Co-operative interactions between NFI and the adenovirus DNA binding protein at the adenovirus origin of replication

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The DNA–protein and protein–protein interactions proposed for the stability of nucleoprotein complexes at the origin of replication in prokaryotes are also thought to impart regulatory precision in eukaryotic DNA replication. This type of specificity can be observed, for example, during adenovirus DNA replication where efficient initiation requires that nuclear factor I (NFI) binds to the origin of DNA replication. Addition of purified NFI stimulates the initiation of adenovirus DNA replication *in vitro* in a reaction that is dependent on the concentration of the adenovirus DNA binding protein (DBP). However, the molecular basis for the synergistic action of NFI and DBP during replication is at present unknown. We report here that DBP increases the affinity of NFI for its binding site in the replication origin. DBP did not, however, increase the affinity of another eukaryotic sequence-specific DNA binding protein, EBP1, for its recognition site. Other single-stranded DNA binding proteins could not substitute for DBP in increasing NFI affinity for its binding site. In addition, DBP was found to alter the binding kinetics of NFI, both by increasing the rate of association and decreasing the rate of dissociation of NFI with the DNA template. The co-operativity between NFI and DBP was also demonstrated on another DNA template, a human NFI site (FIB2), suggesting that this interaction is of general occurrence and not restricted to the adenovirus origin of replication.

Key words: adenovirus/DNA binding protein/nuclear factor I/replication

Introduction

Initiation of DNA replication is an event which requires a remarkable degree of specificity. In prokaryotes this appears to be a direct result of the formation of a large nucleoprotein complex at, for example, the *Escherichia coli* origin of DNA replication, *oriC*, and at the bacteriophage λ origin of replication (Echols, 1986). These replication complexes are thought to be stabilized by protein–protein and protein–nucleic acid interactions. Replication mechanisms in eukaryotes are not so well characterized, but adenovirus type 2 DNA can be replicated *in vitro* by the action of three viral and three cellular proteins (Nagata *et al.*, 1983; Pruijn *et al.*, 1986; Kelly *et al.*, 1988). Nuclear factor I (NFI), a sequence-specific DNA binding protein from HeLa cells, stimulates the initiation of adenovirus DNA replication upon

binding to its recognition site within the adenovirus origin of replication (Figure 1; Nagata *et al.*, 1982; Guggenheimer *et al.*, 1984; Rawlins *et al.*, 1984; De Vries *et al.*, 1985; Hay, 1985; Leegwater *et al.*, 1985). This protein is also known to bind to GCCAAT sequences upstream of several eukaryotic genes (Jones *et al.*, 1987) and is in fact a heterogeneous group of polypeptides that can activate both transcription and DNA replication (Jones *et al.*, 1987; Santoro *et al.*, 1988). Recently, cDNAs for human NFI have been isolated and used to identify a single gene producing multiple mRNAs (Santoro *et al.*, 1988). As a result of alternative splicing at least three different classes of NFI have been observed. The proteins specified by these mRNAs all contain a conserved DNA binding domain linked to additional polypeptide sequences (Santoro *et al.*, 1988). Isolation of cDNAs producing a distinct liver-specific rat NFI, which also contains the conserved DNA binding domain, suggests that alternative splicing may control the tissue-specific expression of distinct forms of NFI (Paonessa *et al.*, 1988). An additional level of complexity in the control of NFI activity is suggested by the finding that several different CCAAT box binding proteins require co-operative interaction of two or more subunits for high affinity, sequence-specific DNA binding (Chodosh *et al.*, 1988).

In adenovirus replication the stimulatory effect of NFI has been reported to be influenced by other cellular factors (Wides *et al.*, 1987) and by the adenovirus-coded DNA binding protein (DBP, De Vries *et al.*, 1985). DBP is a multifunctional protein which binds preferentially to single-stranded DNA in a sequence-independent fashion (Schechter *et al.*, 1980) and is involved in transformation (Ginsberg *et al.*, 1974), gene expression (Carter and Blanton, 1978; Klessing and Grodzicker, 1979; Babich and Nevins, 1981; Handa *et al.*, 1983), as well as in viral DNA replication (Van der Vliet *et al.*, 1977; Horwitz, 1978; Nagata *et al.*, 1982). To investigate the role of DBP in viral DNA replication further we have used DNase I and dimethyl sulphate (DMS) footprinting to determine the influence of DBP on interactions between NFI and its recognition site in the adenovirus origin of DNA replication. We show here that a specific co-operative interaction exists between NFI and DBP, which results in an increased binding affinity of NFI for its recognition site within the replication origin. Moreover, the association and dissociation kinetics of NFI with the DNA template are altered in the presence of DBP

Fig. 1. Sequence of the adenovirus type 2 origin of DNA replication (Ad-ITR). The adenovirus core region and recognition sites of NFI (De Vries *et al.*, 1987) and NFIII (Prujn *et al.*, 1986) are represented. Arrows indicate G-residues protected against methylation on binding of NFI (De Vries *et al.*, 1987).

Fig. 2. Increase in NFI recognition site occupancy in the presence of DBP. A 3'-labelled fragment containing the adenovirus 2 origin of replication (Ad-ITR) was excised from plasmid pEX and incubated with increasing quantities of purified NFI in the absence (A) or presence (B) of 2.5 μ l (0.75 μ g) of purified DBP and then subjected to DNase I digestion. This fragment was also incubated with increasing quantities of DBP alone (C). A 5'-labelled fragment containing part of the SV40 enhancer was excised from plasmid pUC1X72 and incubated with increasing amounts of the enhancer binding protein, EBP1 (Clark *et al.*, 1988), in the absence (D) or presence (E) of 2.5 μ l (0.75 μ g) DBP and then subjected to DNase I digestion. Cleavage products were separated on a 12% polyacrylamide sequencing gel followed by autoradiography. Numbers to the left of the DNase I profiles indicate bases at the boundaries of the recognition sites (De Vries *et al.*, 1987; Clark *et al.*, 1988).

with a resulting increase in the equilibrium binding constant of NFI.

Results

Effect of DBP on NFI recognition site occupancy

The effect of DBP on the binding of NFI to its recognition site within the adenovirus origin of DNA replication was initially investigated using DNase I footprinting. A 3'-labelled DNA fragment containing the NFI binding site (see Figure 1) in the adenovirus 2 replication origin (Ad-ITR) was incubated with increasing amounts of purified NFI in the absence and presence of purified DBP for 60 min at 0°C and then subjected to digestion with DNase I. An increase in protection of the NFI binding site with increasing amounts of NFI is evident in Figure 2A. In the presence of DBP, however, the amount of NFI required to protect the NFI recognition site fully from DNase I digestion is reduced by 5- to 10-fold. The addition of 5 μ l (2 ng) NFI alone results in incomplete protection of the recognition site from DNase digestion (Figure 2A). However, in the presence of 2.5 μ l (0.75 μ g) DBP complete protection of the recognition site from DNase digestion is observed with 1 μ l (0.4 ng) NFI (Figure 2B). Addition of increasing amounts of DBP alone to the Ad-ITR had no observable qualitative effect on the products of DNase I digestion (Figure 2C), although increasing the concentration of DBP increases the amount of DNA remaining uncleaved by DNase I. At very high concentrations of DBP cleavage of the Ad-ITR by DNase I is completely inhibited (data not shown).

To determine that the effect of DBP was specific to NFI

we examined its effect on the binding of EBP1, a protein isolated from HeLa cells which recognizes a specific DNA sequence in the SV40 enhancer (Clark *et al.*, 1988). Binding of EBP1 to the SV40 enhancer results in protection from DNase I cleavage of phosphate bonds between positions 233 and 252 in the SV40 genome (Figure 2D). The addition of DBP to the enhancer-containing DNA fragment, however, had no effect on the DNase I cleavage pattern (Figure 2E), and it did not increase the apparent affinity of EBP1 for its recognition site. In fact a slight decrease in apparent affinity was observed in the presence of DBP (Figure 2E). Therefore, DBP did not increase the binding affinity of another eukaryotic sequence-specific DNA binding protein for its recognition site.

DBP – NFI specificity

To quantitate the effect of DBP on NFI recognition site occupancy, increasing amounts of DBP and a sub-optimal concentration of NFI (1 μ l, 0.4 ng, 41% site occupancy) were incubated with a ^{32} P-labelled fragment containing the Ad-ITR and DNase I footprinting was carried out (Figure 3A). Scanning densitometry of T-residues 29 and 39 was used to determine occupancy (Borowiec *et al.*, 1987) of the NFI recognition site as a function of DBP concentration and the results are represented graphically in Figure 3B. An obvious increase in NFI site occupancy is evident with increasing amounts of DBP (Figure 3A). However, under the conditions of this experiment there appears to be an optimal amount of DBP required for 100% occupancy of the NFI recognition site (1 μ l NFI:5 μ l DBP, 0.4 ng NFI:1.5 μ g DBP). At higher concentrations of DBP DNase I

Fig. 3. Specificity of NFI recognition site occupancy in the presence of DBP and other single-stranded DNA binding proteins. Increasing amounts of DBP (A and B), HeLa cell single-stranded DNA binding protein (C), gene 32 protein of phage T4 (D) or *E. coli* single-stranded DNA binding protein (E) were added to a 3'-labelled fragment of the Ad-ITR in the presence of a fixed amount of NFI (1 μ l, 0.4 ng). At equilibrium samples were treated with DNase I and processed by gel electrophoresis as described in Figure 2. Numbers to the left of the DNase I profiles indicate bases along the top strand of the Ad-ITR as determined by comparison with Maxam–Gilbert sequence ladders. Protein concentrations were adjusted to the same concentration as DBP. Scanning densitometry on T-residues 29 and 39 were used to calculate NFI site occupancy as a fraction of complete protection against DNase I cleavage (B) (Borowiec *et al.*, 1987; 1.0 represents 100% site occupancy; $\square$, T29; $\blacksquare$, T39).

sensitivity is apparent within the NFI recognition site (Figure 3A). It is possible, therefore, that above a certain DBP concentration NFI is excluded from its binding site (Figure 3B). In addition, at 1 μ l (0.3 μ g) and 2.5 μ l (0.75 μ g) DBP alterations in the rate of DNase I cleavage are evident both outside and on the boundary of the NFI recognition site, resulting in several hypersensitive phosphate bonds (Figure 3A). At present the significance of these changes is not clear.

To investigate further the specificity of the DBP induced increase in NFI site occupancy we substituted DBP with other sequence-independent DNA binding proteins that bind preferentially to single-stranded DNA. An amount of NFI (0.4 ng) which gave 41% site occupancy was incubated with a 3'- ^{32} P-labelled fragment of the Ad-ITR in the presence of three different concentrations of the HeLa cell ssb (Wobbe *et al.*, 1987), T4 gene 32 protein (Chase and Williams, 1986) and *E. coli* ssb (Chase and Williams, 1986), and NFI site occupancy determined by DNase I footprinting as described previously. It is clear from Figure 3C–E that none of these proteins will substitute for DBP in increasing NFI site occupancy and in some circumstances NFI binding is reduced (Figure 3D). The enhanced binding of NFI to its recognition site, therefore, appears to be specific to DBP.

Base contact analysis

A number of explanations for the influence of DBP on the binding of NFI to its recognition site are possible, one of which is that the specificity of NFI for its binding site has altered. To test this idea interactions of NFI with G-residues

within its recognition site were investigated by DMS protection. Labelled DNA fragments representing the top or bottom strand of the Ad-ITR were incubated with NFI alone, DBP alone, or NFI + DBP and then subjected to methylation by DMS. The results presented in Figure 4 demonstrate enhanced protection from methylation of G-residues 26, 27 and 34 of the top strand and G-residues 35 and 36 of the bottom strand of the Ad-ITR in the presence of both NFI and DBP. DBP alone does not alter methylation of the Ad-ITR nor does it significantly alter the specific base contacts of NFI on the Ad-ITR, but it does enhance the binding affinity of NFI (Figure 4).

Effect of DBP on NFI association

It appeared possible that DBP increased the binding affinity of NFI with its recognition site by either increasing the rate of association or decreasing the rate of dissociation of NFI with its binding site. To determine the effect of DBP on the rate of NFI association a fixed amount of NFI (2 ng, 5 μ l) was incubated with a 5'-labelled fragment containing the Ad-ITR over an increasing time period in the presence and absence of DBP (1.5 μ g, 5 μ l), followed by DNase I footprinting. The results in Figure 5A and B clearly demonstrate that DBP dramatically increases the association rate of NFI with its binding site. Densitometry on T-residue 29 was used to determine NFI recognition site occupancy as a function of time and a graphic representation of the results is shown in Figure 5C. In these experiments the

Fig. 4. Base contact analysis of NFI and DBP by methylation protection. 5'- and 3'-labelled fragments of the top and bottom strands of the Ad-ITR respectively were incubated in the presence of DBP alone (2.5 μ l, 0.75 μ g), NFI alone (1 μ l, 0.4 ng), or DBP + NFI (0.75 μ g + 0.4 ng), exposed to DMS and cleaved at G-residues by piperidine. Cleaved fragments were separated on a 12% polyacrylamide sequencing gel followed by autoradiography. Numbers to the side of the methylation profiles indicate G-residues protected against methylation by NFI binding (see Figure 1).

DNase I concentration was increased and the DNase I digestion time reduced to 15 s. In the presence of NFI alone 90% site occupancy is achieved after 15 min incubation (Figure 5A). In contrast, on addition of NFI, DBP and DNase I to the Ad-ITR, 67% NFI site occupancy occurs over the 15 s DNase I digestion time (Figure 5B). Addition of DNase I after incubation for 60 s in the presence of NFI and DBP indicates that the recognition site is fully occupied (100% site occupancy, Figure 5C). A detailed kinetic analysis of NFI binding has determined the association rate constant of NFI k_a as $1.04 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (P.H.Cleat and R.T.Hay, manuscript in preparation). From the present findings the increase in association rate of NFI induced by DBP increases this rate constant by >10 -fold.

Effect of DBP on NFI dissociation

In view of the rapid increase in the NFI association rate induced in the presence of DBP, we also investigated the effect of DBP on the rate of NFI dissociation from its binding site. In these experiments a saturating concentration of NFI was incubated with a labelled fragment containing the Ad-ITR in the absence and presence of DBP for 60 min at 0°C. An excess of unlabelled competitor DNA containing

the NFI binding site was then added to all incubations and NFI site occupancy determined over an increasing time period by DNase footprinting (Figure 6A and B). Scanning densitometry of T-residue 29 was used to quantitate NFI dissociation and the results are represented graphically in Figure 6C. DBP has an obvious effect in decreasing the rate of NFI dissociation from its binding site (Figure 6A and B). The slopes of lines of best fit through the two sets of values in Figure 6C reveal that DBP reduces the dissociation rate of NFI by >5 -fold. Subsequent kinetic analysis of the interaction between NFI and its binding site has determined the dissociation rate constant and an equilibrium binding constant for this interaction and indicates that in the presence of DBP the equilibrium binding constant of NFI is increased by 60-fold (P.H.Cleat and R.T.Hay, manuscript in preparation).

NFI–DBP interaction on a human NFI site

To investigate if the co-operative interaction between NFI and DBP occurs on NFI sites other than the site within the adenovirus origin of replication we carried out DNase I footprinting with a sub-optimal concentration of NFI on a 5'-labelled fragment containing a human genomic NFI site (Adhya *et al.*, 1986) in the absence and presence of DBP. It can be seen from Figure 7 that there is an increase in NFI occupancy of the site (between bases 37 and 64) with an increase in DBP concentration up to 1.5 μ g DBP (5 μ l). At 3 μ g DBP (10 μ l) this site becomes more sensitive to DNase digestion, as seen previously with the Ad-ITR DNA template (see Figure 3B). Prominent DNase hypersensitive bands are observed at certain ratios of NFI to DBP; a situation also apparent on the Ad-ITR. These results suggest that the co-operative interaction between NFI and DBP is not unique to the adenovirus origin of replication, but may be of more general significance.

Stimulation of DNA replication by NFI and DBP

Although biochemical and genetic evidence (reviewed by Hay and Russell, 1989) has demonstrated that DBP is involved in the elongation stage of adenovirus DNA replication, its role in initiation of replication is less well defined. We have, therefore, examined the ability of DBP to stimulate initiation and elongation of the first 26 bases, in the presence of a non-saturating concentration of NFI. Using viral cores as templates we demonstrate that, as expected, initiation (formation of the pTP–dCMP complex) and extension to the first G-residue in the genome is dependent on the presence of NFI (Figure 8). Addition of DBP to these reactions stimulates elongation to the 26th nucleotide and also formation of the pTP–dCMP complex 3- to 4-fold. Thus, it is clear that in the presence of non-saturating amounts of NFI, DBP can increase the frequency of initiation and the rate of elongation.

Discussion

The binding of NFI to its recognition site within the adenovirus replication origin is a prerequisite for efficient viral replication (Nagata *et al.*, 1982; Guggenheimer *et al.*, 1984; Rawlins *et al.*, 1984; De Vries *et al.*, 1985; Hay, 1985; Leegwater *et al.*, 1985). The magnitude of NFI stimulation of the replication elongation reaction is known to be influenced by the concentration of the adenovirus DNA

Fig. 5. Effect of DBP on the rate of association of NFI. A 5'-labelled fragment of the Ad-ITR was incubated with NFI (2 ng, 5 µl) over an increasing time period at 20°C in the absence (A) or presence (B) of DBP (1.5 µg, 5 µl), followed by DNase I footprinting. Scanning densitometry on T-residue 29 was used to determine NFI recognition site occupancy as a function of time (C). Numbers to the left of the DNase I profiles indicate bases at the boundaries of the NFI recognition site.

binding protein (De Vries *et al.*, 1985; Lindenbaum *et al.*, 1986). Rosenfeld *et al.* (1987) have reported previously that DBP is not required for initiation of replication. More recently other workers have demonstrated a stimulatory effect of DBP in the initiation reaction (Kenny and Hurwitz, 1988). Here we demonstrate that DBP can stimulate the frequency of initiation when NFI is present in non-saturating amounts. Since the effect we describe is to increase NFI site occupancy we would not expect to observe a stimulatory effect of DBP if NFI was already present in saturating amounts. This observation may therefore offer an explanation for the apparent discrepancy in the literature. DBP is also known to form a specific interaction with the adenovirus DNA polymerase (Field *et al.*, 1984; Lindenbaum *et al.*, 1986) stimulating polymerase activity between 10- and 100-fold. This effect appears to be achieved by dramatically increasing polymerase processivity (Field *et al.*, 1984), and requires catalytic as opposed to stoichiometric amounts of DBP (Lindenbaum *et al.*, 1986). In the present study an analysis of the effect of DBP on NFI interactions with its binding site in the adenovirus replication origin has revealed that the affinity of NFI for its recognition site is significantly increased in the presence of DBP. DBP appears to be required in stoichiometric amounts in this case. Furthermore, DBP both increases the rate of NFI association with and decreases the rate of its dissociation from the DNA template. The observations that NFI site occupancy is not increased by other proteins with DNA binding properties similar to DBP, and that the binding of another sequence-specific DNA binding protein (EBP1) is not influenced by DBP, strongly

Fig. 6. Influence of DBP on the rate of dissociation of NFI. A 5'-labelled fragment of the Ad-ITR was incubated with a saturating quantity of NFI (2 ng, 5 µl) in the absence (A) or presence (B) of DBP (1.5 µg, 5 µl) for 60 min at 0°C. A 100-fold excess of competitor DNA (25 ng of a double-stranded oligonucleotide of the NFI recognition site) was then added to all incubations and DNase I footprinting carried out after increasing time at 20°C. Numbers to the left of the DNase I profiles indicate bases at the boundaries of the NFI recognition site. Scanning densitometry on T-residue 29 was used to determine NFI recognition site occupancy as a function of time and lines of best fit plotted through the two sets of values (C) (■, NFI + DBP; □, NFI).

Fig. 7. Increase in occupancy of a human NFI site in the presence of DBP. A 5'-labelled fragment containing a human genomic NFI site (FIB2) was excised from plasmid pC22 (Adhya *et al.*, 1986) and incubated with a sub-optimal amount of NFI (1 μ l, 0.4 ng) alone or in the presence of increasing amounts of DBP. DNase I footprinting was then carried out and cleaved fragments separated on a 12% polyacrylamide sequencing gel followed by autoradiography. Numbers to the left of the DNase I profile indicate bases at the boundaries of the NFI recognition site.

suggest a specific functional interaction between NFI and DBP. This interaction may be of more general importance, however, in that it can be seen also on a human genomic NFI site.

A possible model for the considerable increase in NFI recognition site occupancy in the presence of DBP could involve a direct protein-protein interaction between these proteins such that the structural configuration of NFI is altered to permit optimal base contacts with a resulting higher binding affinity. However, we have no direct evidence to suggest that a protein-protein interaction occurs between NFI and DBP, although such an interaction cannot be discounted as yet. Such interactions have been strongly implied in other systems and Zheng *et al.* (1987) have demonstrated that the general transcription factor BTF3 binds to RNA polymerase B via a protein-protein interaction.

The most probable alternative model to a protein-protein interaction may be that DBP alters the DNA in such a way that binding of NFI to its recognition site is facilitated. NFI is thought to bind as a dimer and to one side of the DNA helix only (De Vries *et al.*, 1987). DBP may bend or distort the NFI binding site in such a way that base-specific contacts by NFI are brought into better alignment. The DNase hypersensitive residues on either side of the NFI binding site and the reappearance of a DNase sensitive residue within the region normally protected (see Figure 3A) suggest that interactions between NFI and DBP may distort the DNA helix. Chemical accessibility experiments are in progress to examine this possibility. Such DNA distortions are now thought to be common and have been demonstrated in binding of the *E. coli* catabolite activator protein to its

Fig. 8. Influence of NFI and DBP on the initiation of adenovirus DNA replication *in vitro*. Reactions (details in Materials and methods) contained [α - ^{32} P]dCTP, ddGTP, dATP, dTTP, adenovirus type 2 cores as template with DBP and NFI as indicated. Reaction products were fractionated by electrophoresis in a 10% SDS-polyacrylamide gel and visualized by autoradiography. Initiation is measured by the formation of the preterminal protein-dCMP complex (In), whereas elongation is assessed by the formation of preterminal protein extended by 26 bases and terminated by ddG (Ex). The band below the initiated product results from proteolysis of pTP in the presence of cores.

recognition site in the *lac* promoter (Liu-Johnson *et al.*, 1986).

Co-operative interactions between two proteins have been demonstrated in the regulation of transcription. The yeast HAP2 and HAP3 activator proteins, for example, have been shown to act co-operatively in transcriptional activation (Olesen *et al.*, 1987). The binding of either protein to its DNA recognition site is dependent on the presence of the other protein and both proteins have been shown to bind to the same recognition sequence in a single DNA-protein complex (Olesen *et al.*, 1987). Similarly, in bacteria the *E. coli* HU protein is known to enhance the binding of both the *lac* repressor and the CAP protein to their binding sites (Flashner and Gralla, 1988). Conversely, the same protein was shown to inhibit *trp* repressor binding. It appears likely that the action of the HU protein is by altering DNA recognition sites to more or less easily recognized structures (Flashner and Gralla, 1988). Recently the transcriptional factor SL1 has been shown to facilitate binding of the sequence-specific DNA binding protein UBF1 to its recognition site in a mammalian system, via complex formation between these two proteins (Bell *et al.*, 1988). Only then can activation of transcription occur. In the present series of experiments we demonstrate co-operativity between a sequence-independent DNA binding protein and a eukaryotic sequence-specific DNA binding protein.

Although NFI binds tightly to its recognition site (K_D

2.1×10^{-11} M; Rosenfeld and Kelly, 1986), its affinity appears to be increased when the template is optimally coated with DBP (Figure 3A and B). Each assay contains 100 ng of poly(dAdT+dGdC) ($3.2 \mu\text{M}$ in base pairs). Assuming that DBP binds non-specifically to 9–11 bases of DNA (Van Amerongen *et al.*, 1987) then optimum NFI site occupancy occurs when the concentrations of DBP ($0.4 \mu\text{M}$) and DBP binding sites are equal. Thus the large excess of DBP required for increased binding of NFI probably indicates that NFI interacts with template-bound rather than free DBP. Consequently, *in vivo* the interaction of DBP with the viral genome may create a DNA template that has a particularly high affinity for NFI with the result that inside the nucleus of an infected cell limiting amounts of NFI may be preferentially associated with the viral genome. Since NFI has been shown to act in both DNA replication and transcription (Jones *et al.*, 1987; Santoro *et al.*, 1988), both of these processes may be stimulated by DBP with the result that viral templates will be favoured over cellular templates. Despite an incomplete model of the interaction of NFI and DBP in the molecular control of adenovirus replication, these results provide an interesting insight into the regulation of viral replication and possibly transcriptional control.

Materials and methods

³²P-labelled DNA fragments

Construction of plasmid pEX containing the adenovirus 2 origin of replication (Ad-ITR) and plasmid pUC1X72 containing the SV40 enhancer have been previously described (Hay and McDougall, 1986; Clark *et al.*, 1988). A 3'-³²P-labelled fragment of the top strand of the Ad-ITR containing the NFI recognition site was prepared by digestion of pEX with *Bam*HI and repair with the Klenow fragment of *E. coli* DNA polymerase I in the presence of [α -³²P]dCTP. DNA was then recut with *Eco*RI. 5'- and 3'-labelled fragments of the top and bottom strands of the Ad-ITR were prepared by digestion of pEX with *Eco*RI and either labelling with [γ -³²P]ATP and polynucleotide kinase (5'-labelled top strand) or repair with Klenow polymerase in the presence of [α -³²P]dATP (3'-labelled bottom strand) and DNA recut with *Bam*HI. A 5'-labelled fragment containing part of the SV40 enhancer was prepared by digestion of pUC1X72 with *Bam*HI and labelling with [γ -³²P]ATP and polynucleotide kinase. DNA was then recut with *Eco*RI. A 5'-labelled fragment containing a human genomic NFI site was prepared by digestion of pC22 (Adhya *et al.*, 1986) kindly supplied by M. Kenny, with *Eco*RI and labelling with [γ -³²P]ATP and polynucleotide kinase. DNA was then recut with *Bam*HI. Labelled fragments were fractionated by electrophoresis in polyacrylamide gels and recovered by electroelution.

Purification of proteins

NFI ($0.4 \mu\text{g/ml}$) was purified from HeLa cell nuclear extracts by ion exchange chromatography on DEAE–Sephacel followed by three rounds of DNA recognition site affinity chromatography (Kadonaga and Tjian, 1986; Rosenfeld and Kelly, 1986). The purity of the preparation was confirmed by silver-staining of SDS–polyacrylamide gels (Laemmli, 1970; Oakley *et al.*, 1980). DBP (0.3 mg/ml) was purified from nuclear extracts of adenovirus 2 infected HeLa cells by phosphocellulose and DNA cellulose chromatography (Schechter *et al.*, 1980). EBPI ($2 \mu\text{g/ml}$) was kindly supplied by L. Clark after purification by DEAE–Sephacel and DNA recognition site affinity chromatography (Clark *et al.*, 1988). HeLa cell ssb (0.2 mg/ml) was kindly supplied by M. Kenny and purified by chromatography on BioRex 70 and DNA–cellulose (Wobbe *et al.*, 1987). *E. coli* ssb was obtained from Pharmacia and gene 32 protein from Boehringer Mannheim.

DNase I footprinting

Labelled DNA (1.5 ng) was incubated with the different protein preparations for 60 min at 0°C in a final volume of $50 \mu\text{l}$ containing 25 mM Hepes (pH 7.5), 5 mM MgCl_2 , 4 mM dithiothreitol (DTT), 100 mM NaCl, $0.5 \mu\text{g}$ bovine serum albumin (BSA) and 100 ng poly(dAdT) + poly(dGdC). Incubation mixtures were then treated with 0.25 U DNase I (Amersham)

for 60 s at 24°C and the reaction stopped by the addition of $200 \mu\text{l}$ 1.5 M mercaptoethanol–0.3 M sodium acetate containing $100 \mu\text{g/ml}$ tRNA. In determination of the NFI association rate DNase I was increased to 0.6 U per reaction and the incubation period reduced to 15 s. DNA was phenol and chloroform extracted, ethanol precipitated and separated on a 12% polyacrylamide–50% urea sequencing gel.

Methylation protection

A 5'-labelled fragment of the top strand or a 3'-labelled fragment of the bottom strand of the Ad-ITR (1.5 ng) was incubated with NFI in the absence or presence of DBP as described under DNase I footprinting. Reaction mixtures were then treated with $1 \mu\text{l}$ DMS, incubated for 60 s at 20°C and the reaction stopped by the addition of $200 \mu\text{l}$ 1.5 M mercaptoethanol–0.3 M sodium acetate containing $100 \mu\text{g/ml}$ tRNA. DNA was phenol and chloroform extracted, ethanol precipitated and dissolved in $50 \mu\text{l}$ 1 M piperidine. After incubation for 30 min at 90°C , samples were lyophilized and DNA separated on a 12% polyacrylamide sequencing gel.

DNA replication in vitro

Assays, in a total volume of $35 \mu\text{l}$, contained 25 mM Hepes, pH 7.5, 5 mM MgCl_2 , 4 mM DTT, 3 mM ATP, 35 μM dATP, 35 μM dTTP, 25 μM ddGTP, 5 μCi [α -³²P]dCTP (3000 Ci/mmol), 200 ng adenovirus type 2 cores (Goding and Russell, 1983), 30 μg cytoplasmic extract (Harris and Hay, 1988), pTP-pol (Rosenfeld *et al.*, 1987), DBP ($2 \mu\text{g}$) and NFI (1 ng). Incubation was at 31°C for 60 min after which the products of the reaction were analysed by SDS–PAGE and autoradiography (Harris and Hay, 1988).

Scanning densitometry

Autoradiograms were scanned with a Vitatron Universal TLD 100 scanning densitometer and specific bases used to calculate NFI site occupancy as a fraction of complete protection against DNase I cleavage (Borowiec *et al.*, 1987).

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