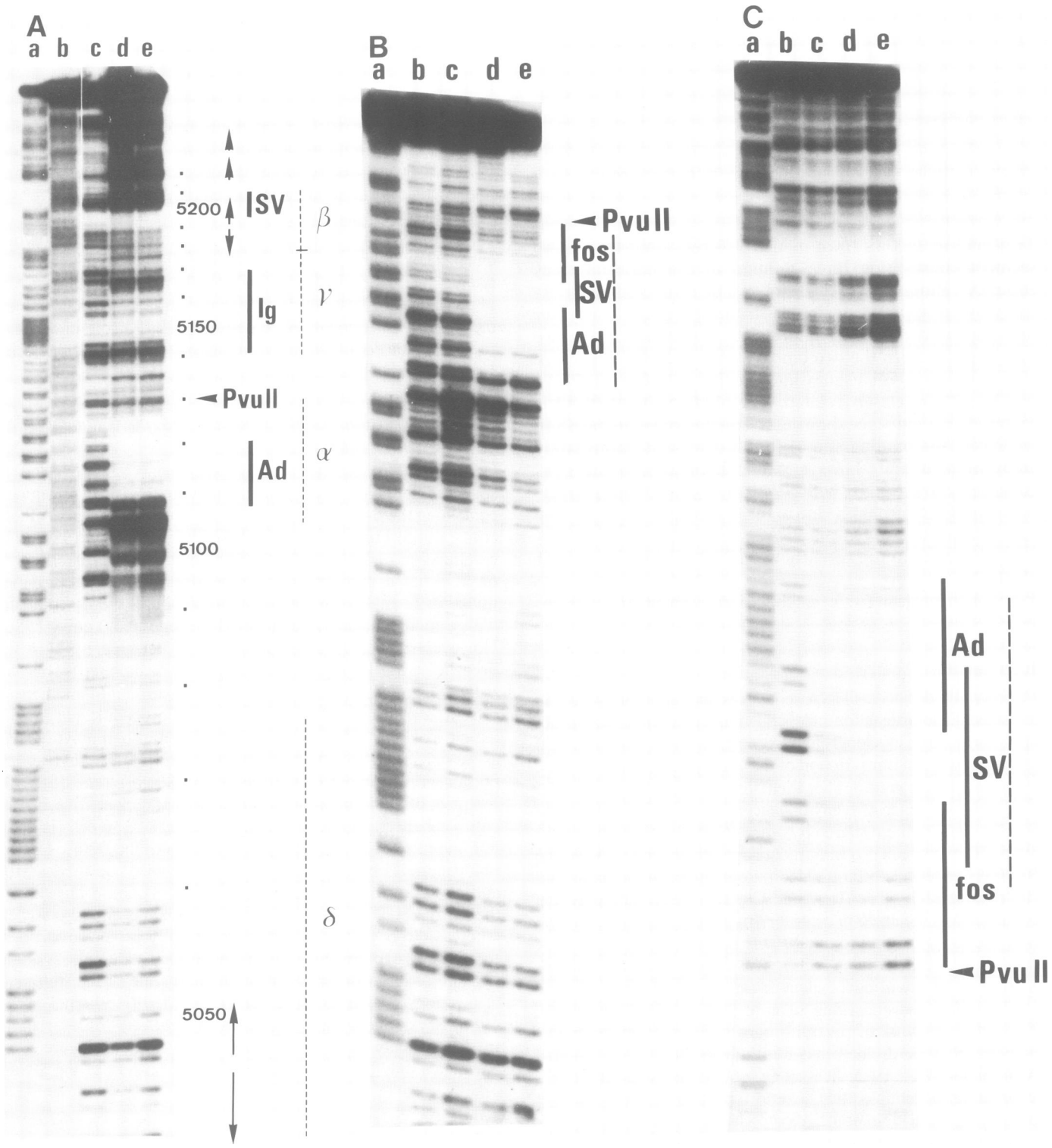

Fig. 2. DNase I footprinting of the polyoma enhancer. **A.** The *BclI*−*ApaI* fragment of polyoma virus containing the enhancer region was 3′ labelled at the *BclI* site and subjected to a DNase I footprinting analysis as described in Materials and methods. G+A and C>T reaction products were loaded in **lanes a** and **b**, respectively. DNA was treated with DNase I in the absence of nuclear extract in **lane c** and in the presence of 1 μg/μl of nuclear extract of 3T6 cells in **lanes d** and **e**. Indicated are the four functional domains, homologies to other enhancers and direct repeats or palindromes. **B.** The *BclI*−*Pvu*II fragment of polyoma containing the α-domain of the enhancer was 3′ labelled at the *BclI* site and subjected to a DNase I footprinting analysis. G+A reaction products were loaded in **lane a**. DNA was treated with DNase I in the absence of nuclear extract in **lane b** and in the presence of respectively 0.25, 0.5 and 1 μg/μl of nuclear extract of 3T6 cells in **lanes c, d** and **e**. Indicated are the homologies to the c-*fos*, SV40 and adenovirus enhancers as are the protected region (discontinuous line). **C.** As **B** but here the *BclI*−*Pvu*II fragment was 3′ labelled at the *Pvu*II site to reveal the footprint of the complementary strand.

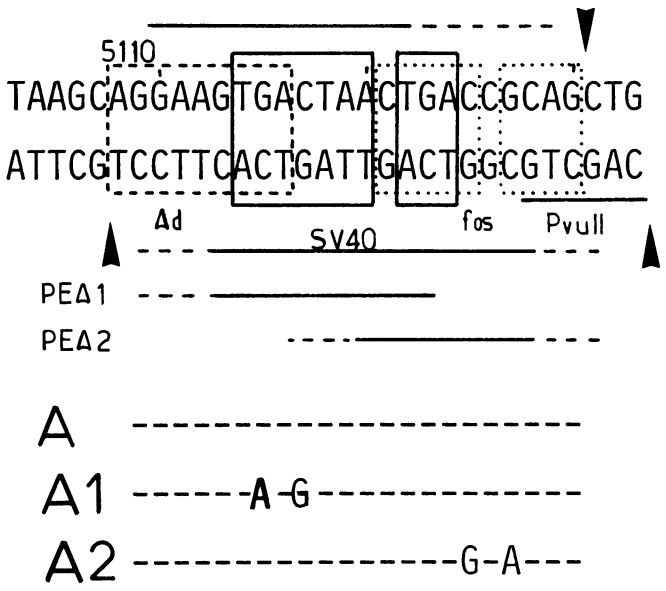

Fig. 3. Nucleotide sequence of the α-domain of the polyoma enhancer. Homologies to the adenovirus, SV40 and c-*fos* enhancers are boxed. Protections against DNase I attack are indicated by continuous or discontinuous lines, hypersensitive sites by arrows. The protection of the lower strand is shown respectively in the absence of competitor, in the presence of competitor A1 (=PEA1) and in the presence of competitor A2 (=PEA2) underneath the sequence. Double-stranded oligonucleotides used in the competition experients are also given at the bottom. Only the upper strand is represented: conserved nucleotides by dashes, mutated nucleotides by letters.

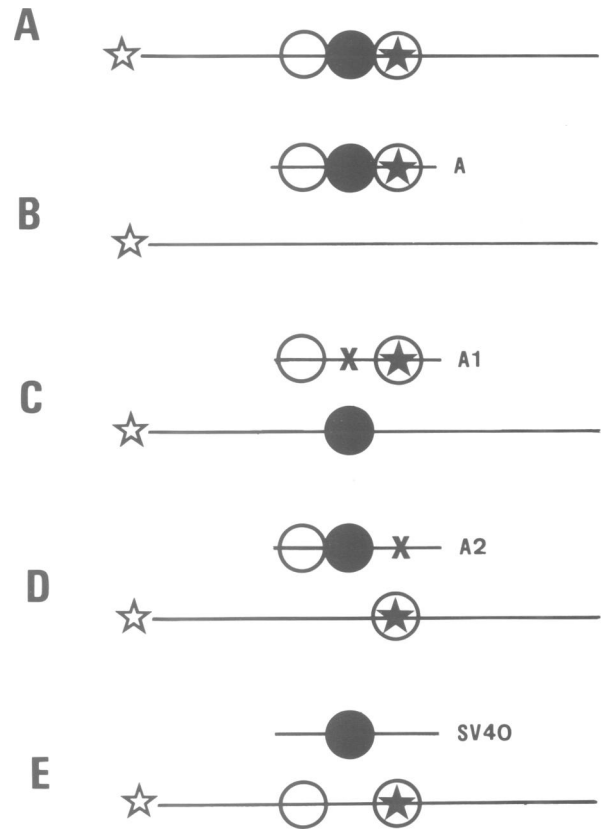

Fig. 4. Representation of competition experiments between double-stranded oligonucleotides and labelled DNA fragments for three different factors. The mutated sites are represented by a cross. The labelled end of the fragment is symbolized by a star and the factor by three distinct circles. In (**A**) no oligonucleotide competitor is added. In (**B**–**E**) excess wild-type or mutant oligonucleotide competitor is added as indicated.

I digestion. In the present work, we concentrated our investigation on the interaction with the α-domain which is entirely contained in the small 107-bp *BclI*–*PvuII* fragment. Since the footprint obtained with this fragment at similar protein concentrations (Figure 2b, c) was identical to that obtained with the large *BclI*–*PvuII* fragment, we used the former fragment in most of our experiments. The protection pattern obtained with both strands is schematized in Figure 3. There is a remarkable coincidence between the protected domain and the 22-bp DNA fragment (nucleotides 5109–5130) that was shown to possess full enhancer activity in, respectively, two copies for viral DNA replication and seven copies for early gene transcription (Veldman *et al.*, 1985). This small DNA sequence contains also three strong overlapping homologies with other enhancers, i.e. the adenovirus enhancer (Hearing and Shenk, 1983), the SV40 enhancer (Zenke *et al.*, 1986) and the c-*fos* enhancer (Treisman, 1985). Thus, the possibility exists that different factors may bind to such a small DNA domain.

At least two factors bind to the α-domain

The precise DNA sequences required for binding of a specific factor can be identified in competition experiments using excess non-radioactive DNA or double-stranded oligonucleotides sharing the same motif. We reasoned that if different factors were binding to the α-domain, it should be possible, by using different competitor oligonucleotides each containing a mutated binding site for one of these hypothetical factors, to compete selectively for the binding of the other factors. This implies, of course, that no strong cooperative effects are involved in factor binding. Figure 4 illustrates the experimental prediction of a situation where three different factors are binding to a DNA segment and wild-type or mutated oligonucleotides, or another viral DNA are used as competitors. We used four small synthetic double-stranded DNA fragments: the first one, A, is the 22-bp fragment used by Veldman *et al.* (1985, kindly provided by G.Veldman), the second one, A1, is similar but contains a double mutation in a sequence shared by the adenovirus and SV40 homologies, the third one, A2, contains a double mutation in the c-*fos* homology and the fourth one is a 24-bp sequence from the SV40 enhancer (nucleotides 109–132) containing the homology to polyoma (see Figure 3). The sequence TCGA was added 5′ to each oligonucleotide to facilitate subsequent cloning or 3′ end labelling.

The results of the competition experiments are shown in Figure 5. Since the non-radioactive oligonucleotides were mixed with the extract before the addition of the labelled fragment (see Materials and methods), the term titration may be more appropriate, assuming that the dissociation rate of these complexes is relatively slow. Nevertheless we will use the term competition for the time being. Two concentrations of cold competitor fragments were used: a 5-fold and a 25-fold molar excess over labelled probe, respectively. We obtained the following results. (i) Using the A double-stranded oligonucleotide (wild-type sequence), the early proximal part containing the c-*fos* homology is already competed out at a 5-fold molar excess while the late proximal part containing the SV40 and adenovirus homologies is fully competed out only at a 25-fold molar excess. (ii) Using the mutated A1 fragment only the early proximal part of the footprint is competed out completely, a faint competition is observed for the late proximal part. (iii) Using the A2 mutated fragment

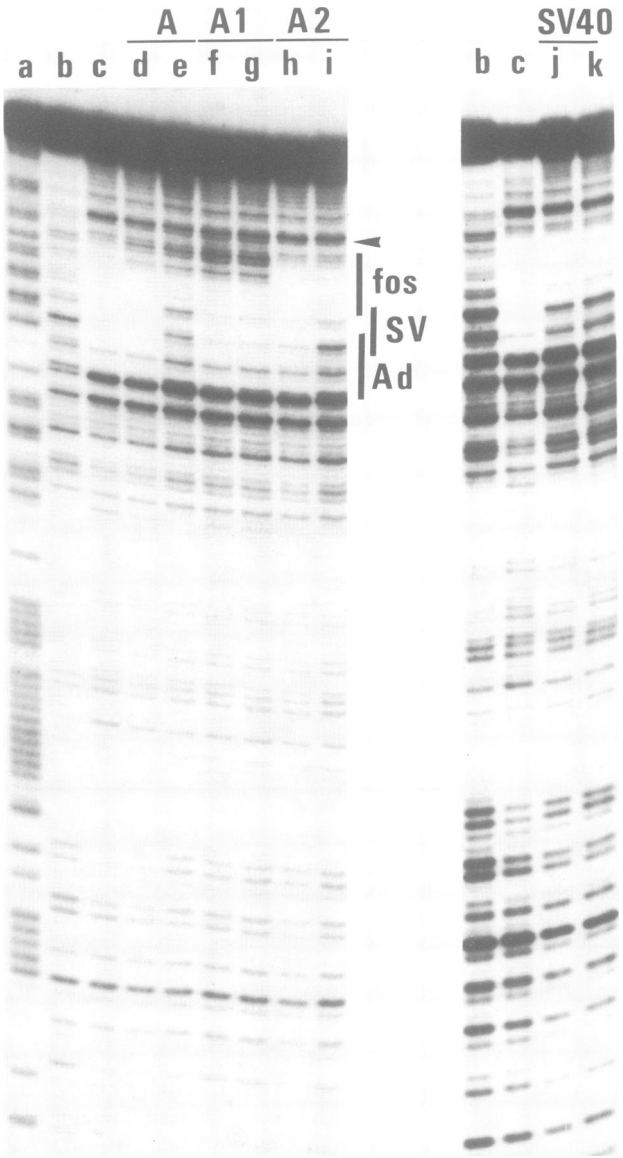

Fig. 5. Competitions with the polyoma enhancer. The *BclI*−*PvuII* fragment was 3′ labelled at the *BclI* site and the footprinting and competition experiments were done as described in Materials and methods. G+A reaction products were loaded in **lane a**. DNA was treated with DNase I in the absence of nuclear extract in **lane b** and in the presence of 1 μg/μl of nuclear extract of 3T6 cells in the following lanes. **Lane c**, no competing oligonucleotide, **lanes d, f, h, j**, 0.25 pmol and **lanes e, g, i, k**, 1.25 pmol of competitor double-stranded oligonucleotides. The nature of the competing oligonucleotides is indicated above the lanes. The sequence of oligonucleotides A, A1 and A2 is given in Figure 3. The SV40 oligonucleotide includes residues 109−132 of the the viral genome.

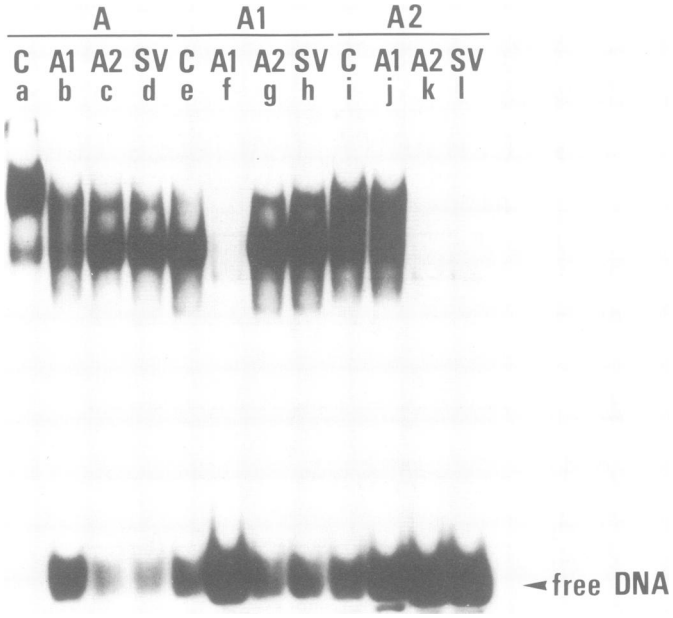

Fig. 6. Gel retardation experiments with the double-stranded oligonucleotides of the α-domain. The A, A1 and A2 fragments were 3′ labelled and subjected to gel retardation experiments with 1 μg/μl of nuclear extract of 3T6 cells as described in Materials and methods. Fragment A was used as probe in **lanes a−d**, fragment A1 in **lanes e−h** and fragment A2 in **lanes i−l**, respectively. Fragment C was used as competitor DNA in **lanes a, e** and **i**, fragment A1 in **lanes b, f** and **j**, fragment A2 in **lanes c, g** and **k** and fragment SV40 in **lanes d, h** *and* **l**, respectively.

only the late proximal part is competed, and (iv) the SV40 fragment competes only with the late proximal part very similarly to the A2 oligonucleotide. We can conclude that the 22-bp A fragment contains the information necessary to bind the factors interacting with the α-domain. At least two factors bind to this domain, the first one binds to the late proximal part of the α-domain and also to the SV40 enhancer, the second one binds to the early proximal part of the α-domain. In the absence of any evidence for a third factor binding to the adenovirus homology, we call the late proximal binding factor PEA1 and the early proximal binding factor PEA2 for polyoma enhancer A binding factors 1 and 2. Competition experiments with SV40 enhancer DNA or mutant oligonucleotides show that the sequences protected against DNase I digestion by both factors are slightly overlapping (see Figure 3). However, since protein binding may exclude access by DNase I to residues not directly interacting with the factor, it is probable that PEA1 and PEA2 contact distinct nucleotides. The absence of cooperativity or competition in the binding of the two factors favor this explanation.

Gel retardation experiments confirm the presence of two DNA binding factors

To confirm the presence of two different factors binding to the α-domain, we used the double-stranded oligonucleotides corresponding to this domain as probes for gel retardation experiments (Garner and Revzin, 1981). Indeed, use of very short DNA fragments greatly improves the results obtained with such assays and permits the detection of factors that do not form stable complexes with longer DNA fragments (M.Raymondjean, personal communication). The 3′-labelled A, A1 and A2 fragments were thus incubated with the nuclear extracts in the presence of excess non-radioactive competitor fragments and subsequently loaded on a non-denaturing polyacrylamide gel as explained in Materials and methods. The wild-type A fragment displays a set of major retarded bands close to the top of the gel (Figure 6, lane a). Competition with the A1 fragment (lane b), or the A2 and SV40 fragments (lane c and d, respectively) causes a displacement of these bands to lower positions. When mutant oligonucleotides were used as probes, the pattern obtained with A2 (lane i) is identical to that seen in lane b for wild-type fragment competed by A1. Similarly, when A1 was used as radioactive probe, the band observed (lane e) was identical to that seen for wild-type fragment competed by A2 or SV40 (lanes c and d).

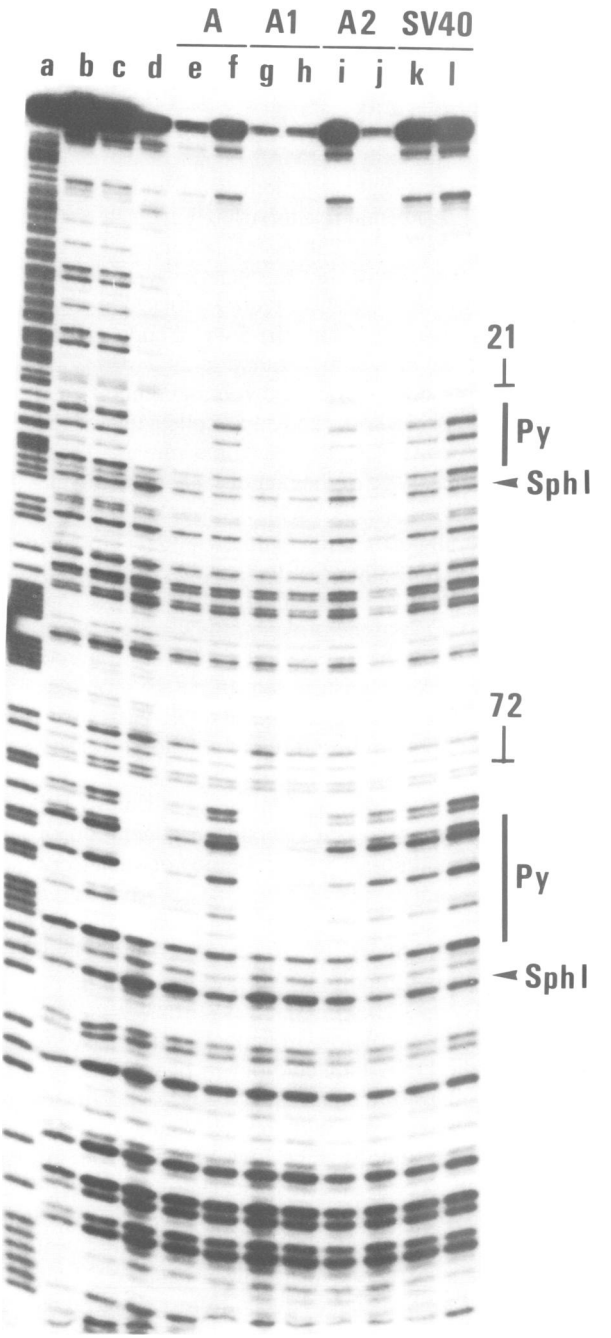

Fig. 7. Competitions with the SV40 enhancer. The *Pvu*II−*Hin*dIII fragment of SV40 containing the origin region was 3′ labelled at the *Pvu*II site. G+A reaction products were loaded in **lane a**. DNA was treated with DNase I in the absence of nuclear extract in **lane b** and **c** and in the presence of 1 μg/μl of nuclear extract of 3T6 cells in the following lanes. 0.25 pmol of the indicated competitor was added in **lanes e, g, i, k** and 2.5 pmol in **lanes f, h, j** and **l**. The limits of the origin-proximal 72-bp repeat and the start of the 21-bp repeats and the polyoma homologies are indicated.

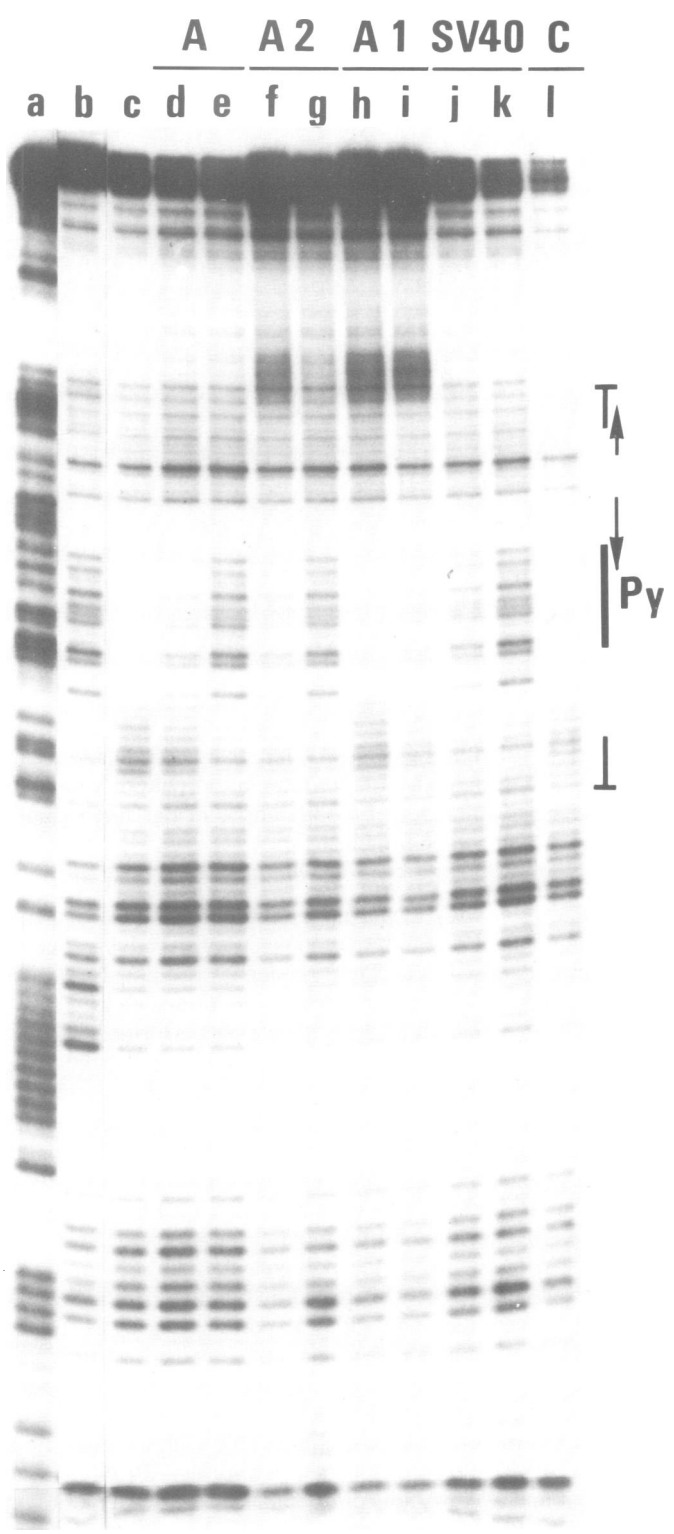

Fig. 8. Competitions with the c-*fos* enhancer. The *Xho*II−*Pst*I fragment containing the c-*fos* enhancer was 3′ labelled at the *Xho*II site. G+A reaction products were loaded in **lane a**. DNA was treated with DNase I in the absence of nuclear extract in **lane b** and in the presence of 1 μg/μl of nuclear extract of 3T6 cells in the following lanes. **Lane c**, no competing oligonucleotide, **lanes d, f, h, j**, 0.25 pmol and **lanes e, g, i** and **k**, 2.5 pmol of the indicated competing double-stranded oligonucleotide. In **lane l**, we included 2.5 pmol of an unrelated double-stranded oligonucleotide. The limits of the c-*fos* enhancer (Treisman, 1985), the palindrome and the polyoma homology are indicated.

The binding on these mutated probes is specifically competed by the homologous oligonucleotides (lanes f and k) and also by the SV40 fragment for the A2 probe (lane l). Altogether this is fully consistent with the independent binding of two different factors as proposed in the preceding section. The retardation pattern observed in lane a also shows that both factors can bind together to the α-domain. Here also a slight competition is observ-

Fig. 9. Comparison of the PEA1 binding sites of polyoma virus, SV40 and c-*fos* enhancers and the PEA2 site of polyoma enhancer. Conserved nucleotides are boxed and homologies between different sites are indicated by vertical lines.

ed for PEA1 binding by oligo A1 (compare free DNA in lane i and j). The complexity of the retardation pattern caused by PEA1 suggests a number of modifications of this factor and/or interactions with other proteins.

PEA1 also interacts with the SV40 and c-fos enhancer

The strong homology displayed by the binding sites of PEA1 and PEA2 to the SV40 and c-*fos* enhancers respectively, and the competition data with the SV40 sequences prompted us to test the interaction of our nuclear extracts with these enhancers. The same type of competition experiments as performed with the polyoma enhancer were done. As expected from the preceding results, PEA1 interacts with the SV40 enhancer (i.e. nucleotides 110−128 for the first of the 72-bp repeats; see Figure 7). Its binding is competed by the polyoma A fragment, the A2 mutant fragment but clearly less by the A1 fragment, in fact less protection of the labelled probe is observed with a 50-fold molar excess of A1 than with a 5-fold molar excess of A.

As shown in Figure 8, the polyoma homology of the c-*fos* enhancer was also clearly protected by our 3T6 cell nuclear extracts. However, the competition experiments gave surprising results. Both A2 and SV40 fragments competed as efficiently as the wild-type A fragment. The accompanying observation that A1 competed clearly less efficiently (competition with a 50-fold molar excess of A1 is at most comparable with a 5-fold molar excess of A2) demonstrates that in reality it is PEA1 and not PEA2 that interacts with the c-*fos* enhancer. In this experiment, we added a 50-fold molar excess of a 40-bp unrelated double-stranded oligonucleotide C in lane l to ensure that the observed competitions are really specific. In fact, we cannot exclude completely that PEA1 is also weakly interacting with the PEA2 binding site of polyoma. This could explain the slight competition for PEA1 binding to the polyoma, SV40 and c-*fos* enhancers displayed by the A1 fragment, in which the PEA1 but not the PEA2 binding site is mutated. This interaction is not too surprising, given the strong sequence homology between the PEA2 site of polyoma and the PEA1 site of c-*fos*. It is intriguing, indeed, that the insertion of 1 bp completely changes the specificity for the recognition of this site by the factor (see Figure 9 and Discussion).

Discussion

We have shown that an enhancer domain as short as the 22 bp long α-domain of the polyoma virus enhancer can bind at least two different proteins that we called PEA1 and PEA2, respectively. Several experimental results strongly indicate that the binding we observe is related to enhancer function. (i) The α-domain was mapped as the core of the A enhancer by Herbomel *et al.* (1984) or as the minimal enhancer required to activate the replication origin (Hassel *et al.*, 1986). (ii) Several copies of the 22-bp double-stranded oligonucleotide function as a strong enhancer when cloned in an enhancerless polyoma genome (Veldman *et al.*, 1985). (iii) W.R.Folk and his colleagues isolated by mutagenesis *in vitro* a series of polyoma mutants defective in enhancer function. In one of the mutants a base change involved the PEA1 recognition site. A viable revertant selected from this mutant restored the PEA1 recognition site (Tang *et al.*, in preparation). The importance of this region is also stressed by the fact that it is duplicated in a number of polyoma strains (Ruley and Fried, 1983). One of the proteins, PEA1, is interacting with the SV40 and c-*fos* enhancers: viral enhancers may constitute a patchwork of cellular regulatory sequences, the proper assembly of which may confer a strong advantage to the virus over cellular genes or the possibility to be expressed in a large variety of cell types.

The internal structure of the α-domain is particularly interesting because it gives us some clues about the generation of larger enhancer regions and possibly the evolution of enhancer binding factors. There is indeed a striking similarity between the PEA1 and PEA2 binding sites: even an almost perfect homology between the PEA1 binding site of c-*fos* and the PEA2 binding site of polyoma, with the important exception of a single base pair insertion in polyoma. This insertion is sufficient to provide an altered recognition specificity, probably by putting two recognition motifs on the DNA molecule out of phase with respect to the interacting groups of the protein. We guess nevertheless from the competition experiments with the A1 mutant that PEA1 conserves some weak affinity for the PEA2 binding site. A plausible evolutionary scheme is that both sites have evolved after duplication from a unique binding site. This would not be surprising because a number of strains have further duplications in this region. Enhancers may be particularly suitable for such rearrangements, perhaps by being excluded from a nucleosomal structure. Different factors interacting with closely related sequences have been described recently for the immunoglobulin heavy chain gene enhancer (Sen and Baltimore, 1986). The existence of such proteins may be explained by duplication and divergence of an ancestral DNA binding factor gene. Another alternative is the existence of different post-translational modifications of a unique factor, conferring slightly divergent DNA recognition specificites or even a system of differential splicing of a common pre-mRNA giving rise to a family of closely related factors. Only in the first case may one predict the existence of gene families for enhancer binding factors.

Different classes of factors may in fact interact with enhancers. Indeed, we have recently characterized the interaction of a factor we called PEB1 with the *Pvu*II-4 fragment containing the β- and γ-domains of the enhancer (Piette and Yaniv, 1986). This has been shown to interact with a large region of ~50 nucleotides in a way similar to the interaction of TFIIIA with the 5S RNA

gene of *Xenopus*. Thus, one group of factors may consist of so-called finger proteins interacting with a rather large DNA domain (Miller *et al.*, 1985; Vincent, 1986), the other may contain proteins of the *lac*-repressor type recognizing smaller DNA motifs (Takeda *et al.*, 1983).

It is interesting to note that although the SV40, c-*fos* and polyoma enhancer bind the same factor, this factor is found in a different context in each case. In c-*fos* a serum-dependent nuclear factor is bound to the palindrome immediately adjacent to the polyoma homology (Treisman, 1986). In SV40, PEA1 may be bound by a Sp1 protein on its origin proximal side (Dynan and Tjian, 1984) and by a factor binding to the SphI motif on its late side in the origin-proximal 72-bp repeat; in the origin-distal repeat, Sp1 may be replaced by a GTGTGGTTT-binding factor. It is probable that PEA1 is similar to the SV40 enhancer binding factor, AP1, characterized in HeLa cells by Lee *et al.* (1987). For both viral enhancers a strong, double-stranded DNase I-hypersensitive site was mapped *in vivo* next to the PEA1 binding site (Herbomel *et al.*, 1981; Cereghini and Yaniv, 1984). These observations suggest that PEA1 function may involve changes in the conformation of DNA, generating strong DNase I-hypersensitive sites.

The fact that different enhancers interact with the same cellular proteins may explain the analogous behaviour of these enhancers in certain physiological situations. Indeed, the polyoma, SV40 and c-*fos* enhancers are weakly active in F9 embryonal carcinoma cells (Sleigh, 1985; Müller, 1986). The SV40 enhancer and c-*fos* expression are also activated after TPA treatment (Imbra and Karin, 1986; Greenberg and Ziff, 1984). It will thus be interesting to analyse the presence of PEA1 binding activity under these conditions and also to look for the presence of PEA1 binding sites in other enhancers regulated in a similar way.

Materials and methods

Preparation of nuclear extract

Protein extracts eluted at 0.4 M NaCl from mouse 3T6 nuclei were prepared as described previously (Piette *et al.*, 1985) and used throughout these experiments.

Labelling and sequencing of DNA fragments

DNA fragments were 3′ labelled with *Escherichia coli* DNA polymerase I, Klenow fragment and sequenced following the chemical degradation method (Maxam and Gilbert, 1980).

DNase I footprinting experiments

The nuclear extract was incubated on ice with 150 ng poly(dI-dC).poly(dI-dC) in 10 mM Hepes pH 8, 17.5% glycerol, 0.1 mM EDTA, 20 mM KCl, 4 mM $MgCl_2$, 2 mM dithiothreitol (DTT) and 4 mM spermidine in a total volume of 10 μl. After 10 min, a few nanograms of 3′-labelled DNA were added and incubation continued for a further 10 min at 20°C. 2 μl of DNase I (50–200 μg/ml) were added subsequently and the reaction was stopped after 1 min by the addition of 12 μl of a solution containing 0.1% SDS, 50 mM EDTA and 200 μg/ml tRNA. 2 μg of protease K were added and digestion continued for 30 min at 42°C. The DNA was phenol extracted, ethanol precipitated and finally loaded on a 8% sequencing gel.

Competition experiments with double-stranded oligonucleotides

Complementary, chemically synthesized oligonucleotides were hybridized in 67 mM Tris, 13 mM $MgCl_2$, 67 mM DTT, 1.3 mM EDTA and 1.3 mM spermidine by incubation for 10 min at 80°C, 10 min at 60°C, 10 min at 37°C and 10 min at 20°C. The indicated amount of double-stranded oligonucleotide was incubated during 10 min on ice together with poly(dI-dC).poly(dI-dC) and nuclear extract before the addition of the labelled fragment. Further procedures were as described for the DNase I footprinting experiments.

Gel retardation experiments

The nuclear extract (20 μg of proteins) was incubated on ice with 1 μg of poly(dI-dC).poly(dI-dC) in 10 mM Hepes pH 8, 17.5% glycerol, 0.1 mM EDTA, 20 mM KCl, 4 mM $MgCl_2$, 2 mM DTT and 4 mM spermidine in the presence of 2.5 pmol of double-stranded competitor oligonucleotides in a total volume of 20 μl. After 10 min ~0.025 pmol of 3′-labelled double-stranded oligonucleotides were added and incubation continued for a further 10 min at 20°C. The reaction mix was immediately loaded on a 8% polyacrylamide gel and run at 160 V in 0.25 × TBE. The gel was subsequently dried and autoradiographed.

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