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Transcription factors AP-3 and AP-2 interact with the SV40 enhancer in a mutually exclusive manner

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The 'core' sequence is critical for efficient transcriptional activity of the SV40 enhancer. Morever, the core was shown to be involved in a signal transduction pathway elicited by treatment of cells with phorbol ester tumor promoters. We report here the identification and characterization of activator protein-3 (AP-3), which recognizes the core element. AP-3 was purified to near homogeneity and identified as a 48K polypeptide. The purified protein is an efficient transcriptional activator *in vitro*. In addition, we show that AP-3 and a second factor that recognizes the SV40 enhancer, AP-2, interact in a mutually exclusive manner. These studies should facilitate understanding of the mechanism by which the SV40 enhancer achieves its characteristic broad cell-type specificity.

Key words: transcription factor/enhancers/transcriptional control/*trans*-activators

Introduction

The SV40 enhancer is a complex *cis*-element comprising multiple sequence motifs acting synergistically (Weiher *et al.*, 1983; Herr and Clark, 1985; Herr and Gluzman, 1985; Zenke *et al.*, 1986; Ondek *et al.*, 1987a,b; Schirm *et al.*, 1987). The first identifiable sequence motif was termed the 'core enhancer element' (the 'core') because of its presence in many other viral and cellular enhancers (Weiher *et al.*, 1983). The core is essential for maximal enhancer activity and viral replication (Weiher *et al.*, 1983; Zenke *et al.*, 1986; Nomiyama *et al.*, 1987). Moreover, the core was shown to act as a tumor-promoter responsive element (Chiu *et al.*, 1987). Mutations which inactivate this element adversely affect its recognition by *trans*-acting factors (Chiu *et al.*, 1987). Hence, the identification of core binding protein(s) is important not only for elucidating enhancer function but also for understanding the involvement of such *trans*-acting factors in the signal transduction pathway elicited by treatment with phorbol-ester tumor promoters. While several factors that interact with the core or nearby sequences were recently described, including c/EBP, EBPI, TEF-2, NFkB, AP-2 and AP-3 (Chiu *et al.*, 1987; Imagawa *et al.*, 1987; Landschulz *et al.*, 1987; Mitchell *et al.*, 1987; Xiao *et al.*, 1987; Clark *et al.*, 1988; Kawakami *et al.*, 1988), a bona fide core recognizing protein capable of stimulating transcription *in vitro* has not been described. Determination of the functional role of these proteins will require their purification to homogeneity in an active form and subsequent analysis in an *in vitro* system. We report here the isolation and characterization of a 48K transcription factor, activator protein-3 (AP-3), which binds to the previously defined core element and is capable of stimulating transcription *in vitro*. In addition, we show that AP-3 and a second factor that recognizes the SV40 enhancer, AP-2 (Imagawa *et al.*, 1987; Mitchell *et al.*, 1987) interact in a mutually exclusive manner.

Results

Purification and characterization of AP-3

We were interested in identifying and characterizing the predominant DNA binding protein present in the epithelial cell lines HeLa and HepG2 that recognizes the core, because we believe such a factor is likely to be responsible for many of the properties characteristic of this element in these cells, e.g. its activation by phorbol esters (Chiu *et al.*, 1987). Using unfractionated HeLa and HepG2 extracts, we first characterized the nucleotide sequences surrounding the SV40 core protected from DNase I digestion (Chiu *et al.*, 1987). Such a footprint conferred by unfractionated extracts should represent recognition by the most abundant or active core binding protein in these cells. This analysis led to the identification of the core recognizing factor, AP-3 (Chiu *et al.*, 1987). To purify AP-3, we prepared an oligonucleotide affinity column containing the originally defined core 5'-TGTGGAAAG-3' (Weiher *et al.*, 1983) and flanking sequences. Purification of AP-3 by conventional liquid chromatography on heparin-agarose and Sephacryl S-300 (Angel *et al.*, 1987) and three successive passes over the core affinity column yielded two predominant protein species with relative molecular masses of 48K and 116K (Figure 1A). The 116K species copurifies with several other DNA binding proteins and possesses no detectable specific DNA binding properties (Imagawa *et al.*, 1987; Mermod *et al.*, 1988). Hence, it was believed that the more abundant 48K polypeptide represented AP-3. To confirm its identity, the 48K polypeptide was eluted from preparative SDS-polyacrylamide gels, renatured (Hager and Burgess, 1980) and examined for DNA binding activity. To minimize the risk of proteolysis and to optimize the chances of identifying weaker or less abundant although potentially relevant DNA binding proteins, we used partially purified AP-3 preparations (Figure 1B). Three specific DNA binding polypeptides were identified (Figure 1C). As expected, the 48K species exhibited core binding properties essentially identical to the unfractionated extract. A small portion of this material was electrophoresed alongside third-pass affinity-purified AP-3 to verify that they were the same protein (Figure 1A). In addition, a 43K polypeptide exhibiting an identical footprint pattern was found. Since the

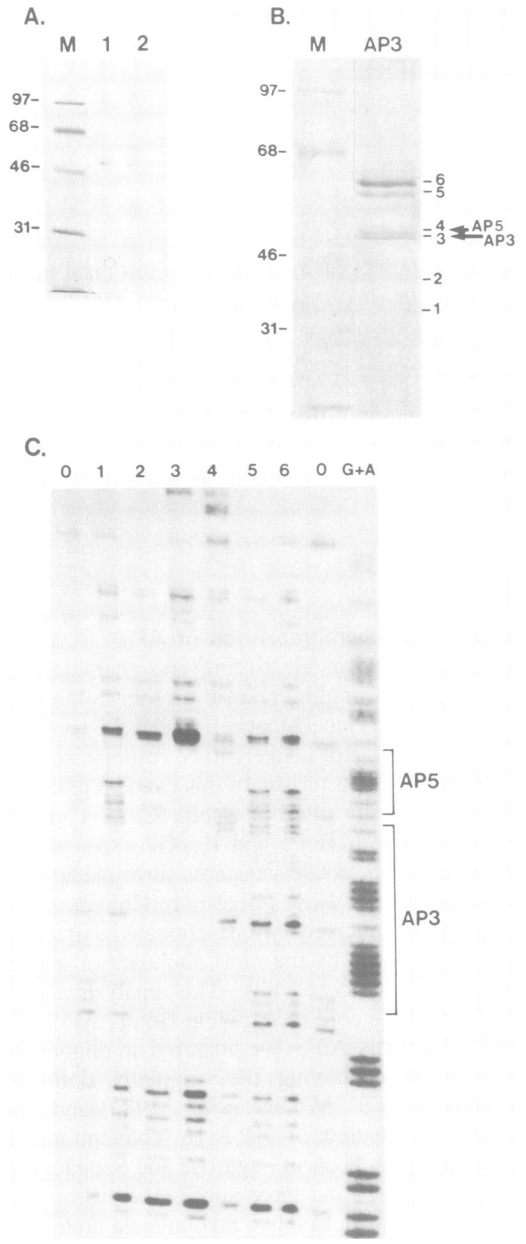

Fig. 1. Purification and identification of AP-3. (**A**) Purification of AP-3 from HeLa S3 whole-cell extracts. **Lanes 1** and **2** contain a small portion of the gel eluted polypeptide corresponding to band 3 of the renaturation experiment (see **panel B**, lane 2) and AP-3 after three cycles of affinity chromatography (100 ng) respectively. Proteins were analyzed by 10% SDS–PAGE and stained with Coomassie blue. M, mol. wt standards. (**B**) SDS–PAGE analysis of partially purified AP-3 subjected to renaturation. Material isolated by a single pass over the oligodeoxynucleotide affinity column was analyzed by 10% SDS–PAGE and stained with Coomassie blue. The various polypeptide bands which were subjected to renaturation are identified by a number on the right. M, mol. wt standards. (**C**) Identification of the active AP-3 DNA binding species. AP-3 (1 μg) partially purified by a single pass over the DNA affinity column was separated by preparative SDS–PAGE. The different polypeptide bands were excised (see **panel B**), eluted and subjected to renaturation. The renatured polypeptides were then examined for binding to the SV40 enhancer by standard DNase I footprinting reactions. Numbers at the top of the figure correspond to the polypeptides identified in panel B.

relevant abundance of the 43K polypeptide varies from one preparation to another, we believe it to be a degradation product of the 48K AP-3 protein. Unexpectedly, we also identified a 50K polypeptide, activator protein-5 (AP-5) (Jones *et al.*, 1987), which protects a sequence within the A element of the enhancer whose sequence resembles the core (Herr and Clarke, 1986), 5′-TGTGGAATGT-3′. Although the DNase I protection pattern of the 50K polypeptide is similar to the recently described A-element binding protein TEF-1 (Davidson *et al.*, 1988), further studies will be needed to determine if these are identical proteins.

Characterization of the AP-3 recognition site

The recognition sequence of AP-3 was further defined by using a series of mutants, pA11–pA15 (Zenke *et al.*, 1986), spanning this region of the enhancer. Binding of purified AP-3 and the initial 0.4 M heparin–agarose fraction to these mutants were tested by DNase I protection (Figure 2). As an internal control, a sufficient amount of purified AP-3 was added resulting in protection of secondary sites present downstream of the core (see below). Mutants pA11, pA12 and pA13 had a deleterious effect on protection of the core by AP-3, while pA14 and pA15 showed little or no effect. Densitometric analysis of the data shown in Figure 2 indicates that the effect of the pA12 mutation was approximately twice as severe as the effect of pA11 and pA13. Interestingly, using the 0.4 M heparin–agarose fraction which contains many different factors, we found that whenever a mutation adversely affected AP-3 binding the overlapping AP-2 site was fully protected. Hence, while both factors appear to be present at saturating amounts, AP-2 binding was observed only when the AP-3 site was inactivated. This and the extensive overlap in their footprints suggested that AP-2 and AP-3 compete for binding to this region of the enhancer.

To characterize the interaction between these factors more precisely, purified AP-2 and AP-3 were titrated against each other and their binding to the SV40 enhancer was monitored. While maintaining a constant concentration of AP-2, addition of small increments of AP-3 resulted in a marked decrease in AP-2 site protection and a corresponding increase in protection of the core by AP-3 (Figure 3A). The same phenomenon occurs during the inverse titration, protection of the core by a constant amount of AP-3 decreases upon addition of small increments of AP-2 (data not shown). These experiments demonstrate that binding of either factor interferes with the ability of the other factor to bind its respective sequence motif. AP-3 appears to be the dominant core binding factor in unfractionated HeLa cell extracts.

Recent transfection studies indicate that, while the isolated core or C element is active in both HeLa and HepG2 cells (Chiu *et al.*, 1987), it lacks biological activity in F9 embryonal carcinoma stem cells (Chiu *et al.*, 1988). To determine whether these differences in biological activity correlate with differences in the abundance of AP-3, we examined extracts of undifferentiated F9 cells for the presence of this factor. As shown in Figure 3B, no 'AP-3-like' protection is observed using the F9 extract. In these cells, the predominant core binding protein exhibits different recognition properties leading to protection of a smaller region than AP-3. In addition, the F9 extract did not exhibit an 'AP-2-like' activity using both the SV40 (Figure 3B) and $hMTII_A$ probes (P.Skroch, unpublished data). These results are also in agreement with the transfection studies showing that the AP-2 element is inactive in these cells (Chiu *et al.*, 1988).

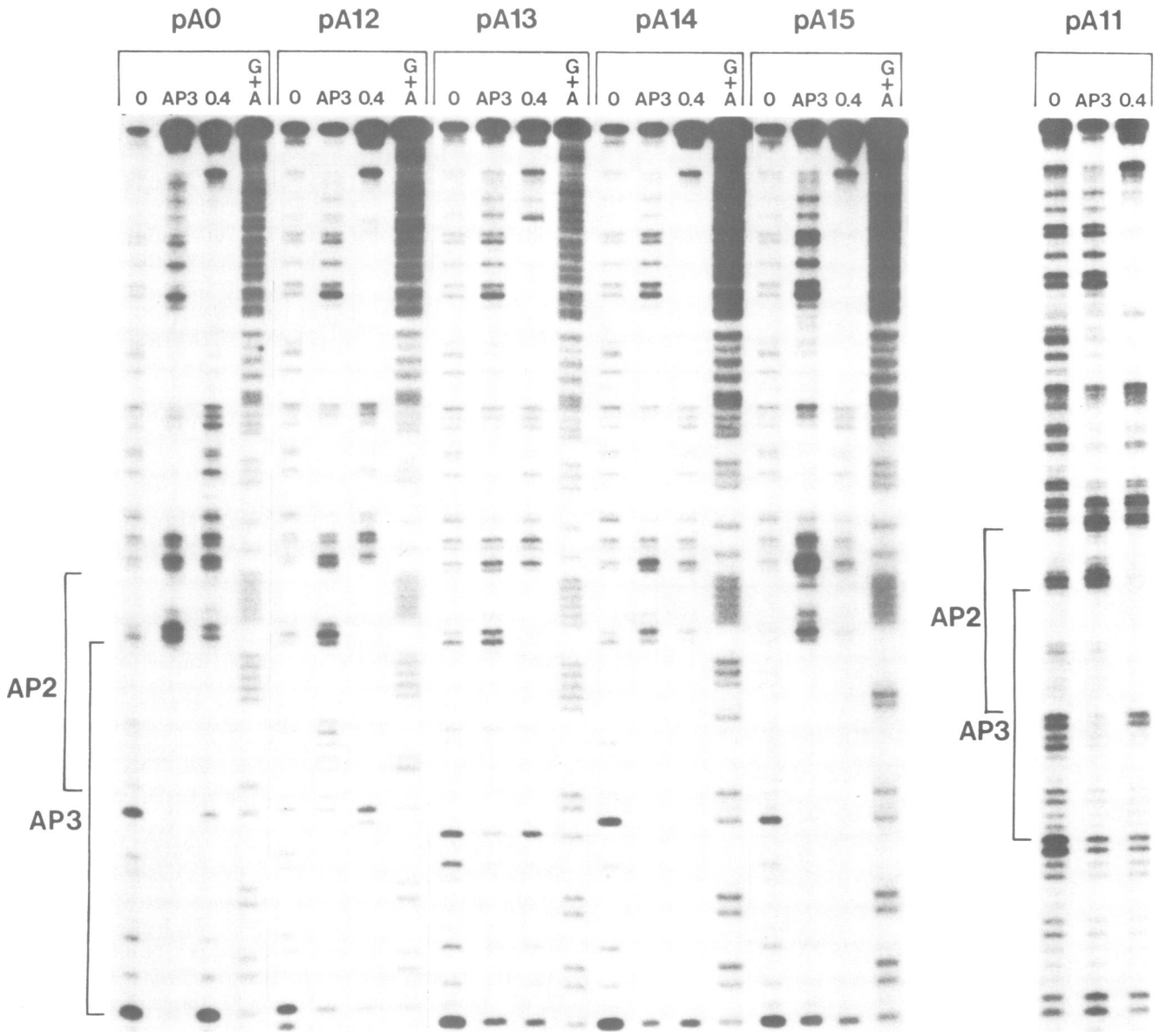

Fig. 2. Analysis of the AP-3 recognition sequence. (**A**) Analysis of AP-3 binding to wild-type and mutant SV40 enhancers. The wild-type, pAO and mutant SV40 constructions, pA11–pA15 (Zenke *et al.*, 1986), which contain point mutations spanning the core region, were used to prepare the DNA probes as described above for the pAO probe. The probes were incubated with either 2–5 ng of third-pass affinity-purified AP-3 or 10–20 μg of HeLa S3 0.4 M heparin–agarose fraction and subjected to standard DNase I protection. Regions protected by purified AP-2 and AP-3 in the wild-type enhancer are indicated to the left of each panel.

To define further the activators recognized by AP-3, we examined its binding to other viral and cellular enhancers. Murine mammary tumor virus, murine Moloney leukemia virus and the interleukin-2 (IL2) enhancers all displayed high-affinity binding sites for AP-3 (Figure 4). We also found each enhancer to contain secondary recognition sites which become protected at higher levels of AP-3. Comparison of these sequences reveals no significant homology to the consensus sequence of the high-affinity sites (Figure 4). The ability of transcription factors to recognize different *cis*-elements, which display remote homology or no homology at all, however, is not uncommon as evidenced by c/EBP (Landschulz *et al.*, 1987), AP-4 (Mermod *et al.*, 1988), AP-2 (Imagawa *et al.*, 1987; Mitchell *et al.*, 1987) and HAP2 (Pfeifer *et al.*, 1987).

AP-3 stimulates transcription in vitro

To demonstrate that AP-3 is a bona fide transcription factor, we tested its ability to stimulate transcription *in vitro*. To minimize potential complications due to the competition between AP-3 and AP-2 for the SV40 core, as well as the other previously mentioned core binding proteins, we opted to use an alternative recognition site for these experiments. The IL2 site I (see Figure 4) was selected because it exhibited high affinity for AP-3 and possessed no obvious overlapping binding sites for other factors, including AP-2 (E.Serfling and F.Mercurio, unpublished data). A multimer (5×) of the IL2 site I was inserted upstream of the tk promoter to generate a test promoter. The enhancerless tk and α-globin promoters were included as controls. Addition of purified AP-3 to a HeLa whole-cell extract depleted of AP-3

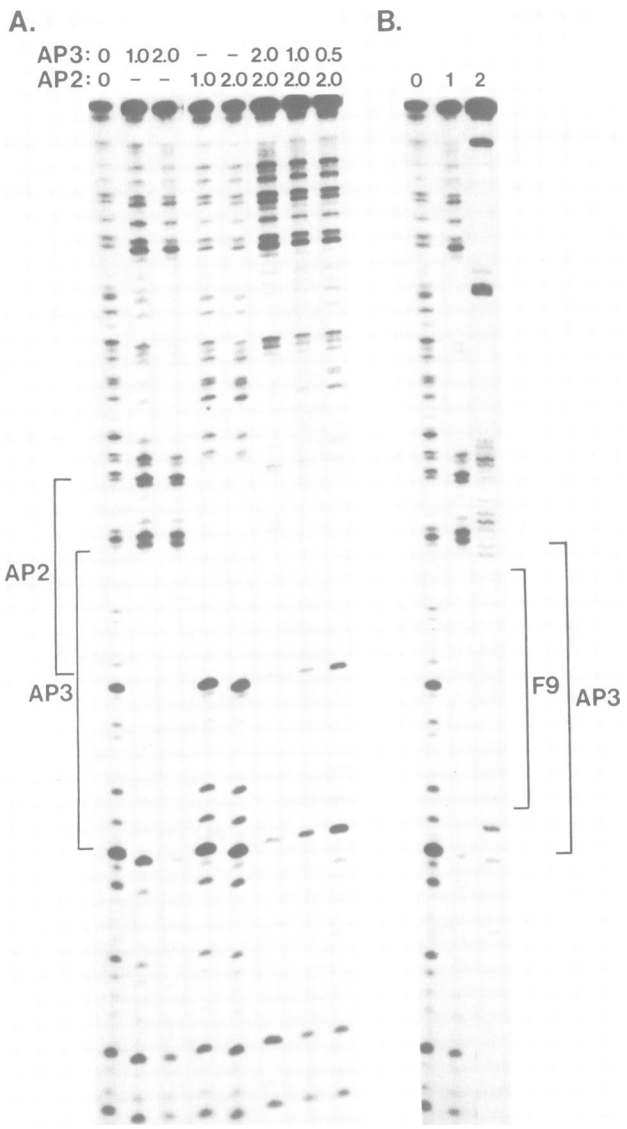

Fig. 3. Analysis of AP-2 and AP-3 interaction with the SV40 core region. (**A**) Purified AP-3 (~1.0 μg/ml) was titrated against a constant amount of purified AP-2 (~1.0 μg/ml) and their respective binding properties monitored by DNase I protection. Probes were generated from the SV40 wild-type pAO construction. The volume of AP-2 and AP-3 added to each reaction is designated at the top of the figure. (**B**) Comparison of the predominant core binding proteins from HeLa and F9 cells. The SV40 pAO probe was incubated with either 50 μg of F9 whole-cell extract (**lane 1**) or 2 ng of purified AP-3 (**lane 2**).

pA Mutants		11 12 13 14 15 GTT TGA TGT CCC AAA
Wildtype SV40	(-249)	GTGTGGAAAGTCCCC (-235)
Primary binding sites:		
SV40I	(-249)	GTGTGGAAAGT (-239)
MMTVI	(-150)	AAGTGGTTTCC (-140)
MMTVII	(-189)	TTATGGTTACA (-179)
MLVI	(-93)	CTGTGGTAAGC (-83)
MLVII	(-169)	CTGTGGTAAGC (-159)
IL2I	(-196)	AGGTGAAATCC (-206)
IL2II	(-88)	ATGTGTAATAT (-78)
Concensus		TGTGGAAA TTT
Secondary binding sites:		
SV40II	(-219)	CAGAAGTATGCA (-208)
SV40III	(-196)	CTCAATTAGTCA (-185)

Fig. 4. Comparison of AP-3 recognition sequences on SV40, murine mammary tumor virus (MMTV), Moloney murine leukemia virus (MLV) and the interleukin-2 (IL2) enhancers. Sequences protected by AP-3 on the various enhancer elements using standard DNase I footprinting are as indicated. Conserved nucleotides between the different binding sites are shown in bold type. In addition, the sequences affected by the various SV40 mutants are shown above and the two secondary AP-3 sites within the SV40 enhancer are shown in the bottom of this panel. Preparation of the various DNA probes used to identify these sites is described in Materials and methods.

stimulated transcription of 5×IL2(I)tkCAT2 ~10-fold, as measured by primer extension (Figure 5). Transcription of the control constructs pBLtkCAT2 and pα-globin was not affected by the addition of AP-3. The results indicate that the purified AP-3 protein is capable of functioning as a transcription factor *in vitro*.

Discussion

The present studies show that the predominant core binding factor in HeLa cells is the 48K transcriptional activator AP-3. Based on identical footprinting patterns, AP-3 is also the predominant factor that recognizes the core in HepG2 cells (Chiu *et al.*, 1987). The functional relationship between AP-3 and other previously mentioned core binding factors is not completely clear. It will require the purification of these factors in a biochemically active form capable of affecting transcription *in vitro*. For example, the AP-3 protein mentioned by Mitchell *et al.* (1987), which also recognizes the core or an adjacent sequence, appears to be different from the AP-3 protein which we have characterized here. Mitchell *et al.* (1987) described AP-3 as a 57K protein, although no direct proof, e.g. renaturation experiments and a description of its purification, was provided. Another core binding protein, whose size is 57K, is TEF-2 (Davidson *et al.*, 1988). However, its relationship to AP-3 is not clear either. This apparent molecular mass is quite different from the size of protein we purified. Other differences between the two AP-3 proteins are in the manner by which they interact with AP-2. Mitchell *et al.* (1987) described that AP-3 binds coordinately with AP-2, yielding more extensive protection of the SV40 enhancer in that region. This is in direct contradiction to the data presented here showing that AP-3 and AP-2 interact with overlapping sites and bind in a mutually exclusive manner. Thus, the AP-3 protein mentioned by Mitchell *et al.* (1987) is likely to be different from the AP-3 protein whose purification and characterization are described here.

Determination of the relatedness between the two AP-3 proteins and TEF-2 addresses a more fundamentally important question regarding which sequence motif within the SV40 enhancer is responsible for properties previously associated with the core element. Subsequent to the characterization of TEF-2, Davidson *et al.* (1988) claimed that the traditional core element is not responsible for the biological functions previously assigned to it; they proposed that the recognition site for TEF-2, 5′-GGGTGTGG-3′,

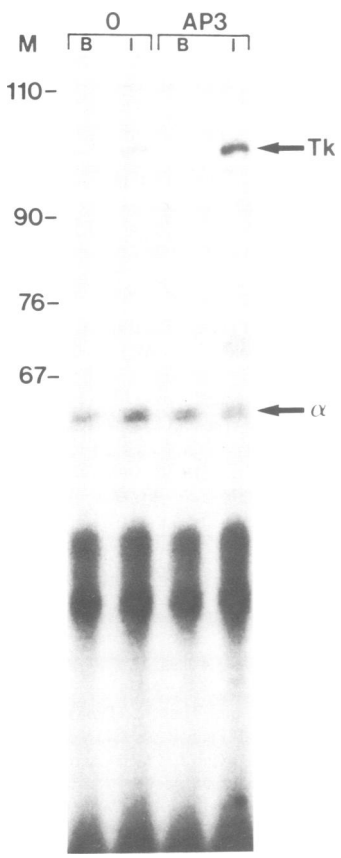

Fig. 5. Stimulation of transcription by affinity-purified AP-3. HeLa S3 whole-cell extracts depleted in AP-3 were prepared as described in Materials and methods. Transcription reactions were performed in a total volume of 50 μl, which included 3.4 μl (14.7 mg/ml) of AP-3-depleted whole-cell extract. 500 ng of either test (I) or control (B) templates, pBL5XIL2(I)tkCAT2 and pBLtkCAT2 respectively, were added to each reaction. 2 μl of purified AP-3 (~2 ng) were added as indicated. tk and α on the right refer to the correctly initiated tkCAT and α-globin transcripts. The migration positions of mol. wt markers (in nucleotides) are indicated on the left.

is actually the more appropriate motif. They show quite convincingly that the three Gs at positions 249, 250 and 251 are required for binding of TEF-2 *in vitro* and efficient enhancer activity of multimers of this element *in vivo*. These data clearly distinguish TEF-2 from AP-3, in that these nucleotides were not included in the sequence used to prepare the AP-3 specific affinity column and therefore are not required for AP-3 binding. Based on their results with TEF-2, these authors have redefined the core sequence and concluded that the TEF-2 recognition site was the functionally important element (Davidson *et al.*, 1988). However, this is not consistent with *in vivo* data obtained utilizing the intact SV40 enhancer. Previous work indicated that mutations of Gs 249, 250 and 251, which completely eliminate binding of TEF-2, had only a modest effect on overall enhancer activity (Zenke *et al.*, 1986). There appears to be a discrepancy regarding *in vivo* enhancer activity which varies according to whether the element is examined as part of the whole enhancer or as a multimerized synthetic sequence cloned upstream or downstream to some test promoter. Previously we have shown that other point mutations (dpm 6), which have a severely incapacitating effect on the activities of both the intact and the isolated C element (Herr and Clarke, 1986), interfere with binding of AP-3 (Chiu *et al.*, 1987). In addition, we have shown that AP-3 is the predominant protein which interacts with the core region of the enhancer in HeLa cells. Thus, before discarding the importance of the core sequence, it will be necessary to demonstrate that AP-3 does not interact with the SV40 enhancer *in vivo* while TEF-2 does.

Another factor that binds to the core sequence of the SV40 enhancer is NFkB (Kawakami *et al.*, 1988). However, in HeLa cells the ability of NFkB to bind DNA is entirely dependent on treatment of the cells with TPA (Sen and Baltimore, 1986). Thus NFkB is unlikely to contribute to the basal activity of the enhancer in unstimulated cells.

It appears likely that several core binding proteins may contribute to overall SV40 enhancer activity in HeLa cells. Identification of a functionally dominant factor *in vivo*, if such a factor exists, will require more extensive studies. The type of interaction we observe for AP-2 and AP-3 could function to expand the host range of the SV40 enhancer as well as for 'fine-tuning' of its activity in response to various environmental cues affecting the activity of these factors. For example, while in some cells AP-3 is the dominant factor interacting with this part of the enhancer, in other cells, where AP-3 is less abundant, AP-2 may be the more important factor. The overlap of multiple core binding factors could well be the main factor contributing to the broad cell-type specificity demonstrated by the SV40 enhancer and may not be unique to the core element. The present studies suggest a possible mechanism by which the modular nature of the SV40 enhancer enables it to undergo complex regulation. We show that AP-3 is a potent transcriptional activator *in vitro* and is capable of activating enhancer-dependent transcription by itself and does not require simultaneous binding of an adjacent enhancer-binding factor. We believe that the purification and characterization of such factors will eventually lead to a complete understanding of enhancer function using *in vitro* systems. Since AP-3 is a cellular factor, it will be even more important to determine its role in the regulation of cellular gene expression.

In contrast to HeLa cells, undifferentiated F9 cells appear to lack AP-3 and contain a different core binding factor. The exact function of this factor will require further purification and characterization. However, based on the lack of biological activity of multimerized core or C element sequences in these cells (Chiu *et al.*, 1987), we suggest that this factor may represent the titratable repressor previously detected by transfection experiments using these cells (Gorman *et al.*, 1985a,b; Sleigh and Lockett, 1985; Sassone-Corsi *et al.*, 1987). On the other hand, other investigators find that in the context of the intact enhancer the core element is required for optimal enhancer activity in F9 cells (Nomiyama *et al.*, 1987). This suggests a different possibility according to which, the core binding factor of F9 cells, in contrast to AP-3, is not capable of stimulating transcription on its own because it has to interact with additional factors recognizing sequences lying outside the isolated C element.

Materials and methods

Cell culture

HeLa S3 cells were grown in spinner flasks in Joklik modified Eagle's medium supplemented with 2.5% fetal calf serum (FCS) and 2.5% calf serum. Some of the HeLa S3 cells were obtained from Don Giard at the MIT Cell Culture Center. F9 embryonal carcinoma stem cells were grown in F12–Dulbecco's modified Eagle's medium supplemented with 10% FCS and 10^{-4} M 2-mercaptoethanol.

Preparation of extracts and purification of AP-3

Whole-cell extracts were prepared as previously described (Angel *et al.*, 1987). F9 whole-cell extracts used for footprinting were prepared from 2–4 ml of packed cell volume. For protein purification, extracts were prepared from 25–50 ml of packed cell volume HeLa S3 cells. Extracts of 8–15 mg protein/ml were loaded on a heparin–agarose column equilibrated with TM buffer consisting of 50 mM Tris–HCl (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, 12.5 mM $MgCl_2$, 20% glycerol, 0.1 M KCl, 1 mM $(Na)_2MoO_4$ and 0.1 mM phenylmethylsulfonyl fluoride. The column was washed with TM containing 0.2 M KCl and eluted with TM buffer containing 0.4 M KCl. The 0.4 M KCl heparin–agarose fraction was loaded on a 400 ml Sephacryl S-300 column and eluted with TM buffer. The presence of AP-3 was monitored by DNase I footprinting and the fractions containing this activity were pooled and applied to a DNA affinity column. The AP-3 affinity column was prepared as described by Kadonaga and Tjian (1986), using double-stranded oligodeoxynucleotide prepared by annealing of two oligodeoxynucleotides, 5′-GATCTGTGGAAAGTCCCA-3′and 5′-GATCTGGGACTTTCCACA-3′, corresponding in sequence to the high-affinity site on the SV40 enhancer. The column was loaded with 30 mg of the S-300 fraction mixed with 200 μg of poly(d[I-C]), washed with 0.1 M KCl–25 mM Hepes (pH 7.9) buffer, and eluted with 0.6 M KCL–25 mM Hepes (pH 7.9) buffer. This step was repeated twice.

Renaturation of AP-3 DNA binding activity was performed as described by Briggs *et al.* (1986) for Sp1, with slight modifications. Approximately 1–2 μg of AP-3 purified by a single cycle of affinity chromatography was precipitated with trichloroacetic acid, resuspended in SDS sample buffer containing 5 mM dithiothreitol, incubated at 37°C for 5 min, and fractionated by SDS–PAGE. One lane was stained with Coomassie blue, and a second nonsliced lane was sliced to separate between the different polypeptide bands. The polypeptides were eluted from the slices in the presence of 50 μg bovine serum albumin and were renatured exactly as described by Briggs *et al.* (1986). The net yield of AP-3 binding activity was ~3%.

DNase I footprinting

Footprinting reactions were performed in a total volume of 50 μl containing 1–2 ng of end-labeled DNA probes (10 000 c.p.m./reaction), as previously described (Angel *et al.*, 1987), except that when purified protein was used, poly(d[I-C]) was omitted. The SV40 wild-type DNA probe used for footprint reactions was generated by digestion of pAO (Zenke *et al.*, 1986) with Asp 718 which cleaves at position −294, labeled by filling-in with the Klenow fragment of *Escherichia coli* DNA polymerase I, and subsequently digested with *Bgl*II which cleaves at position −70. SV40 mutant DNA probes pA11 to pA15 (Zenke *et al.*, 1986) were prepared as described for the wild-type pAO probe. The MMTV probe was labeled by filling-in of the *Hin*dIII site at position −236 of pHC8-CAT (Cato *et al.*, 1988) and subsequent digestion with *Sma*I at position −72. The MLV probe was labeled by filling-in of the *Xba*I site at position 8113 of pLPLM (Yee *et al.*, 1987) and subsequent digestion with *Cla*I at position 7674. The IL2 probe was labeled by filling-in of the *Hin*dIII site at position −293 of pBLIL2(−293/−7)tkCAT2 (Serfling *et al.*, 1989) and subsequent digestion with *Bam*HI at position −7.

In vitro transcription

HeLa whole-cell extracts were prepared as described under cell culture and depleted of AP-3 by batch incubation with the core oligodeoxynucleotide-affinity resin. Transcription reactions were performed in a total volume of 50 μl, which included 3.4 μl (14.7 mg/ml) of AP-3-depleted whole-cell extract and 20 μl of a nucleotide triphosphate mix, as previously described (Bodner and Karin, 1987). Five hundred nanograms of either the test or control templates, pBL5XIL2(I)tkCAT and pBLtkCAT2 respectively, were added to each reaction. As an internal control, 200 ng of pαglobin were also added to each reaction. Two microliters of highly purified AP-3 (~2 ng) were added as indicated. pBL5XIL2(I)tkCAT was kindly provided by Dr Edgar Serfling, who constructed it by inserting five copies of chemically synthesized IL2 enhancer segment, −191 to −215, containing the high-affinity IL2 I site into pBLtkCAT2 (Serfling *et al.*, 1989).

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