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**A cellular protein binds to a conserved sequence in the adenovirus type 2 enhancer**

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**ABSTRACT**

A sensitive gel retention assay has been utilized to detect proteins from uninfected Hela nuclei which interact with the adenovirus type 2 enhancer. This assay has been employed to monitor fractionation of nuclear extracts. Three enhancer binding factors were resolved by chromatography on DEAE-Sepharose and one of the factors was further purified by chromatography on heparin-Sepharose. DNase protection experiments have shown that the heparin-Sepharose fraction contains a factor which binds predominantly to the conserved sequence GTGG<sup>AAA</sup> present at position 160 in the adenovirus type 2 genome and found in many viral and cellular enhancers. Protection of this sequence from DNase I digestion was abolished by competition with a synthetic duplex oligonucleotide spanning bases 144-181. This region corresponds to the sequence defined by Hen *et al.* (1) as possessing enhancer function. Competition experiments indicated that the enhancer binding factor also bound, albeit with reduced affinity, to multiple sites in the Ela upstream region located between positions 192 and 353. Within the sequences which compete are regions with homology to the high affinity site at position 160. The enhancer binding factor also binds with high affinity to sequences within the SV40 enhancer demonstrating that this factor interacts with sequences common to both the adenovirus and SV40 enhancers.

**INTRODUCTION**

Enhancers are DNA sequences which constitute part of the transcriptional control system of eukaryotes and their viruses. At present enhancers are distinguished from other promoter elements by their ability to activate transcription of linked genes in a position and orientation independent fashion. While enhancers have been shown to activate multiple linked genes over large distances they tend to activate proximal promoters to a greater extent than distal promoters. The activity of enhancers can be regulated in a tissue and species specific manner, the result of which is cell type specific expression of enhancer linked genes (reviewed in ref. 2).

The control of enhancer activity is likely to be the result of interactions between DNA sequences within the enhancer, regulatory proteins and the transcriptional machinery. Evidence for the existence of cellular

trans-acting factors which activate enhancers has come from competition experiments in vivo (7) and in vitro (8, 9, 10, 11). Genomic footprinting methods have been used to identify contacts between B cell specific factors and the immunoglobulin gene enhancer in vivo (12) and in isolated nuclei (13). A similar approach has identified contacts in isolated nuclei between cellular factors and the inducible enhancer of the human  $\beta$ -interferon gene (14). Recently footprinting techniques (15) and gel retention assays (16, 17) have been used to detect cellular proteins, present in nuclear extracts, which bind to the SV40 enhancer (18), polyomavirus enhancer (19, 20), insulin gene enhancer (21) and the immunoglobulin gene enhancer (22).

Transcription of the adenovirus Ela gene is regulated by DNA sequences with enhancer like properties located 200-400 bp upstream of the transcriptional start site (23, 24, 25, 1, 26). However, within this region different assays for enhancer activity have defined two separate enhancer elements (23, 24, 1). One element (enhancer A in figure 1) has been localized within a 24 bp region positioned approximately 160 bp from the adenovirus type 2 left end (1). The other element (enhancer B in Figure 1) is more complex and appears to contain two functionally distinct domains. One domain (repeated at 200 and 300 bp from the left end) regulates Ela transcription in vivo. The second domain (consisting of 4 repeated sequences located between 250 and 280) regulates activity of all early transcription units (24).

Here we demonstrate that specific nuclear proteins from uninfected Hela cells interact with the adenovirus Ela enhancer defined by Hen et al.(1). A gel retention assay has been used to follow fractionation of the nuclear extract. Competition experiments and DNase footprinting have established that proteins present in enriched fractions interact with the conserved sequence TGTGG<sup>AAA</sup><sub>TTT</sub> present in the adenovirus, SV40 and other viral and cellular enhancers.

## MATERIALS AND METHODS

### Plasmids and Labelled Fragments

pEX (27) contains the left terminal adenovirus type 2 Xba I fragment in which the terminal C of the genome is part of a reconstructed Eco RI site (28) and contains a Bam HI linker inserted into the Hph I cleavage site at the internal boundary of the left inverted terminal repeat. pMKD 231 (29) contains the SV40 T-antigen gene and control region inserted into the Eco RI site of pBR322. In addition this plasmid contains a Bam HI site inserted

between the enhancer and 21 base pair repeats. This plasmid was a kind gift from R.D. Everett MRC Virology Unit, Glasgow, Scotland. Labelled fragments containing the adenovirus Ela enhancer were prepared by first cleaving pEX with Bam HI. [ $\gamma$ - $^{32}$ P] ATP (Amersham specific activity 3000 Ci/mmol) and polynucleotide kinase were used to 5'-end label dephosphorylated DNA. [ $\alpha$ - $^{32}$ P] dATP (Amersham specific activity 3000 Ci/mmol), the other three unlabelled dNTPs and Klenow polymerase were used to 3'-end label DNA. Secondary cleavage with Acc I, Bal I, or Sac II generated fragments of 94 bp, 170 bp and 257 bp respectively. Fragments were purified on 6% polyacrylamide gels and electroeluted (30). A 101 bp Pvu II-Bam HI fragment from pUC9 (31) was 3'-end labelled and purified as described above.

#### Competitors for Gel Retention Assays

The following fragments were isolated from the Ela gene region present in pEX: 111 bp Eco RI-Bam HI; 94 bp Bam HI-Acc I; 170 bp Bam HI-Bal I; 76 bp Acc I - Bal I; 163 bp Acc I-Sac II; 87 bp Bal I-Sac II; 98 bp Sac II-Pvu II; 171 bp Pvu II-Pvu II (Figure I). A 164 bp Bam HI-Pvu II fragment containing two copies of the SV40 enhancer was isolated from pMKD 231 and a 101 bp Pvu II-Bam HI fragment was isolated from pUC9. Fragments were electroeluted from 6% polyacrylamide gels as described above. Concentration of eluted DNA fragments was determined on a small polyacrylamide gel stained with ethidium bromide. Complementary single-stranded synthetic oligonucleotides spanning bases 144-181 from the left end of the Ad 2 genome (oligo A) were kindly provided by C. Goding, Marie Curie Memorial Foundation Research Institute, Oxted, England. Complementary single stranded synthetic oligonucleotides spanning bases 18-41 (NF1 oligo) and 195-211 (oligo B) were made using a Biosearch Model 8600 DNA synthesiser in the MRC Virology Unit, Glasgow. Complementary oligonucleotides were annealed by first heating to 100°C in 0.3M NaCl, 10 mM Tris HCl pH 8.0, 1 mM EDTA followed by slow cooling.

#### Preparation and Fractionation of HeLa Nuclear Extract

HeLa cells were grown in suspension in minimal essential medium containing 7.5% newborn calf serum.

Nuclei from  $2 \times 10^{10}$  HeLa cells were isolated (32) and washed three times with 25 mM Tris HCl pH 7.5, 5 mM KCl, 0.5 mM MgCl<sub>2</sub>, 0.5 mM DTT, 0.2M sucrose. The nuclei were resuspended in the same buffer containing 0.35M NaCl and the suspension stirred on ice for 30 minutes. Particulate material was removed by centrifugation at 100,000 x g for 60 minutes. The supernatant was adjusted to 0.3M NaCl and applied to a DEAE-Sepharose (Pharmacia)

column equilibrated with buffer A (25 mM Tris HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 20% glycerol) containing 0.3M NaCl. The flow through was dialysed against buffer A containing 0.05M NaCl and the fine precipitate formed removed by centrifugation at 100,000 x g for 30 minutes. The supernatant was then applied to a second DEAE-Sepharose column equilibrated with buffer A containing 0.05M NaCl. After washing with two column volumes of buffer A containing 0.05M NaCl, bound material was eluted with a linear gradient of 0.05-0.3M NaCl. Fractions (32-36) containing enhancer binding activity were pooled, diluted to 0.1M NaCl with buffer A and applied to a column of heparin-Sepharose (Pharmacia) equilibrated with buffer A containing 0.1M NaCl. Bound proteins were step eluted with buffer A containing 0.1, 0.3, 0.5, 0.7 and 1.0M NaCl. Samples were frozen in liquid N<sub>2</sub> and stored at -70°C. All procedures were performed at 0-4°C. Protein concentration was determined by the method of Bradford (39).

### Gel retention assay

Assays contained 0.1 ng labelled probe ( ~ 10,000 cpm), 2 µg of unlabelled DNA (a 1:1 mixture of poly d(A-T): poly d(G-C), (Pharmacia) 20 mM Hepes NaOH pH 7.5, 1 mM DTT, 1 mM EDTA, 100 mM NaCl and 1-3 µl of protein fraction in a final volume of 25 µl. After 30 minutes at 20°C 5 µl of 50% glycerol, 10 mM Hepes NaOH pH 7.5, 0.1% bromophenol blue was added and the entire reaction loaded onto a 6% (80:1 acrylamide:bisacrylamide) polyacrylamide gel containing 50 mM Tris borate pH 8.3, 1 mM EDTA. Electrophoresis was conducted at 200 V for one hour. Gels were fixed in 10% acetic acid, dried and exposed to Kodak XAR5 X-ray film at -70°C with an intensifying screen.

### DNase protection

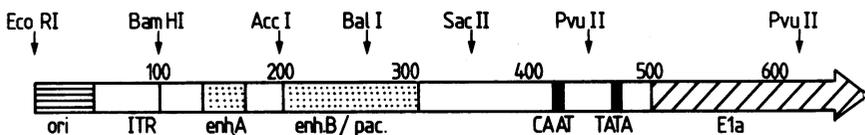
The reaction contained 0.1 ng labelled probe ( ~ 10,000 cpm), 2 µg of unlabelled DNA (a 1:1 mixture of poly d(A-T):poly d(G-C), 27 mM Hepes NaOH pH 7.5, 1.5 mM DTT, 0.5 mM EDTA, 100 mM NaCl, 7% glycerol and various amounts of protein fraction in a total volume of 50 µl. After 30 minutes at 20°C 0.5 unit of DNase I (Amersham) was added in 5 µl of the above reaction buffer containing 20 mM MgCl<sub>2</sub>. The reaction was allowed to proceed for 60 seconds at 20°C and was stopped by the addition of 25 µl 1M NaCl, 20 mM EDTA, 1% SDS, 150 µg/ml proteinase K. After 60 minutes at 37°C DNA was precipitated with ethanol, washed with 70% ethanol, dried and redissolved in 5 µl 98% formamide, 20 mM NaOH, 1 mM EDTA with bromophenol blue and xylene cyanol FF. DNA was denatured at 100°C for 2 minutes, cooled on ice and fractionated on an 8% polyacrylamide gel containing 50% urea. The gel was

fixed in 10% acetic acid, dried and exposed to X-ray film at  $-70^{\circ}\text{C}$  in the presence of an intensifying screen. Sequence markers were provided by subjecting end labelled fragments to G + A or C + T specific reactions (33).

## RESULTS

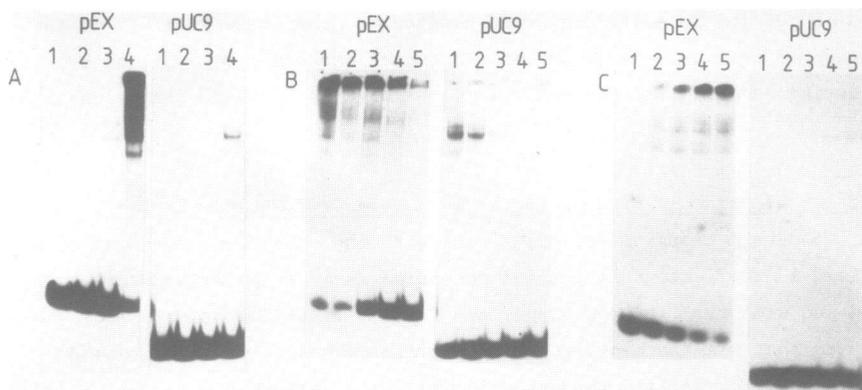
### Detection of Enhancer Binding Proteins in HeLa Cell Nuclear Extracts

To detect proteins which interact with the adenovirus enhancer we have utilized a modification of the gel retention assay originally described by Fried and Crothers (16) and Garner and Revzin (17). The principal of the assay is that DNA fragments to which protein is bound have a decreased electrophoretic mobility through polyacrylamide gels when compared to unbound DNA fragments. Non-specific interactions between the labelled DNA fragment and cellular proteins are eliminated by the inclusion of a large excess of unlabelled non-specific DNA. Cell extracts prepared from HeLa cells were tested for proteins which bound to a 170 bp probe (from Bam HI to Bal I) which contains the enhancer A and copies of each element present in enhancer B (Fig. 1). To monitor non-specific binding a 101 bp labelled probe was prepared from pUC9 (Pvu II-Bam HI). HeLa cells were fractionated into nuclei and cytosol and the nuclei extracted with increasing concentrations of sodium chloride. Incubation of labelled probe with bovine serum albumin results in the detection of a discretely migrating labelled fragment (Fig. 2A, lanes 1) characteristic of free DNA. Cytoplasmic extracts and 0.15M sodium chloride extracts of nuclei displayed little binding to either the enhancer or non-specific probe (Figure 2A, lanes 2 and 3). However incubation of 0.35M sodium chloride extracts of nuclei with the adenovirus enhancer probe resulted in the appearance of labelled species with decreased



**Figure 1.** 5' flanking region of adenovirus type 2 E1a gene.

The left end of the adenovirus type 2 genome present in pEX is shown along with restriction enzyme cleavage sites utilized in this study. Sequences possessing enhancer like properties are indicated by the stippled area. Enhancer A (Enh. A) was defined by Hen *et al.* (1). Enhancer B which overlaps a sequence required for packaging (Enh. B/pac) was defined by Hearing and Shenk (24). The adenovirus type 2 origin of replication (38) is indicated by horizontal shading, the CAAT and TATA homologies by filled boxes and the E1a coding region by diagonal shading.



**Figure 2.** Detection of enhancer binding proteins in HeLa cell nuclear extracts.

Labelled probes containing the adenovirus enhancer region (pEX, Bam HI to Bal I) or a fragment of *E. coli* DNA (pUC9, Pvu II to Bam HI) were incubated with cell extracts in the presence of 2  $\mu$ g unlabelled DNA, a 1:1 mixture of poly d(A-T) and poly d(G-C), followed by electrophoresis through a 6% polyacrylamide gel.

A: Fragments were incubated with 1, 5  $\mu$ g bovine serum albumen; 2, 5  $\mu$ g of cytoplasmic extract; 3, 5  $\mu$ g of 0.15M NaCl nuclear extract; 4, 5  $\mu$ g of 0.35M NaCl nuclear extract.

B: Fragments were incubated with 5  $\mu$ g 0.35M NaCl nuclear extract in the presence of increasing concentrations of NaCl: 1, 0.05M; 2, 0.1M; 3, 0.15M; 4, 0.2M; 5, 0.25M.

C: Fragments were incubated under standard assay conditions with 1  $\mu$ g, 2  $\mu$ g, 3  $\mu$ g, 4  $\mu$ g and 5  $\mu$ g of 0.35M NaCl nuclear extract (lanes 1-5).

electrophoretic mobility (Figure 2A, pEX lane 4). Incubation of these extracts with the non-specific probe resulted in a small fraction of the labelled probe being shifted to a more slowly migrating species (Figure 2A, pUC9 lane 4). The quantitative and qualitative differences observed between the enhancer containing probe and the non-specific probe suggest the presence of nuclear proteins which bind specifically to the adenovirus enhancer. Extraction of nuclei with concentrations of sodium chloride greater than 0.35M did not increase the amount of enhancer specific binding proteins present in the extract (data not shown). Non-specific binding in these assays was competed out by the presence of a large excess of poly d(G-C) and poly d(A-T). DNA of high sequence complexity (e.g. Sheared *E. coli* DNA and pUC9 DNA) were unsuitable for this purpose as they also diminished specific binding (data not shown), presumably as a result of competition for the enhancer specific factor.

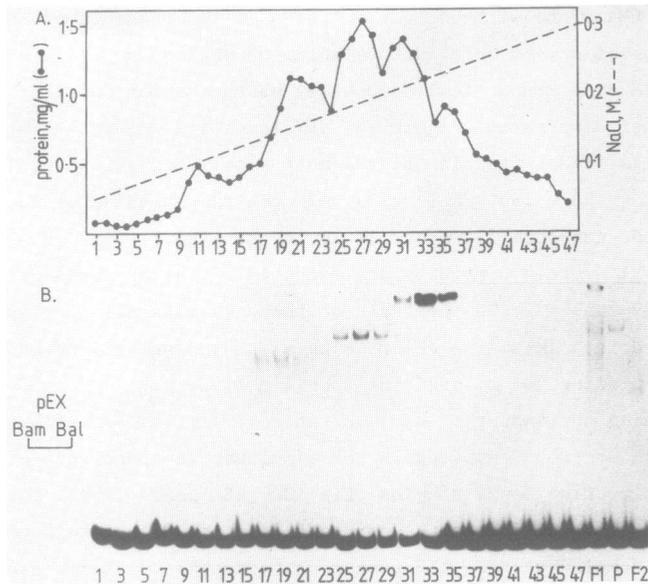
The stability of these DNA-protein complexes was tested by incubating

the labelled DNA probe with nuclear extracts in the presence of increasing concentrations of sodium chloride. Binding of nuclear proteins to the enhancer containing probe slowly decreases as the sodium chloride concentration is increased (Figure 2B, pEX lanes 1-5). Even at 0.25M sodium chloride a significant fraction of the most slowly migrating species is still present (Figure 2B, pEX lane 5). In contrast binding of nuclear proteins to the non-specific probe is rather sensitive (Fig. 2B, pUC9 lanes 1-5) and no DNA-protein complexes are detected in the presence of 0.2M NaCl (Fig. 2B, pUC9 lane 4). On the basis of these results all subsequent assays were conducted in 0.1M sodium chloride as a compromise between reducing non-specific binding and maintaining specific binding.

Increasing the amount of nuclear extract relative to a fixed amount of labelled enhancer probe resulted in the simultaneous appearance of all three more slowly migrating bound species (Fig. 2C, pEX lanes 1-5). This suggests that the different bound species represent distinct DNA-protein complexes and that one complex was not the precursor to another. At low protein concentration binding to the pUC9 probe was negligible when compared to the enhancer containing probe (Fig. 2C, compare lanes 2). These initial results suggested that nuclei from Hela cells contained proteins which bound specifically to the adenovirus enhancer.

#### Fractionation of the Nuclear Extract

To separate the various enhancer binding species the nuclear extract was fractionated by column chromatography. To remove nucleic acids the nuclear extract was passed over DEAE-Sepharose in 0.3M sodium chloride. All enhancer binding activity was present in the column flow through (Fig. 3B, lane F1). After dialysis to 0.05M sodium chloride a fine precipitate was removed by centrifugation. Although the bulk of enhancer binding activity remained soluble a proportion of one species was removed by centrifugation (Fig. 3B, lane P). The clarified extract was then applied to DEAE-Sepharose and bound proteins eluted with a linear gradient of sodium chloride. Fractions were monitored for enhancer binding and non-specific binding by the gel retention assay. Use of the 170 bp enhancer containing probe indicates that a separation of the three major enhancer binding species has been effected by this chromatographic step (Fig. 3B). In comparison, binding to the non-specific probe was minimal (data not shown). Fractions 32-36 contained an activity responsible for formation of the most abundant and most slowly migrating bound complex (Fig. 3B). These fractions were applied to a heparin sepharose column and the bound proteins eluted by



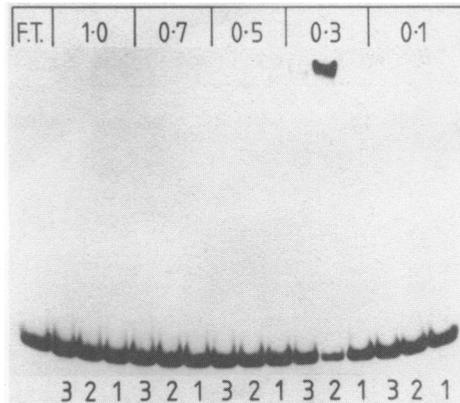
**Figure 3** Fractionation of HeLa cell nuclear extract on DEAE Sepharose.

HeLa cell nuclear extract was passed over a DEAE Sepharose column in 0.3M NaCl, processed as described under "Experimental Procedures" and loaded onto a DEAE-Sepharose column at 0.05M NaCl. The column was developed with a linear gradient between 0.05M and 0.3M NaCl. Fractions sampled are indicated below each figure. F1 represents the material passing through the first DEAE-Sepharose column, F2 material passing through the second DEAE Sepharose column and P material which precipitated after dialysis into 0.05M NaCl. A. protein concentration in each sample was determined by the method of Bradford (39). B. Fractions were incubated with the Bam HI to Bal I fragment from pEX, and DNA-protein complexes resolved on a 6% polyacrylamide gel.

stepwise increases in the sodium chloride concentration. Fractions were tested for enhancer binding activity in the gel retention assay using the 170 bp Bam HI to Bal I probe. All enhancer binding activity bound to the column and was eluted in a single step with 0.3M sodium chloride (Fig. 4).

Specificity of Enhancer Binding Proteins

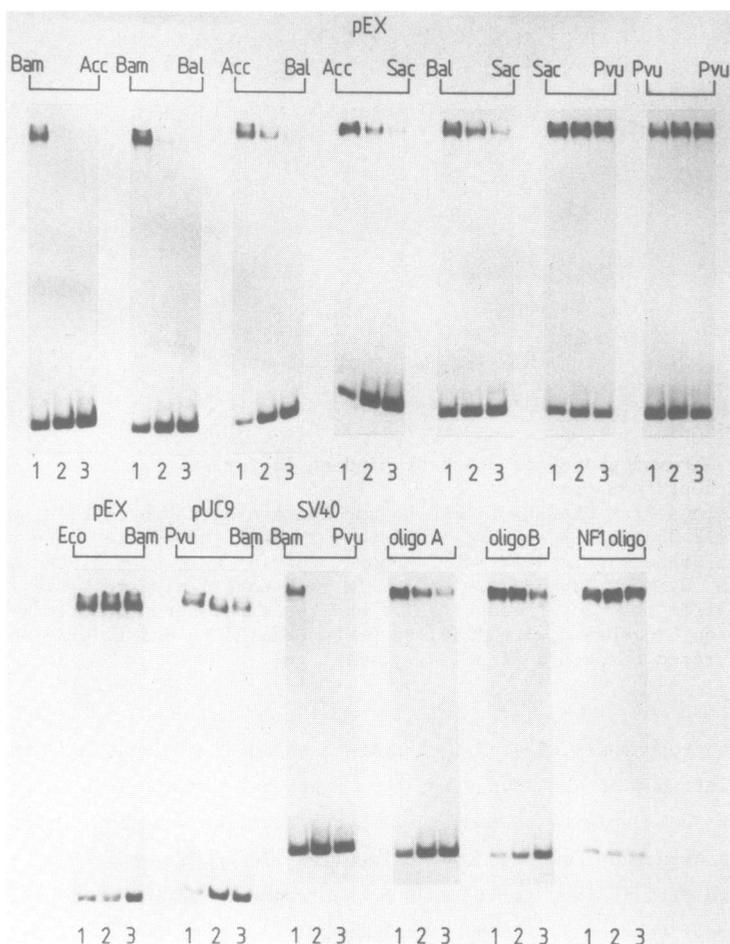
To determine the binding specificity of factors present in fractions containing enhancer binding activity competition experiments were performed. A constant non-saturating amount of enhancer binding fraction (2 µg, DEAE-Sepharose fraction) was incubated with a constant amount of enhancer containing probe (170 bp, Bam HI to Bal I) and increasing amounts of various unlabelled DNA fragments. Free and bound labelled DNA species were resolved



**Figure 4** Chromatography of enhancer binding factor on heparin-Sepharose.

Fractions from DEAE-Sepharose column containing enhancer binding activity (32-36) were pooled, diluted to 0.1M NaCl and applied to a column of heparin-Sepharose in 0.1M NaCl. Bound proteins were step eluted with 0.1M, 0.3M, 0.5M, 0.7M and 1.0M NaCl. In each case 1  $\mu$ l samples were withdrawn from the flow through (F.T.) and the first three fractions eluting at each step, incubated with labelled probe (Bam HI to Bal I, pEX) and electrophoresed through a 6% polyacrylamide gel.

by gel electrophoresis (Fig. 5). Fragments spanning nucleotides 1 to 622 from the left end of the adenovirus type 2 genome, present in pEX, were tested for their ability to compete with the labelled enhancer containing probe. As expected the homologous unlabelled 170 bp fragment (Bam HI to Bal I) competed efficiently, as did a 94 bp subfragment (Bam HI to Acc I). The 163 bp (Acc I to Sac II), 76 bp (Acc I to Bal I) and 87 bp (Bal I to Sac II) fragments also competed for binding of the labelled probe, albeit with reduced efficiency. Fragments containing the inverted terminal repeat (Eco RI to Bam HI), the "CAAT box" (Sac II to Pvu II) and the "TATA box" and Ela mRNA cap site (Pvu II to Pvu II) failed to compete. To test whether the factor which bound to the adenovirus enhancer also bound to other viral enhancers competition experiments were performed with a 164 bp (Bam HI to Pvu II) fragment containing two copies of the SV40 enhancer. This fragment competed very efficiently for the factor which binds to the adenovirus enhancer (Fig. 5). The enhancer binding fraction therefore recognises sequences common to both the adenovirus and SV40 enhancers. To our surprise the 101 bp (Pvu II-Bam HI) fragment isolated from pUC9, which had been used as a control for non-specific binding, also competed, although at a reduced

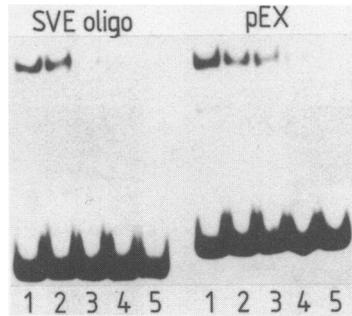


**Figure 5** Competition analysis of enhancer binding protein specificity.

Reactions contained 0.25 ng labelled DNA probe (Bam HI to Bal I, pEX), 2  $\mu$ g of unlabelled DNA (poly d(A-T), poly d(G-C)) and 2  $\mu$ g of protein from fraction 31 of the DEAE-Sepharose column. In addition reactions contained 0.25 ng (lane 1), 5 ng (lane 2) and 12.5 ng (lane 3) of unlabelled purified fragments, the origin of which is indicated above each panel. Oligo A, Oligo B and NFI oligo are described in "Experimental Procedures". Reactions were analysed by electrophoresis through 6% polyacrylamide gels.

level. A possible explanation for this observation is given in the discussion.

To define more precisely the sequences recognised by the enhancer binding proteins double stranded synthetic oligonucleotides corresponding to



**Figure 6** Competition analysis with double strand synthetic oligonucleotide from the SV40 enhancer

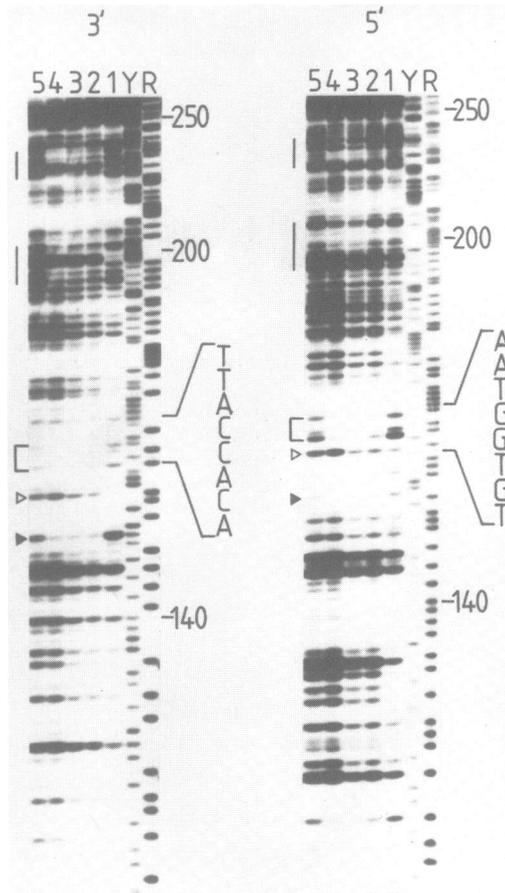
Reactions contained 0.25ng labelled DNA probe (SVE oligo; BamHI to ACCI, pEX), 2µg of unlabelled DNA (poly d (A-T), poly d (G-C) and 2 µg of protein from fraction 31 of the DEAE-Sephadex column. In addition reactions contained 0 (lane 1), 0.25 ng (lane 2), 1.25 ng (lane 3), 2.5 ng (lane 4) and 6.25 ng (lane 5) of the unlabelled double strand synthetic oligonucleotide

5' - GATCTAGGGTGTGGAAAGTCCCG - 3'  
3' - ATCCACACCTTTCAGGGCCTAG - 5'

Reactions were analysed by gel electrophoresis.

defined regions from the left end of the adenovirus type 2 genome were constructed and employed in competition assays as described above. Synthetic double stranded fragments spanning bases 144-181 (oligo A) and 195-211 (oligo B) both competed to a limited extent for the enhancer binding fraction, although only when present in a large molar excess. A synthetic fragment (NF1 oligo, base 18-41) which contained the binding site for nuclear factor I (34) failed to compete.

Sequences within the SV40 enhancer which were responsible for the observed competition were also identified by competition experiments. A double strand synthetic oligonucleotide containing bases 236-253 from the SV40 enhancer (SVE oligo) was labelled and incubated with the enhancer binding fraction. Free and bound species were resolved by gel electrophoresis (figure 6). A specific DNA-protein complex was observed that could, as expected, be competed by an excess of the double strand unlabelled oligonucleotide. A 94bp sub fragment from pEX (Bam HI to Acc I, figure 1) which contains the adenovirus enhancer also bound to proteins in the DEAE fraction (figure 6, pEX, lane 1). Formation of the DNA-protein complex was eliminated by incubation with the double strand synthetic oligonucleotides corresponding to sequences in the SV40 enhancer which contained the 'core' consensus sequence (figure 6, pEX, lanes 2-5).



**Figure 7** DNase I protection of the adenovirus enhancer by the enhancer specific factor.

The 257 bp Bam HI to Sac II fragment, from pEX which contains enhancer A and part of enhancer B was labelled with  $^{32}$ P at the Bam HI site as described in "Experimental Procedures". The 3' labelled fragment (3') and 5' labelled fragment (5') were incubated with increasing amounts of the heparin-Sepharose purified enhancer binding factor in the presence of 2  $\mu$ g unlabelled DNA (poly d(A-T), poly d(G-C)). Reaction mixtures were treated with DNase I and electrophoresed in an 8% denaturing polyacrylamide gel. A + G (R) and C + T (Y) specific cleavage reactions of the labelled fragments were electrophoresed in parallel as markers. 1, no added protein; 2, 5  $\mu$ g heparin fraction; 3, 9  $\mu$ g heparin fraction; 4, 18  $\mu$ g heparin fraction; 5, 18  $\mu$ g heparin fraction and 100 ng synthetic duplex oligonucleotide spanning bases 144-181. The major protected area against DNase I cleavage is indicated by the bracket. Bonds protected against DNase I ( $\blacktriangledown$ ) or displaying enhanced DNase I cleavage ( $\nabla$ ) are also shown. Additional areas of altered DNase I cleavage are indicated by vertical bars. The sequence homologous to the 'core' consensus of enhancers is indicated.



**Figure 8** Summary of DNase protection data.

The sequence of the 5' flanking region of the adenovirus type 2 Ela gene between positions 141-249 (250 to 348 bases upstream from the Ela capsite) is shown. Bonds protected from DNase I cleavage (▼) or displaying enhanced cleavage by DNase I (▽) in the presence of the enhancer specific factor are indicated. Repeated sequences I and II (9 out of 11 match) are indicated by brackets.

#### Binding Site for the Enhancer Specific Protein

The binding site for the enhancer specific protein was determined by DNase footprinting (15) and DNase footprinting in the presence of competing synthetic duplex oligonucleotides. Probes for footprinting both strands were prepared by labelling 5' and 3' termini at the Bam HI site in pEX (Fig. 1) followed by secondary cleavage with Sac II. Increasing quantities of the heparin fraction were bound to the probe, lightly digested with DNase I and the digestion products fractionated on a denaturing polyacrylamide gel (Fig. 7, lanes 1-4). A number of areas show altered sensitivity to DNase I, the most prominent of which is centred on position 160. On both strands a protected bond is followed by an enhanced cleavage which is followed by a small area of protection (159-163 on 5' labelled strand, 157-161 on 3' labelled strand). Other less prominent areas of protection and enhanced cleavage are evident centred on position 200 and position 235 (Fig. 7, summarized in Fig. 8).

As an additional means of defining the binding site the labelled probe was incubated with the heparin fraction in the presence of a large molar excess of a synthetic duplex oligonucleotide spanning bases 144-181 (oligo B) prior to DNase I digestion. Protection of bonds 159-163 on 5' strand and 157-161 on 3' strand is abolished by the oligonucleotide (Fig. 7, lanes 5). Although the enhanced cleavages at 157 (5' strand) and 153 (3' strand) are reduced in intensity it is clear that incubation with the duplex oligonucleotide does not completely abolish the enhanced cleavage by DNase I. The presence of the duplex oligonucleotide simultaneously abolishes the protected zones centred on positions 200 and 235. However the enhanced cleavages in these regions are not markedly reduced by the presence of the duplex oligonucleotide. A prominent example is the enhanced cleavage on the 3' strand at position 197 (Fig. 7, lanes 5).

**Table I** Homologies among fragments which compete for binding of the enhancer specific factor.

		G T G G <sup>A A A</sup>	'core' consensus	
G A T G T G G T A A A		↑ ↑ ↑	Ad 2, 156-166	7/7
G T T G T A G T A A A			Ad 2, 233-243	6/7
G C T G T G G A A T G			SV40, 273-263	7/7
G G T G T G G A A A G			SV40, 250-240	7/7
G A A G T G A C A A T			Ad 2, 200-210	5/7
C C G G T G T A T A C			Ad 2, 186-196	6/7
G A A G T G A A A T C			Ad 2, 300-310	6/7
T C T G T G T T A C T			Ad 2, 317-327	6/7
T G T G T G G A A T T			pUC9, 425-435	7/7

The sequence surrounding the predominant binding site for the enhancer specific factor was compared to sequences present on fragments which could compete for binding and to the 'core' consensus sequence present in many viral and cellular enhancers (3). The location of the sequence is indicated, as is the extent of homology to the 'core' consensus.

**DISCUSSION**

We have utilized a sensitive gel retention assay to detect HeLa cell nuclear proteins which bind to the adenovirus enhancer. This assay has been used to follow the fractionation of the nuclear extract and indicates that at least three enhancer binding species can be detected and resolved by chromatography on DEAE Sepharose. One of these species has been shown by DNase footprinting to bind predominantly to the 'core' consensus sequence GTGG<sup>AAA</sup>, present at position 160 in the adenovirus type 2 genome and found in many viral and cellular enhancers (3, 35). Protection of this sequence from DNase I digestion was abolished by competition with a duplex synthetic oligonucleotide corresponding to bases 144-181 from the left of the adenovirus type 2 genome. This region corresponds to the sequence defined by Hen *et al.* (1) as possessing enhancer function. The role of the 'core' consensus sequence in binding of the factor was further strengthened by experiments in which double strand synthetic oligonucleotides containing the 'core' consensus sequence present in the SV40 enhancer efficiently competed with the adenovirus type 2 enhancer for binding of the factor.

Competition experiments have also demonstrated that in addition to these high affinity binding sites proteins responsible for DNA-protein complex formation also bind to other sites with reduced but significant affinity (Figure 4, summarized in Table 1). While effective competition was observed with a fragment containing the 'core' consensus (Bam HI to Acc I) significant competition was also observed with other fragments from the Ela

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upstream region. Sequences between positions 192 and 267 (Acc I to Bal I fragment) and 267 and 353 (Bal I to Sac II fragment) also compete, albeit to a lesser extent. Other Ela upstream sequences outwith these regions fail to compete. Within the sequences which compete are regions with homology to the high affinity site at 160: sequences 233 to 243 have a 9 out of 11 match with sequences 156 to 166. In other areas the homology is less striking (Table 1). DNase footprinting supports the conclusion that these sequences indeed bind the enhancer specific factor as bonds within the region 224-238 show reduced and enhanced cleavage with DNase I in the presence of the enhancer specific factor.

Competition experiments (figure 4) demonstrated that the enhancer binding factor bound weakly to a pUC9 derived fragment that contained a perfect match to the 'core' consensus thus indicating that sequences other than the 'core' consensus are required for high affinity binding. These studies indicate that factor(s) in the enhancer binding fraction bind to a number of related sequences with a range of affinities. It is not clear if this binding is due to a single binding factor or a number of related factors. The multiplicity of binding factors is suggested by the resolution of three binding species by chromatography on DEAE Sepharose. Competition experiments and the use of small labelled subfragments in gel retention assays indicate that these three species recognise similar sequences (data not shown). Whether they represent separate protein species or processed forms of the same protein remains to be determined.

The role of these enhancer binding factors in enhancer activity is suggested by the finding that sequences from the left end of the adenovirus type 2 genome were shown to stimulate transcription when linked to a heterologous promoter in vivo. Deletion analysis indicated that sequences between positions 155 to 178 which contain the 'core' consensus sequence and bind the enhancer specific factor, were responsible for the bulk of the observed enhancer activity. Removal of the binding site for the enhancer specific factor drastically reduced enhancer activity (1). However it has been shown that removal of sequences between positions 106-195 from the Ad 5 variant dl 309 had no detrimental effect on virus viability or Ela transcription measured at 5 hours post infection (23). In fact the mutant virus Ad 5 dl 309-6 lacking these sequences, reproducibly generated slightly more Ela mRNA than the parent virus (23). These apparently conflicting results can be reconciled by the finding that the Ela gene products can negatively regulate viral (4, 5) and cellular enhancers (6) which contain the 'core'

consensus sequence. Thus it is possible that the enhancer element defined by Hen et al. (1) is active early in infection prior to the accumulation of Ela proteins. In the presence of Ela protein this enhancer is then repressed. Removal of the site responsible for repression may therefore stimulate Ela transcription as in the case of Ad 5 dl 309-6 (23). How Ela proteins negatively regulate enhancers is not known but the enhancer binding factor described here represents a potential target for Ela action.

Although the factor described here may be important for enhancer function it is clear that interaction of this factor with a single enhancer element is not sufficient for enhancer function. A considerable body of evidence indicates that enhancer function requires the interaction of proteins with multiple enhancer motifs (36). Enhancer activity can also be generated by duplicating the motifs found in enhancers (37). These findings suggest that enhancer activity requires the formation of a large DNA-protein complex in which a limited set of proteins interact at various points along the enhancer. To establish the role of the enhancer specific factor described here will require its complete purification and an assay for its activity in vitro.

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