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THE ROLE OF SEQUENCE-SPECIFIC DNA-BINDING PROTEINS IN ADENOVIRUS DNA REPLICATION

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SUMMARY

In prokaryotes it is well established that proteins which recognise defined DNA sequences are involved in the control of gene expression and replication. Cellular proteins in eukaryotes which may perform a similar function have been identified by their interactions with control regions of the human adenovirus genome.

Immediately after infection a small region (E1a) at the left end of the adenovirus genome is expressed. Proteins coded by the E1a region transcriptionally activate the viral early genes. The products of a number of these early genes are directly involved in replication of the viral DNA. DNA sequences which are required for efficient E1a transcription and for the initiation of DNA replication have been identified by mutational analysis. Cellular proteins which recognise these sequences were detected using a sensitive gel retention assay. The basis of this assay is that during electrophoresis DNA-protein complexes migrate more slowly through a polyacrylamide gel than free DNA. In this way a cellular protein which binds to a conserved sequence in the adenovirus enhancer has been identified and partially purified. Cellular proteins which bind to adenovirus type 2 and 4 origins of replication have also been fractionated from nuclear extracts of uninfected HeLa cells. The roles of these proteins in adenovirus replication will be discussed.

INTRODUCTION

In prokaryotes the power of genetics has been used to identify regulatory regions of the genome and the proteins which interact with them. The inability to utilise this genetic approach for an analysis of animal cells has led to an alternative approach in which animal viruses have been used as models to study the regulatory processes of their hosts. Because of their relative simplicity the papovavirus SV40 and the human adenoviruses have been extensively utilised for this purpose. In these instances conventional genetics and site directed mutagenesis have identified regions of the viral genomes which are involved in the regulation of viral transcription and DNA replication. Since these viruses rely heavily on the host cell to carry out these processes our approach has been to identify and characterise cellular proteins which interact with regulatory sequences present in the human adenovirus genome.

Immediately after infection of human cells with adenovirus type 2 the E1a transcription unit, located at the left end of the genome, is expressed. Proteins coded by the E1a region transcriptionally activate other viral early genes, a number of which are directly involved in replication of the viral DNA. The product of the E2a region is the single strand specific DNA binding protein (DBP) while the products of the E2b region are the viral DNA polymerase (pol) and the precursor to terminal

protein (pTP). The pTPpol complex and DBP then participate in the initiation of DNA replication which takes place at the terminally-located origins of DNA replication. In the case of adenovirus type 2 a number of cellular proteins have also been shown to participate in the initiation reaction *in vitro*.

In this communication we report on the identification of a cellular protein which binds to the adenovirus type 2 enhancer and the role of cellular proteins in the initiation of adenovirus type 2 and type 4 DNA replication.

MATERIALS AND METHODS

Cells and viruses

HeLa cells were grown in suspension in minimal essential medium containing 7 % newborn calf serum. The 293 cell line (Graham *et al.* 1977) was grown in 'Glasgow-modified Eagles medium' containing 10 % foetal calf serum. Adenovirus types 2 and 4 were grown and titrated on 293 cells by the method of Williams (1970). Virus was purified on a glycerol/caesium chloride gradient (Mautner & Willcox, 1974) followed by centrifugation to equilibrium in a second caesium chloride gradient. Virion DNA was extracted by the method of Pettersson & Sambrook (1973).

Plasmids and labelled fragments

The plasmids utilised in these studies have all been described previously: pEX (Hay & McDougall, 1986); pHR18, p4A2, pHR1 (Hay, 1985*b*); pC4, pD21 (Adhya *et al.* 1986). Labelled fragments containing the adenovirus type 2 E1a enhancer were prepared by first cleaving pEX with *Bam*HI. [γ-^{32}P]ATP (Amersham, specific activity 3000 Ci mmol^{-1}) and polynucleotide kinase were used to label dephosphorylated DNA. [α-^{32}P]dATP (Amersham, sp. act. 3000 Ci mmol^{-1}), the other three unlabelled dNTPs and Klenow polymerase were used to label the 3'-ends of the DNA. Secondary cleavage with *Acc*I, *Bal*I or *Sac*II generated fragments of 94, 170 and 257 base pairs (bp), respectively. Fragments were purified on 6 % polyacrylamide gels and electroeluted. Complementary oligonucleotides were annealed by first heating to 100 °C in 0·3 M NaCl, 10 mM Tris·HCl, pH 8·0, 1 mM EDTA followed by slow cooling. Double-stranded oligonucleotides were labelled with [α-^{32}P]dATP and Klenow polymerase as described above.

Cell fractionation

Nuclei and cytosol were prepared from uninfected HeLa cells as described previously (Challberg & Kelly, 1979). The enhancer binding protein fraction was obtained by chromatography of the nuclear extract on DEAE-Sepharose and heparin-Sepharose (Barrett *et al.* 1987). Nuclear fractions stimulating adenovirus type 2 DNA replication were prepared as previously described (Nagata *et al.* 1983; Pruijin *et al.* 1986).

Analytical procedures

Gel retention assays (Barrett *et al.* 1987), DNase protection experiments (Galas & Schmitz, 1978), dimethyl sulphate (DMS) protection experiments (Gilbert *et al.* 1976) and analysis of DNA replication *in vivo* (Hay, 1985*a*) have all been described previously.

RESULTS

Detection of enhancer binding fractions in HeLa cell nuclear extracts

DNA sequences required for efficient E1a transription have been located between the cap site and 400 bases upstream. In addition to the 'TATA' and 'CAAT' homologies DNA sequencies with enhancer-like properties have been located

200–400 bases upstream of the transcriptional start site (Hearing & Shenk, 1983, 1986; Weeks & Jones, 1983; Hen *et al.* 1983; Imperiale *et al.* 1983).

Two distinct elements have been recognised within this region. One region (enhancer A in Fig. 1) was localised to a 24 bp region between nucleotides 155 and 179 from the left end of the genome (Hen *et al.* 1983). The other region possessing enhancer activity (enhancer B in Fig. 1) is more complex and appears to contain two functionally distinct domains. One repeated domain regulates E1a transcription whereas the second repeated domain modulates the transcription of all early transcription units (Hearing & Shenk, 1983, 1986).

As a first step in understanding the mechanism of enhancer action we have attempted to detect cellular proteins which interact with the adenovirus enhancer. To detect proteins which bind to a specific DNA sequence we have employed a modification of the gel retention assay originally described by Fried & Crothers (1981) and Garner & Revzin (1981). The basis of this technique is that a DNA–protein complex will migrate more slowly during electrophoresis in a polyacrylamide gel than free DNA. To exclude non-specific interactions between the labelled DNA fragment and cellular proteins a large excess of unlabelled non-specific DNA is included in the binding reaction (Strauss & Varshavskey, 1984). The probe utilized to detect enhancer-binding proteins consisted of a 170 bp DNA fragment (from *Bam*HI to *Bal*I, Fig. 1) which contains enhancer A and copies of each element present in enhancer B. To assess non-specific binding a 101 bp labelled probe from pUC9 (*Pvu*II to *Bam*HI) was utilized. As a source of proteins 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 (BSA), cytoplasmic extracts and 0·15 M NaCl extracts of nuclei displayed little binding to either the enhancer or non-specific probe (data not shown). However incubation of the 0·35 M NaCl nuclear extracts with the enhancer probe resulted in the appearance of more slowly migrating labelled species (Fig. 2(A)). Incubation of 0·35 M NaCl extracts with the non-specific probe did not result in the accumulation of appreciable quantities of the more slowly migrating species (Fig. 2(B)). Extraction of nuclei with concentrations of NaCl greater than 0·35 M did not increase the

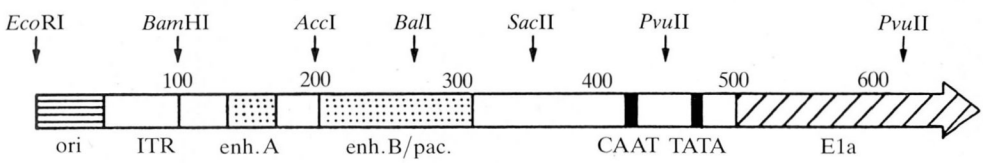

Fig. 1. 5′ flanking region of adenovirus type 2 E1a gene. The left end of the adenovirus type 2 genome present in pEX is shown along with restriction enzyme cleavage sites utilized in this study. Sequences possessing enhancer like properties are indicated by the stippled area. Enhancer A (enh. A) was defined by Hen *et al.* (1983). Enhancer B which overlaps a sequence required for packaging (enh. B/pac) was defined by Hearing & Shenk (1986). The adenovirus type 2 origin of replication (Hay, 1985*b*) is indicated by horizontal shading, the CAAT and TATA homologies by filled boxes and the E1a coding region by diagonal shading.

enhancer binding activity of the extracts. To compete out non-specific binding a mixture of the two copolymers poly [d(G-C)] and poly[d(A-T)] was utilized. DNA of high sequence complexity (e.g. sheared *Escherichia coli* DNA) was found to be unsuitable as a competitor as it reduced binding to the enhancer containing probe, presumably as a result of the presence of sequences in *E. coli* DNA that were similar to the sequences present in the adenovirus enhancer. These preliminary observations thus identify a nuclear protein derived from HeLa cells which binds specifically to the adenovirus enhancer.

Fractionation of the nuclear extract

To obtain a preparation of nuclear protein with increased enhancer binding activity, and to remove other binding activities the HeLa cell nuclear extract was fractionated by ion exchange chromatography. Nucleic acids were first removed by

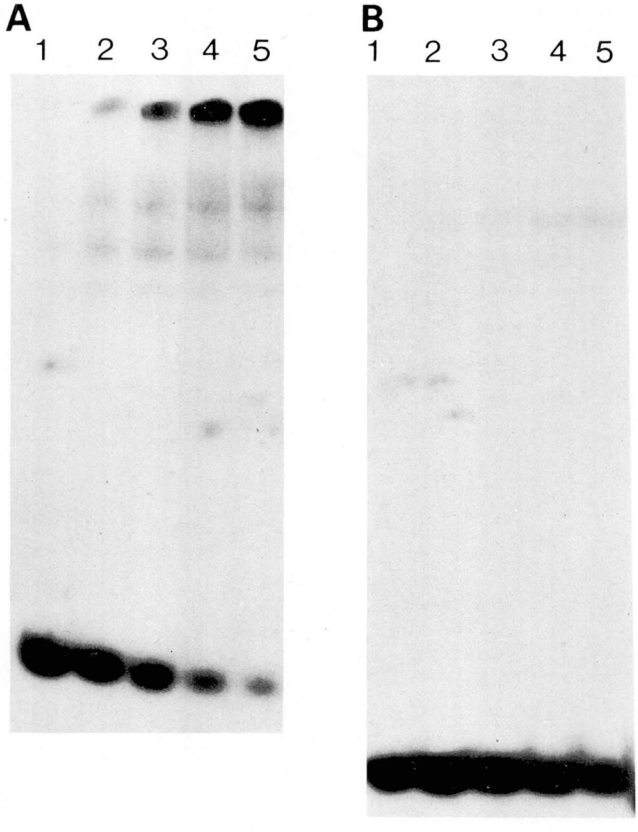

Fig. 2. Labelled probes containing (A) the adenovirus enhancer region (pEX, *Bam*HI to *Bal*I) or (B) a fragment of *E. coli* DNA (pUC9, *Pvu*II to *Bam*HI) were incubated with 1, 2, 3, 4 and 5 μg of 0·35 M NaCl nuclear extracts (lanes 1–5) in the presence of 2 μg unlabelled DNA (a 1:1 mixture of poly[d(A-T)] and poly[d(G-C)], DNA–protein complexes were resolved by electrophoresis through a native 6% polyacrylamide gel.

passing the nuclear extracts over DEAE-Sepharose in 0·3 M NaCl. At this salt concentration nucleic acids are retained on the column while the bulk of the nuclear protein, including the enhancer binding protein, are present in the column flow through. The NaCl concentration was reduced to 0·05 M by dialysis and protein was applied to a DEAE Sepharose column equilibrated with 0·05 M NaCl. Bound proteins were eluted with a linear gradient of NaCl from 0·05 to 0·3 M. Alternate fractions were tested for the presence of enhancer-binding protein by the gel retention assay (Fig. 3). Binding was quantitated by excising the more slowly migrating bound species and determining the radioactivity by scintillation counting. The enhancer-binding species eluted from DEAE Sepharose at 0·25 M NaCl (Fig. 3) and could be further purified on heparin Sepharose and native DNA-cellulose, where it eluted at 0·3 M NaCl in both cases (data not shown).

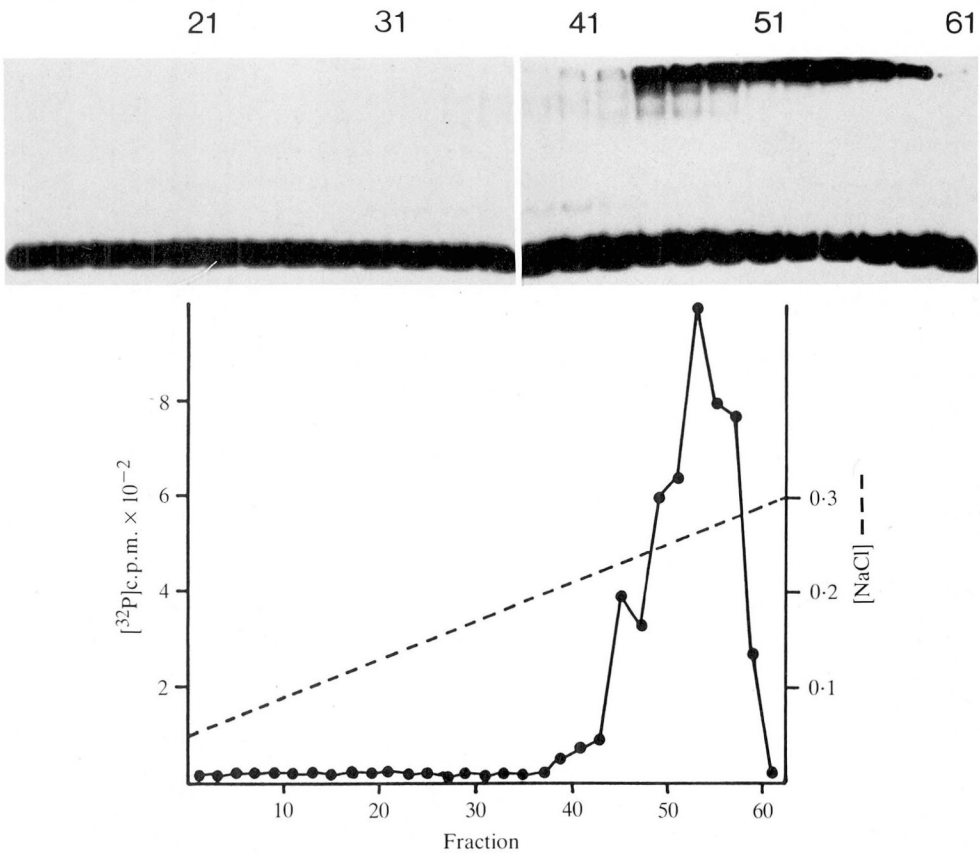

Fig. 3. Fractionation of HeLa cell nuclear extract. Nuclear extract was loaded onto DEAE Sepharose and bound proteins eluted with a gradient of NaCl from 0·05 to 0·3 M. In the upper panel fractions were incubated with the *Bam*HI-*Acc*I fragment from pEX and DNA protein complexes resolved on a polyacrylamide gel. Quantitation of free and bound species was obtained by excising fragments from the polyacrylamide gel and determining the radioactivity present by liquid scintillation counting (lower panel).

Defining the recognition site of the enhancer-binding protein

Three techniques were used to define the recognition site of the enhancer-binding protein namely competition assays, DNase footprinting, and DMS footprinting. Competition assays were performed by incubating a constant, non-saturating amount of enhancer-binding fraction (2 μg DEAE-Sepharose fraction) with a constant amount of the probe containing the enhancer sequence (0·5 μg, 170 bp *Bam*HI to *Bal*I, see Fig. 1) and increasing amounts of various unlabelled DNA fragments. Free and bound labelled DNA species were resolved by gel electrophoresis. The homologous unlabelled fragment (*Bam*HI to *Bal*I), as expected, competed for binding of the labelled probe as did the adjacent fragment which contains part of enhancer B (*Acc*I to *Sac*II), albeit with reduced efficiency. Fragments containing the 'TATA box' and E1a mRNA cap site (*Pvu*II to *Pvu*II), the 'CAAT box' (*Sac*II to *Pvu*II) and the inverted terminal repeat (*Eco*RI to *Bam*HI) failed to compete. Unlabelled fragments containing the SV40 enhancer, the polyoma virus enhancer and the Rous sarcoma virus enhancer competed efficiently for binding. All fragments which competed for binding contained sequences homologous to the 'core' consensus sequence recognised in many viral and cellular enhancers (Laimins *et al.* 1982; Weiher *et al.* 1983). Double-stranded synthetic oligonucleotides of 30 bp which correspond to the regions on the SV40 and adenovirus genomes which contain this site also competed for binding, suggesting that this sequence may be the target for the enhancer-binding protein (data not shown).

To investigate this possibility the sequences bound by the enhancer-binding protein were determined by DNase footprinting (Galas & Schmitz, 1978). DNA fragments containing the adenovirus enhancer were prepared which contained either a single 5′ or 3′ labelled terminus. Increasing quantities of a heparin-Sepharose fraction of enhancer-binding protein were bound to the probe, lightly digested with DNase I and the digestion products fractionated on a denaturing polyacrylamide gel. The most prominent area of altered DNase I sensitivity is centred on position 160. On both strands a protected bond is followed by a small area of enhanced cleavage which is followed by a small area of protection (159–163 on top strand, 157–161 on bottom strand, Fig. 4).

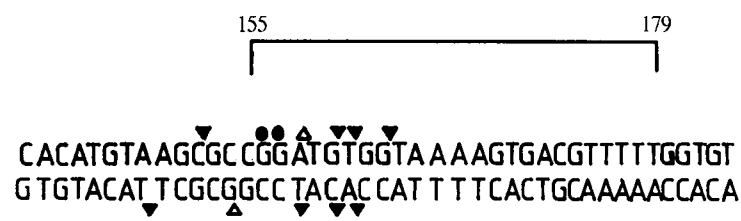

Fig. 4. Summary of DNase and DMS protection data. The sequence upstream from the adenovirus type 2 E1a gene between positions 142–184 is shown. Bases protected from methylation (●) and bonds protected from DNase I cleavage (▼) or displaying enhanced cleavage by DNase I (△) in the presence of the enhancer binding fraction are indicated. The boundary of sequences with enhancer activity is shown with a bracket (Hen *et al.* 1983).

To determine which G residues in the adenovirus enhancer were in contact with the enhancer-binding protein methylation protection experiments were performed (Gilbert *et al.* 1976). 5′ and 3′ end labelled probes containing the adenovirus enhancer were incubated with increasing quantities of enhancer-binding protein (heparin-Sepharose fraction) and briefly exposed to dimethyl sulphate. DNA chains containing modified guanine residues were cleaved at the point of modification by treatment with piperidine and fractionated on a denaturing polyacrylamide gel. On the top strand two guanine residues at positions 155 and 156 are clearly protected from methylation, whereas no guanine residues are protected on the bottom strand (Fig. 4).

Sequence required for adenovirus replication in vivo

Using a transfection assay it had previously been demonstrated that sequences required *in vivo* for adenovirus type 2 replication were located within the terminal 45 bp of the genome, whereas sequences required *in vivo* for adenovirus type 4 replication were located within the terminal 18 bp of the viral genome (Hay, 1985*a*,*b*). In adenovirus type 2, *in vitro* studies have demonstrated that the terminal 18 bp of the genome containing a highly conserved region (bases 9–18), constitutes the minimal origin of replication (*ori*). Located between bases 19 and 45 is the binding site for nuclear factor 1 (NF1), a cellular protein which stimulates adenovirus type 2 replication *in vitro* (Nagata *et al.* 1983). However, there may be other protein binding domains within the adenovirus type 2 origin of replication which are as yet unrecognised. To investigate this possibility and further define the role of NF1 in adenovirus type 2 replication we have performed *in vivo* transfection experiments with a series of hybrid plasmids, which contain the minimal *ori* sequence fused, in each orientation, to a cellular NF1 binding site (Adyha *et al.* 1985). The plasmids utilised in these experiments are as follows: 1, pHR18 contains the terminal 18 bp, of the viral genome which is identical in adenovirus types 2 and 4; 2, p4A2 contains the terminal 140 bp, of the adenovirus type 4 genome, which does not contain a binding site for NF1; 3, pHR1 contains the terminal 103 bp, of the adenovirus type 2 genome; 4, pC4 contains the terminal 18 bp, of the viral genome linked to a cellular NF1 binding site; 5, pD21 is similar to PC4 but with the cellular NF1 binding site in the opposite orientation. Each plasmid was linearised with *Eco*RI to expose the origin of replication and transfected into 293 cells either with adenovirus type 2 or type 4 DNA as helper. After 72 h DNA was extracted and digested with *Dpn*I to cleave any unreplicated DNA (Hay, 1985*a*). Replicated DNA was detected by Southern (1975) blotting using nick-translated pUC8 DNA. Since adenovirus type 4 only requires the terminal 18 bp of the viral genome for *ori* function all plasmids replicate with equal efficiency, when co-transfected with adenovirus type 4 DNA (Fig. 5(A)). However, a quite different situation is apparent with adenovirus type 2. Whereas plasmids containing the complete adenovirus type 2 origin of replication replicate efficiently (Fig. 5(A), track 3) plasmids containing the

terminal 18 bp are replicated very poorly (Fig. 5(A), track 1). Addition of a cellular NF1 binding sites, in either orientation, only partially restores replication activity (Fig. 5(A), tracks 4 and 5). A similar level of replication is also observed with the plasmid containing the adenovirus type 4 terminal sequences (Fig. 5(A), track 2) which does not contain an NF1 binding site. These data suggest that the NF1 binding site in combination with the minimal *ori* does not constitute a fully active replication origin and that additional DNA sequences also present at the adenovirus type 4 terminus are required. Comparison of the adenovirus type 2 and type 4 DNA sequences revealed that bases 39 to 53 in the adenovirus type 4 genome had a 12 out of 15 match with the corresponding region in the adenovirus type 2 genome, suggesting that this might constitute the site of action of an additional protein involved in adenovirus type 2 replication.

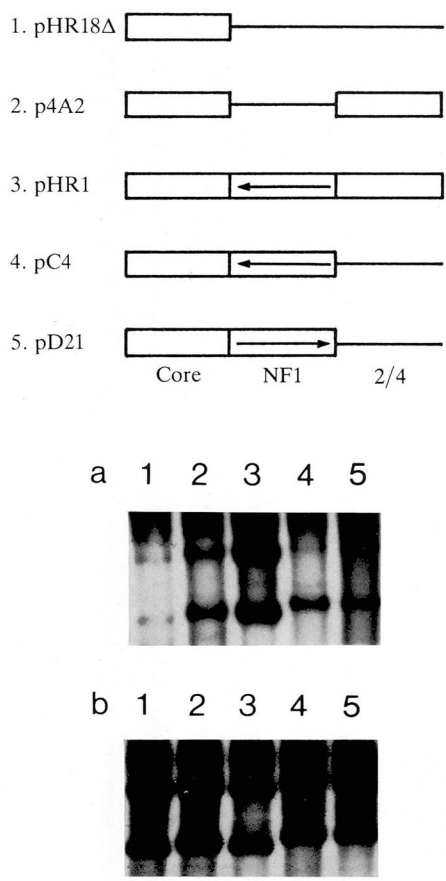

Fig. 5. The upper panel shows a diagram of the sequence elements present in each plasmid. In the lower panel the plasmids indicated above were cleaved with *Eco*RI to expose the adenovirus terminus and cotransfected into 293 cells with adenovirus type 2 (A) or adenovirus type 4 (B) DNA. After 72 h DNA was extracted, digested with *Dpn*I and analysed by southern blotting using nick translated pUC8 DNA as probe. Each track contains DNA extracted from equivalent numbers of cells ($\approx 3 \times 10^5$).

Purification of a cellular protein which binds to the adenovirus type 2 and type 4 inverted terminal repeats

As mentioned above, the DNA sequence between bases 39 and 53 of the adenovirus type 4 genome was conserved in adenovirus type 2 and potentially represents the site of action of a protein involved in viral DNA replication. To investigate this possibility two complementary oligonucleotides of 31 bases were synthesised, which contained the adenovirus type 4 DNA sequence between bases 33 and 59. The complementary oligonucleotides had 4 base 5′ extensions and could therefore be conveniently labelled with [α^{32}P]dNTPs and the Klenow fragment of *E. coli* DNA polymerase I. The labelled double-stranded oligonucleotides were purified by polyacrylamide gel electrophoresis and used as probes in gel retention assays. Incubation of 0·35 M-NaCl nuclear extracts with the labelled double strand oligonucleotide resulted in the appearance of a more slowly migrating labelled species. As indicated above, this is indicative of a specific interaction between a DNA-binding protein and its target DNA sequence. This binding activity was therefore purified by ion exchange chromatography on DEAE Sepharose followed by phosphocellulose. The pooled phosphocellulose peak was applied to denatured DNA cellulose and bound protein eluted with a linear gradient of NaCl. Binding activity was monitored by gel retention assay using the double-stranded synthetic oligonucleotide as a probe (Fig. 6). The activity eluting from the denatured DNA cellulose was dialysed to remove NaCl and used for further studies. DNase protection experiments indicated that the protein protected sequences between bases 37 and 55 on the adenovirus type 4 genome and the corresponding region on the adenovirus type 2 genome (data not shown). The binding fractions were also capable of stimulating adenovirus type 2 DNA replication *in vitro* (3- to 4-fold) when nuclear factor 1 was also present. However, although the binding site is also present on the adenovirus type 4 inverted terminal repeat (ITR) adenovirus type 4 replication *in vitro* does not respond to the addition of the binding fraction (see below). This confirms the previously determined origin requirements for adenovirus type 4 DNA replication *in vivo* (Hay, 1985*b*). Recently a protein called nuclear factor III (NFIII), which stimulates adenovirus type 2 replication *in vitro* and has the same binding characteristics as above was described (Pruijn *et al.* 1986).

Initiation of adenovirus type 4 replication in vitro

To investigate the surprising differences between adenovirus type 2 and type 4 DNA replication we have developed an *in vitro* system for the replication of adenovirus type 4 DNA, which mimics the differences we have previously observed *in vivo*.

HeLa cells infected with adenovirus type 4 in the presence of hydroxyurea, were fractionated into cytosol and nuclei. Nuclei were further extracted with 0·15 M NaCl as previously described for adenovirus type 2 (Challberg & Kelly, 1979). To measure initiation in these extracts we have followed the transfer of dCMP onto the preterminal protein, which is the first synthetic event in viral DNA replication. In

these assays viral template DNA and cell extract are incubated in the presence of [α-^{32}P]dCTP. [^{32}P]dCMP transferred to preterminal protein is then detected by autoradiography following SDS–polyacrylamide gel electrophoresis (Desiderio & Kelly, 1981). In contrast to adenovirus type 2, incubation of infected cell nuclear extract with adenovirus type 4 cores or terminal protein-linked genomic DNA (DPC) did not result in significant transfer of dCMP onto the preterminal protein. However, incubation of template containing the adenovirus type 4 ITR with cytoplasmic extract resulted in the transfer of [^{32}P]dCMP onto the preterminal protein (Fig. 7). In addition to template extract and dCTP, the reaction required optimal concentrations of ATP and $MgCl_2$. Unlike adenovirus type 2, where the addition of uninfected cell nuclear extract to infected cell cytosol stimulates replication, we have found that uninfected cell nuclear extract inhibited initiation when added to adenovirus type 4 infected cell cytosol. Quantitatively the transfer of dCMP observed with adenovirus type 4 cytosol alone was similar to that observed when adenovirus type 2 cytosol was supplemented with uninfected cell nuclear extract.

Although on a molar basis viral cores and DPC were the most efficient templates, plasmids containing the adenovirus type 4 ITR were also active, provided that they

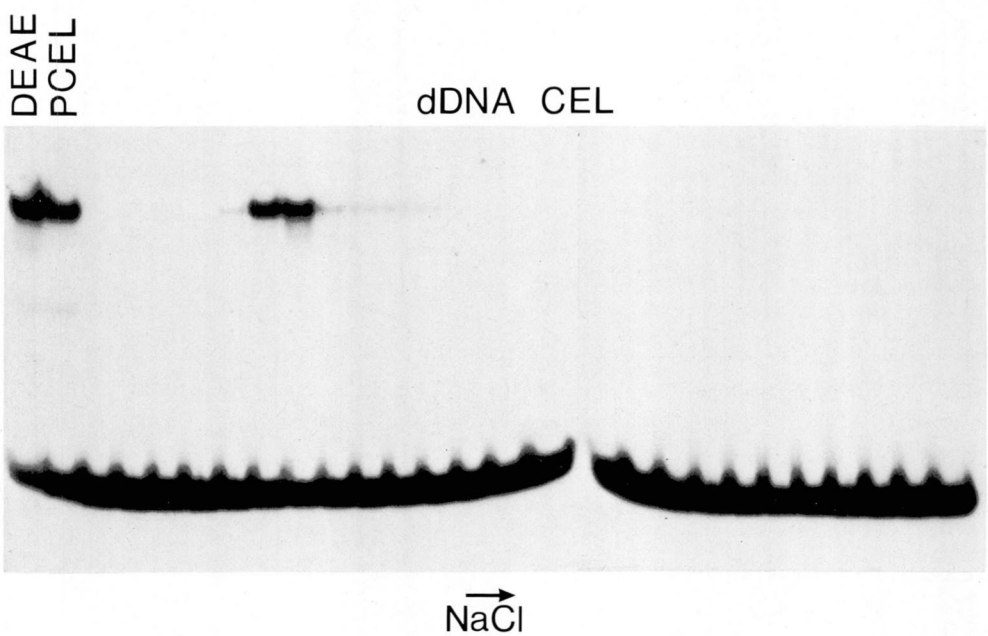

Fig. 6. Isolation of an activity which binds to the adenovirus type 4 ITR. Nuclear extract was fractionated on DEAE Sepharose and phosphocellulose and fractions which bind to a double strand synthetic oligonucleotide, corresponding to positions 33–59 in the adenovirus type 4 ITR, detected by gel retention assay. The peak of activity from the phosphocellulose column was loaded onto a denatured DNA cellulose column and bound protein eluted with a linear gradient of NaCl (0·1 to 1·0 M). Fractions were tested in the gel retention assay for binding to the double-stranded synthetic oligonucleotide.

were linearised to expose the viral terminus (Fig. 7). The linearised vector (pUC9) did not support complex formation. To define the DNA sequence requirement for intiation of adenovirus type 4 replication *in vitro* the template efficiencies of plasmids which contain deleted ITRs (Hay, 1985*b*) were tested. Plasmids containing the terminal 18 bp of the viral genome supported initiation to the same extent as the complete adenovirus type 4 ITR, whereas deletions extending into the terminal 18 bp abolished initiation. These data are in complete agreement with previously described *in vivo* studies (Hay, 1985*b*), and suggest that unlike adenovirus type 2, adenovirus type 4 is not dependent on host cell nuclear factors I and III for initiation of DNA replication.

One possibility however is that the presence of inhibitors in the nuclear extract could mask the effect of stimulatory nuclear factors. Nuclear extracts from uninfected HeLa cells were therefore fractionated by ion-exchange chromatography on DEAE Sepharose and the individual fractions added to the adenovirus type 4 infected cell cytosol and assayed for intiation activity. One peak of inhibitory activity was detected which was separated from a peak of stimulatory activity. This activity did not co-elute with the previously described NFIII. The stimulatory activity was active with viral cores, DPC and was equally active with plasmids containing the entire adenovirus type 4 ITR or the terminal 18 bp. Purified NFIII, which binds to

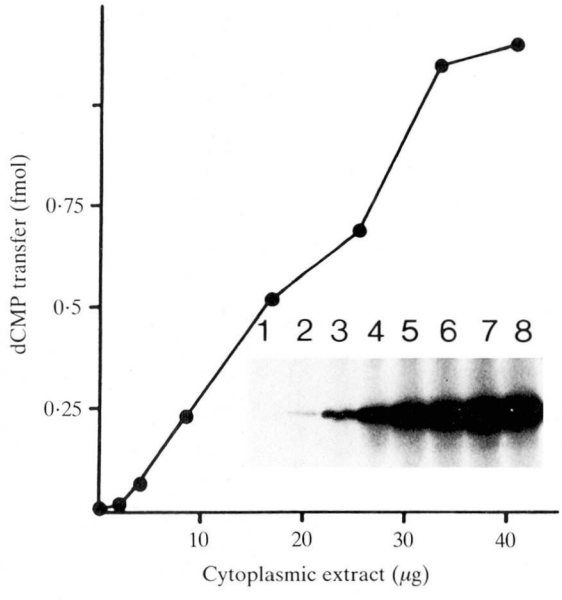

Fig. 7. Initiation of adenovirus type 4 replication *in vitro*. Increasing quantities of cytoplasmic extract from adenovirus type 4 infected HeLa cells were incubated with *Eco*RI cleaved p4A2 template DNA (50 ng), $MgCl_2$, ATP and [α^{32}P] dCTP at 37 °C for 60 min. The reaction products were digested with micrococcal nuclease and fractionated on an SDS polyacrylamide gel. dCMP transfer onto preterminal protein was determined by autoradiography (insert) and quantitated by determining the radioactivity in excised gel slices (graph).

the adenovirus type 4 ITR, did not stimulate the initiation reaction using viral cores, DPC or plasmids containing the adenovirus type 4 ITR.

DISCUSSION

In this communication we have studied a variety of cellular proteins which bind to specific DNA sequences located at the left end of the adenovirus genome. One of these proteins interacts with the adenovirus type 2 enhancer and is presumably involved in the control of viral transcription. The other cellular proteins investigated interact with sequences within the viral origin of DNA replication. *In vivo* and *in vitro* studies support the notion that these proteins are involved in the initiation of viral DNA replication.

To identify these proteins we have utilized a sensitive gel retention assay which detects DNA-protein complexes formed between a radioactively labelled target DNA sequence and a protein which recognises the target sequence. Thus we have detected a HeLa cell nuclear protein which binds to the adenovirus enhancer. Competition experiments with restriction enzyme fragments and duplex synthetic oligonucleotides, DNase protection, and DMS protection experiments indicate that the protein binds predominantly to the 'core' consensus sequence GGATGTGG$^{AAA}_{TTT}$ present at position 160 in the adenovirus type 2 genome and found in many viral and cellular enhancers (Laimins *et al.* 1982; Weiher *et al.* 1983). That this cellular protein may be utilized by a number of different viruses is suggested by the finding that binding sites for this protein exist in the enhancers of SV40, polyoma virus and the Rous sarcoma virus.

The role of the enhancer-binding protein in transcriptional activation is suggested by the finding that sequences from the left end of the adenovirus type 2 genome could stimulate transcription when linked to a heterologous promoter *in vivo*. An analysis of deletion mutants indicated that sequences between positions 155 and 178, which contains the 'core' consensus sequence and recognition site for the enhancer-binding protein, were largely responsible for enhancer activity. Removal of the recognition site for the enhancer-binding protein drastically reduced transcriptional activity (Hen *et al.* 1983). It should be noted that removal of sequences between positions 106–195 in an adenovirus type 5 variant had no effect on viral viability or E1a transcription (Hearing & Shenk, 1983). Interpretation of these data may be complicated by the finding that products of the E1a gene can negatively modulate enhancer-stimulated transcription (Borrelli *et al.* 1984; Velcich & Ziff, 1985).

In other viral systems a role of the 'core' consensus sequence in enhancer activity has been demonstrated. Removal or mutagenesis of this sequence in the SV40 enhancer leads to a decrease in transcriptional activity both *in vivo* and *in vitro* (Weiher *et al.* 1983; Wildeman *et al.* 1984; Sassone-Corsi *et al.* 1985; Zenke *et al.* 1986). In addition, revertants of SV40 mutants with defective enhancers contained duplications of the 'core' consensus sequence which restored transcriptional activity (Herr & Glutzman, 1985; Herr & Clarke, 1986).

Although the enhancer-binding protein may be important for transcriptional activation it is unlikely that interaction of this factor alone with the enhancer could explain the dramatic stimulation of transcription mediated by an enhancer. In fact the evidence suggests that transcriptional activation *via* enhancers requires the interaction of proteins with multiple enhancer motifs (Zenke *et al.* 1986). Thus enhancer-mediated transcriptional activation probably requires the formation of a large DNA–protein complex in which a limited set of proteins interact at various points along the enhancer.

The role of cellular sequence-specific DNA-binding proteins in adenovirus DNA replication has been investigated by utilizing two complementary approaches. To define the DNA sequences required for DNA replication an *in vivo* transfection assay (Hay *et al.* 1984) is utilized. Once the essential sequences have been defined, proteins which interact with them are identified using a gel retention assay. These sequence-specific DNA-binding proteins are then purified and tested for their ability to stimulate viral DNA replication *in vitro*. Previous *in vivo* (Hay, 1985*a*,*b*) and *in vitro* studies (Nagata *et al.* 1983) had indicated that nuclear factor I (NFI) played a crucial role in the replication of adenovirus type 2 DNA. The involvement of another cellular protein in adenovirus type 2 DNA replication was suggested by the finding that plasmids which contained the minimal *ori* and an NFI binding site did not replicate as efficiently as a plasmid which contained the minimal *ori*, the NFI binding site and an additional conserved sequence. This conserved sequence, located between positions 37 and 51 in the adenovirus type 2 genome, has a 12 out of 15 match with the corresponding region in the adenovirus type 4 genome. We isolated a protein which bound to this region and found that in the presence of NFI it could stimulate adenovirus type 2 replication. Pruijn *et al.* (1986) have isolated a protein with these characteristics and designated it nuclear factor III (NFIII). Although this site is present in the adenovirus type 4 ITR it does not appear to be required for DNA replication as plasmids containing the terminal 18 bp of the genome replicate as well as plasmids containing the complete ITR. These results were confirmed by testing the purified NFIII in an adenovirus type 4 *in vitro* replication system which we developed. No stimulation of initiation of adenovirus type 4 replication was observed using levels of NFIII which gave maximal stimulation of adenovirus type 2

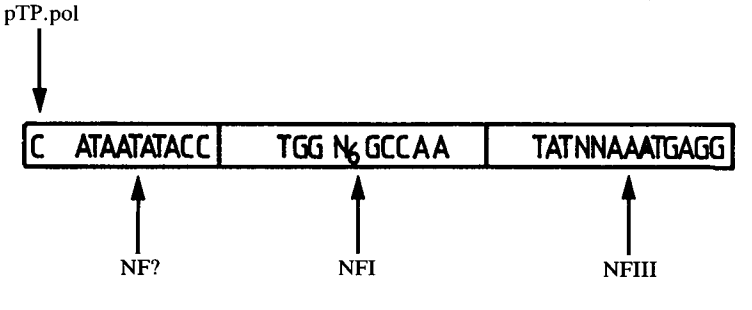

Fig. 8. Diagrammatic representation of the DNA sequence elements present in the adenovirus type 2 origin of replication and the proteins which may interact with them.

replication. It is therefore clear that although the adenovirus type 4 ITR contains a binding site for the protein, NFIII plays no role in adenovirus type 4 DNA replication. In adenovirus type 2 NFIII does appear to have some role in replication although it seems to be less important than NFI. In fact, adenovirus type 2 genomes containing only a 45 bp left ITR give rise to infectious progeny with wild-type growth characteristics (Hay & McDougall, 1986). An additional nuclear factor which stimulates adenovirus type 4 replication *in vitro* was also identified. Since it stimulates initiation on plasmids containing only the terminal 18 bp of the genome it is possible that this DNA sequence is the site of action of this protein.

DNA–protein interactions which may be involved in adenovirus DNA replication are summarised in Fig. 8. As these sites are adjacent, it seems likely that interactions between the proteins will be crucial for the initiation event.

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