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Adenovirus DNA replication *in vitro*: site-directed mutagenesis of the nuclear factor I binding site of the Ad2 origin

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ABSTRACT

The template requirements for efficient adenovirus DNA replication were studied *in vitro* in a reconstituted system with cloned DNA fragments, containing the Ad2 origin region, as templates. Replication is enhanced by nuclear factor I, a cellular protein that binds specifically to the Ad2 origin. This stimulation is shown to be strongly dependent on the concentration of the adenovirus DNA binding protein. Using synthetic oligonucleotides we have constructed plasmids with base substitutions in the nuclear factor I binding region. Footprint analysis and competition filter binding studies show that two of the three small blocks of conserved nucleotides in this region are involved in the binding of nuclear factor I. The binding affinity can be influenced by the base composition of the degenerate region just outside these two blocks. *In vitro* initiation and DNA chain elongation experiments with the mutants demonstrate that binding of nuclear factor I to the Ad2 origin is necessary for stimulation. However, binding alone is not always sufficient since a mutation which only slightly disturbs binding is strongly impaired in stimulation of DNA replication by nuclear factor I.

INTRODUCTION

The adenovirus genome consists of a linear double-stranded DNA molecule of about 36.000 base pairs with a 55-kDa terminal protein (TP) covalently attached to both 5'-termini (1, 2). The origins of replication are contained within the inverted terminal repeats (ITR) of 103 base pairs (for Ad2) and initiation of replication can take place on both ends. The development of a cell-free DNA replication system (3) has made possible an extensive investigation of the factors involved in replication and the mechanisms by which replication proceeds (reviewed in 4, 5, 6, 7).

A characteristic feature of adenovirus replication is the protein-priming mechanism by which initiation of DNA synthesis takes place. *In vitro* studies have shown that replication starts with the formation of a covalent bond between the 80-kDa precursor of the terminal protein (pTP) and dCMP, the first nucleotide in the new DNA chain (8, 9, 10). This complex is subsequently elongated to a new chain of genome length by a strand displacement mechanism.

Displaced strands are converted into duplex molecules by unknown mechanisms. In addition to the pTP, the initiation reaction requires the virus encoded 140-kDa Ad DNA polymerase that is isolated in a complex with pTP (11, 12). The third virus encoded protein involved in replication is the 72-kDa DNA-binding protein (DBP) that is absolutely required for elongation of the pTP-dCMP complex (7, 13, 14). A reconstituted system consisting of these three viral proteins and purified DNA-TP of Ad2 as template can sustain a basal level of pTP-dCMP complex formation and subsequent elongation (12, 15). Fractionation of extracts of uninfected HeLa cells allowed the identification of a nuclear factor (nuclear factor I, 16, 17) and a cytoplasmic factor (18) that stimulate pTP-dCMP complex formation and subsequent elongation in the reconstituted system. Nuclear factor I was shown to be a site specific DNA-binding protein that binds to the Ad2 origin and protects a part of the origin against DNase I digestion (17, 19, 20; Fig. 1).

An important step in the investigation of the template requirements for efficient replication was the observation that replication *in vitro* could be studied by making use of cloned Ad2 terminal fragments, devoid of TP, as template (21, 22). This allowed the use of *in vitro* mutagenesis techniques to determine the viral sequences that are required for replication. In the origin region four blocks of nucleotides are highly conserved between the different adenovirus serotypes (Fig. 1). By making use of deletion mutants evidence has been presented that the first conserved block (nucleotides 9-18) is required for specific binding of the pTP-pol complex to the origin of DNA replication (23). Furthermore, it was shown that the first 18 bp of Ad2 are required to support a limited amount of replication *in vitro* (24, 25, 26). These results suggest that binding of pTP-pol to the first conserved block is absolutely necessary for replication. Conserved blocks II, III, and IV are situated in the region to which nuclear factor I binds. Experiments with deletion mutants indicated that binding of nuclear factor I to this region is strictly correlated to its ability to stimulate initiation and subsequent elongation. The internal right boundary of the region necessary for nuclear factor I binding and stimulation of replication has been placed between nucleotides 37 and 48 (19, 27).

In this report we describe the introduction *in vitro* of single and double base substitution mutations, by oligonucleotide directed mutagenesis, into the nuclear factor I binding region. The effects of the mutations on the stimulating effect of nuclear factor I on replication have been tested in an *in vitro* initiation and elongation system. A quantitative analysis of the

binding of nuclear factor I to the set of mutants has been performed by a competition filter binding assay. By making use of DNase I footprinting, binding of nuclear factor I to the mutants has been studied qualitatively.

MATERIALS AND METHODS

Recombinant DNA clones

XD-7 was a gift of J. Corden and consists of the 1338 bp XbaI E-fragment of Ad2 that has been cloned into the EcoRI site of pBR322 (28). MXE-1 and MXE-2 were constructed by cloning the insertion of XD-7 into the EcoRI site of M13mp8. ssMXE-1 contains the l-strand fragment and ssMXE-2 contains the r-strand fragment of Ad2. Xpm2223, Xpm2426, Xpm34A, Xpm34C, Xpm3637, and Xpm46G were constructed by cloning the EcoRI insertions of the MXE-1 (2426, 3637) and MXE-2 (2223, 34A, 34C, 46G) derived pointmutants (described below) into the EcoRI site of pBR322.

Oligonucleotides

18-mer oligodeoxynucleotides were synthesized as described (29). 5'-phosphorylation of oligonucleotides was performed as follows: (I) When used as probe in screening for mutants, oligonucleotides were labeled by incubating 20 pmol oligonucleotide (140 ng) for 45 min at 37° C in a reaction volume of 25 µl containing 5 units T4 polynucleotide kinase (Boehringer), 40 pmol [γ-^{32}P]ATP (spec. act. 3180 Ci/mmol, New England Nuclear). 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol. T4 polynucleotide kinase was inactivated by incubation during 15 min at 68° C. [γ-^{32}P]ATP was removed by chromatography on Sephadex G-25 in 50 mM ammoniumbicarbonate (pH 7.8). (II) When used as primer for complementary strand synthesis, oligonucleotides, containing the non-complementary nucleotide in the middle, were 5'-phosphorylated under the same conditions, except that 1 µg oligonucleotide and 100 µM ATP (final concentration) were used.

Site directed mutagenesis

0.2 pmol (600 ng) of ssMXE-1 or ssMXE-2 and 8 pmol of phosphorylated oligonucleotide were heated for 3 min at 80° C and allowed to cool to 30° C in a buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 75 mM NaCl (final volume 10 µl). Complementary strand was synthesized by adding 5 units of E. coli DNA polymerase I (Klenow fragment, Boehringer) and adjusting the buffer to 50 µM ATP, 50 µM dNTP's, 7.5 mM DTT, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 75 mM NaCl (final volume 20 µl). After 30 min incubation at 30° C, 1.8 unit of T4 DNA ligase (Boehringer) was added and the reaction was allowed to proceed for 40 hr at 12° C. $CaCl_2$ treated E. coli strain JM101 cells were transformed

with 5 µl of the ligation mixture described above (60 min, 0° C; 2 min, 42° C). Then 20 ml of medium (L-broth) and 1 ml of exponentially growing JM101 cells were added and growth was continued for 90 min at 37° C. After centrifugation a phage titration of the supernatant was done and plates containing 200-300 plaques were prepared. By this procedure the occurrence of mixed DNA populations was avoided (30).

Screening by plaque hybridization

We made use of the observation (31) that mutants can be detected due to the decreasing effect of mismatches on the thermal stability of heteroduplexes between short oligonucleotides and ssDNA (32). Plates were screened directly for mutants as follows: Two replicafilters of each plate were made by adsorption of plaques to nitrocellulose filters for 10 min at 4° C followed by 10 min of denaturation in 0.1 N NaOH, 1.5 M NaCl and 10 min of neutralization in 0.2 M Tris-HCl (pH 7.5), 0.3 M NaCl, 0.03 M NaCitrate (pH 7.0). After preincubation of the filters for 1 hr at 12° C in 6 x NET (1 x NET is 0.15 M NaCl, 1 mM EDTA, 15 mM Tris-HCl (pH 7.5)), 5 x Denhardt (1 mg/ml Ficoll, 1 mg/Ml polyvinylpyrolidone, 1 mg/ml BSA), 0.5% Nonidet P40 and 50 µg/ml denaturated calf thymus DNA in a volume of 25 ml, 4 pmol (15 µCi) of the [^{32}P]-labeled oligonucleotide used to introduce the desired mutation was added and hybridized overnight at 12° C. Filters were washed 5 times for 20 min in 6 x NET, 0.5% Nonidet P40 at 0° C and then washed two times for 10 min in 6 x NET, 0.5% Nonidet P40, one filter at the critical temperature and the other ten degrees below this temperature. The critical temperature was determined in a separate experiment, by constructing a melting curve for each oligonucleotide, and chosen 4° C above the temperature at which no hybridization with wildtype DNA could be detected anymore. Positive plaques were selected and single-stranded recombinant M13 DNA was isolated as described (33). The sequences of all mutants were verified by DNA sequencing according the dideoxy chainterminator method (34).

DNA isolation and purification

Plasmid and M13 RF DNA was isolated as described (35) and purified on a CsCl gradient. The DNA was further purified by RNase treatment, phenol extraction and filtration over a 1 ml Sepharose 4B column.

DNA replication *in vitro*

The purification of DBP (36), pTP-pol (23), nuclear factor I (20), and cytoplasmic RNA (18) has been described. The amounts of DBP and RNA are given in µg, pTP-pol and nuclear factor I are given in units as described (23, 20). Reaction mixtures (12.5 µl) contained 25 mM Hepes-KOH (pH 7.5), 5 mM $MgCl_2$,

1 mM ATP, 5 mM creatine phosphate, 5 µg/µl creatine kinase, 40 µM each of dGTP, dATP, and dTTP, 2.5 mM [α-^{32}P]dCTP (80 Ci/mmol), 600 µM aphidicolin, 1 µg RNA, 2.7 µg DBP, 1 mU pTP-pol, 0.1 mU nuclear factor I and 30 ng of Eco RI digested plasmid DNA (6.5 fmol). After 60 min of incubation at 37° C the reaction was terminated by adding 1.5 µl stopmix (1% SDS, 1 x TBE, 35% sucrose, 0.1% bromophenol blue). Reaction products were analyzed by electrophoresis on a 1% agarose gel containing 0.1% SDS. Electrophoresis was carried out in 0.5 x TBE, 0.1% SDS.

pTP-dCMP complex formation *in vitro*

Reaction mixtures (25 µl) contained 25 mM Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 5 mM creatine phosphate, 5 µg/µl creatine kinase, 40 µM ddATP, 1.25 µM [α-^{32}P]dCTP (640 Ci/mmol), 600 µM aphidicolin, 2 µg RNA, 1.1 µg DBP, 2 mU pTP-pol, 0.2 mU nuclear factor I and 60 ng of EcoRI digested plasmid DNA. After 2 hr of incubation at 30° C, 5 units of Staphylococcus aureus nuclease (Boehringer) were added and incubation was continued for 1 hr at 30° C. Proteins were precipitated with 20% trichloroacetic acid and samples were separated by electrophoresis on 10% polyacrylamide-SDS gels (37).

DNase I footprinting and filter binding assay

The footprinting and filter binding procedures are described extensively elsewhere (20). In short, EcoRI digested plasmid DNA's were 5'-phosphorylated with [γ-^{32}P]ATP and digested with PvuII. For footprinting experiments the origin containing EcoRI-PvuII fragment (454 bp) was isolated from polyacrylamide gels. 1-2 ng EcoRI-PvuII fragment (2.7-5.4 fmol, 0.01 µCi) was incubated in 25 µl for 60 min at 0° C with 0.5 mU nuclear factor I in a buffer containing 50 mM or 150 mM NaCl, followed by DNase I digestion (50 sec, 24° C, 0.1 U), phenol extractions and polyacrylamide gel electrophoresis. Filter binding experiments were performed as competition experiments with labeled XD-7 DNA (described above) and unlabeled competitor DNA, digested with EcoRI. Binding of nuclear factor I to the DNA was done by 60 min of incubation at 0° C in a buffer containing 150 mM NaCl, followed by filtration over nitrocellulose (Millipore, HA), recovery of the filter bound material and agarose gel electrophoresis.

RESULTS

Site directed mutagenesis of the Ad2 origin

In order to determine the DNA sequences that play a role in Ad2 replication, single and double base substitution mutations (pointmutations) were introduced into the nuclear factor I binding region of the Ad2 origin using

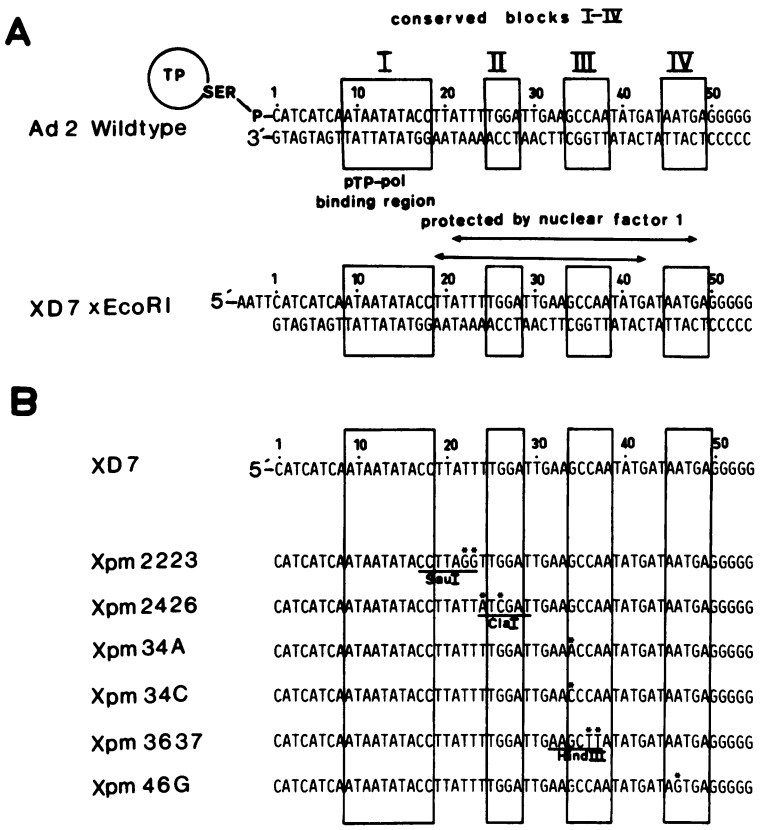


Figure 1 DNA sequence of the wildtype Ad2 origin region and 6 constructed mutants. A. Comparison of the left terminus of Ad2 as present in virion DNA with the terminal sequence from XD-7 DNA digested with EcoRI. Nucleotides that are conserved in most human serotypes are boxed. The region of the 1-strand that is described to be protected by nuclear factor I against DNase I digestion is indicated, either 19-42 (19, 20) or 21-48 (17). B. Sequence of the pointmutants. The plasmids have been named XpmN, where N stands for the position of the base substitution(s).

the oligonucleotide-directed mutagenesis procedure (reviews 38, 39, 40). Part of the sequence of the constructed mutants is shown in Fig. 1. Mutations have been introduced both in conserved blocks II, III, and IV, and in the degenerate region between block I and II. In three cases a new restriction site has been created by a double pointmutation. Xpm34A, Xpm34C, and Xpm46G were constructed by using a mixture of oligonucleotides, containing all three nucleotides that are not complementary to wildtype DNA at the position of the nucleotide that had to be substituted. At position 34 only two of the three

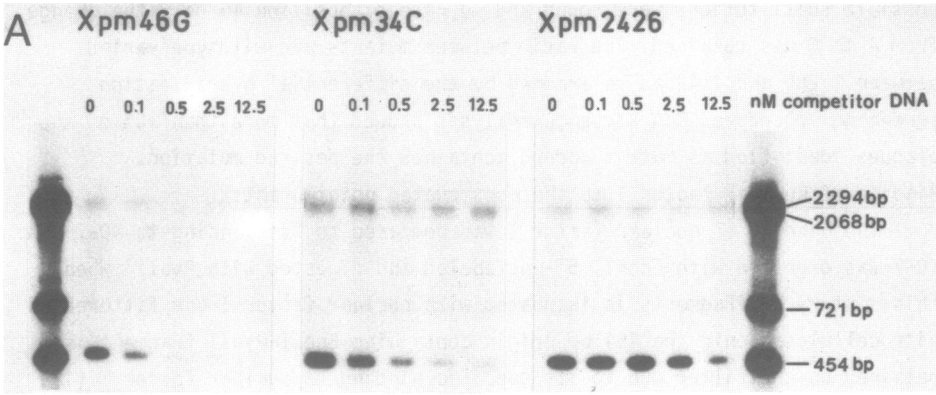
possible substitutions were found and in case of position 46 only the change from A to G was obtained. The ratio between mutants and wildtype varied between 1:200 and 1:40 as determined by the differential hybridization screening procedure. DNA sequence analysis showed that more than 90% of the plaques identified as mutant indeed contained the desired mutation.

Binding of nuclear factor I to the constructed pointmutants

The binding of nuclear factor I was compared to its binding to XD-7 DNA. XD-7 was digested with EcoRI, 5'-endlabeled and digested with PvuII. When this mixture of fragments is incubated with nuclear factor I and filtered on nitrocellulose, only the 454 bp origin containing EcoRI-PvuII fragment is retained on the filter due to its specific binding to nuclear factor I (17, 20). The binding of nuclear factor I to the set of pointmutants was quantitated by addition of increasing amounts of unlabeled EcoRI digested mutant DNA as competitor.

In Fig. 2A the results for three mutants are shown. Xpm46G competes like wildtype DNA, Xpm34C is a weaker competitor, and Xpm2426 competes only at high concentrations (125-fold excess). This is similar to aspecific pBR322 competitor DNA. In Fig. 2B the results of all mutants are summarized. The data are presented as the ratio of competitor to input DNA required to achieve a 50% reduction in the amount of labeled DNA-protein complex. The double pointmutations in the conserved blocks II (Xpm2426) and III (Xpm3637) reduced the binding affinity for nuclear factor I about 100-fold. Substitution mutations at position 34 (conserved block III) revealed that the effect of a mutation on the binding affinity for nuclear factor I can depend on the nature of the base pair by which the GC base pair is replaced. An AT base pair at this position gives a stronger decrease in the ability of nuclear factor I to bind to the DNA than a CG base pair. Substitution of the AT base pair at position 46 (conserved block IV) by a GC base pair has no effect on the binding to nuclear factor I. Surprisingly we found that a double pointmutation at positions 22 and 23, which is situated in a degenerate region that was shown to lay outside the minimal region required for efficient binding to nuclear factor I (20), decreased the binding to nuclear factor I approximately 5-fold.

A qualitative picture of the sequence specific interaction between nuclear factor I and the adenovirus origin can be obtained by making use of DNase I footprinting techniques. It has been established that nuclear factor I protects nucleotides 19-42 (19, 20) or 21-48 (17) of the l-strand of the left end Ad2 origin from DNase I digestion. This region includes conserved blocks II, III, and in the case of 21-48 also block IV. In Fig. 3 the footprinting



B

	50% COMPETITION RATIO COMPETITOR TO INPUT DNA
Xpm2223	5
Xpm2426	> 125
Xpm34A	25
Xpm34C	5
Xpm3637	80
Xpm46G	1
XD7	1
pBR322	> 125

Figure 2 Comparison of the binding affinity of nuclear factor I for wildtype XD-7 DNA and mutant XpmN DNA. A. Competition filter binding of Xpm46G, Xpm34C and Xpm2426. 5'-endlabeled XD-7 DNA (0.1 nM, digested with EcoRI and PvuII) is competed for binding to 0.02 mU of nuclear factor I by increasing amounts of unlabeled competitor DNA, digested with EcoRI. The amount of competitor DNA is indicated in nM above the lanes and ranges from 0 to 125-fold excess. Specific binding to nuclear factor I was quantitated by cutting out of gel the 454 bp origin containing fragment, followed by determination of radioactivity by Cerenkov radiation. In this way for all mutants a competition curve was constructed and the factor of excess competitor DNA that must be added to obtain 50% competition was calculated. In B these results are given.

results of four pointmutants are shown. The 454 bp EcoRI-PvuII fragments of XD-7 DNA and of the pointmutants were 5'-endlabeled at the EcoRI site, isolated and partially digested with DNase I in the absence or the presence of nuclear factor I, followed by electrophoresis of the products on a denaturing polyacrylamide gel. In the presence of nuclear factor I nucleotides 19 to 42 of XD-7 DNA are protected against DNase I as described in detail elsewhere (20). Xpm46G (lane e) and Xpm2223 (lane d) show the same protection pattern

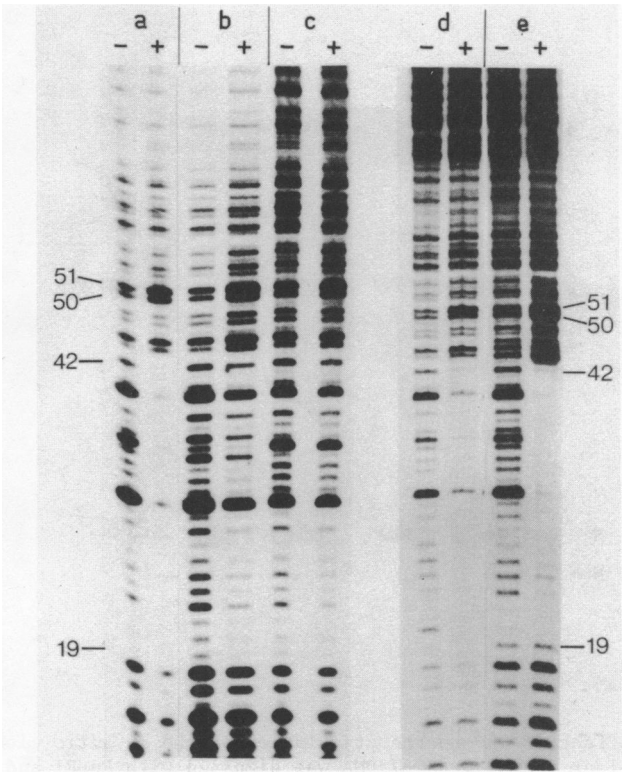


Figure 3 DNase I footprinting patterns of XD-7 DNA compared to XpmN DNA. The origin containing 454 bp EcoRI-PvuII fragment (2.0 fmole) of XD-7 DNA and the mutants were endlabeled at the EcoRI site and partially digested with DNase I in the absence (-) or the presence (+) of nuclear factor I. Products were analyzed on a 10% denaturing polyacrylamide gel. Lane a: XD-7 DNA; lane b: Xpm34A; lane c: Xpm34C; lane d: Xpm2223; lane e: Xpm46G. For lanes a, b and c DNase I digestion was performed in a buffer containing 50 mM NaCl, for lanes d and e this was 150 mM NaCl. The position of the protected region is indicated, the numbers refer to the position on the Ad2 sequence.

as wildtype DNA. However, protection of Xpm2223 is less efficient, which agrees with the results of the filter binding experiment. The nuclear factor I binding region of mutants Xpm34A, Xpm34C, Xpm2426, and Xpm3637 is not protected at 150 mM NaCl (results not shown). When tested at a less stringent salt concentration (50 mM NaCl), similar to the concentrations used for the *in vitro* DNA replication tests, Xpm34A (lane b) and Xpm34C (lane c) show a protection pattern like XD-7 (lane a) although only a low percentage of the

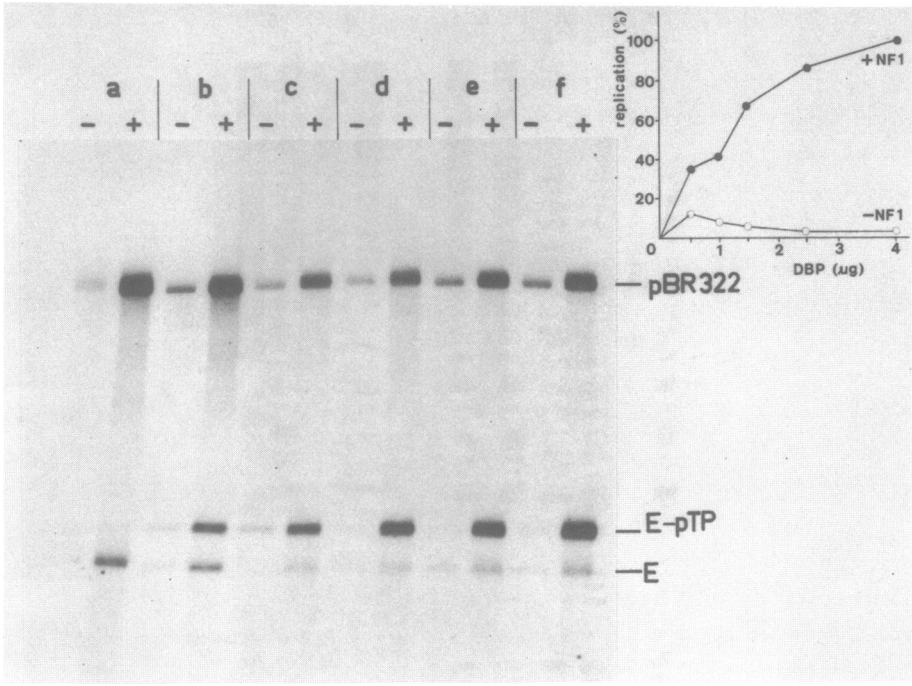


Figure 4 The effect of DBP on the stimulation of the *in vitro* elongation reaction by nuclear factor I. XD-7 DNA was digested with EcoRI and used as template in the *in vitro* replication reaction under the incubation conditions described in the materials and methods section. Replication was performed in the absence (-) or the presence (+) of nuclear factor I at increasing concentration of DBP. Replication was quantified by determining the amount of [α-^{32}P]dCTP incorporated into the replication band (E-pTP) by Cerenkov radiation. Lane a: no DBP; lane b: 0.5 µg DBP; lane c: 1.0 µg DBP; lane d: 1.5 µg DBP; lane e: 2.5 µg DBP; lane f: 4.0 µg DBP. The stimulation of replication by nuclear factor I at each DBP concentration can be deduced from the insert. The level of replication is indicated in percentages where replication in the presence of nuclear factor I and 4.0 µg DBP is taken as 100 percent.

DNA molecules is protected. Xpm2426 and Xpm3637 are not protected at all (results not shown). An interesting feature of the binding of nuclear factor I to the wildtype Ad2 origin is the increased sensitivity for DNase I digestion around positions 50 and 51. This is also observed for Xpm2223, Xpm34A, Xpm34C, and Xpm46G. We therefore conclude that single and double pointmutations in the nuclear factor I binding region of the Ad2 origin affect the affinity for nuclear factor I, but do not alter the DNase I footprinting pattern in the cases presented here.

Nuclear factor I stimulation is dependent upon the DBP concentration

We were interested in the effects of the pointmutations on DNA replication. Using TP-free plasmid DNA, initiation *in vitro* only takes place when the origin is exposed at the end of a DNA molecule (21, 22). Therefore template DNA was digested with EcoRI to yield a 1343 bp fragment, containing the left end origin of Ad2 at its end, and a 4364 bp pBR322 fragment. DNA chain elongation is assayed by incubating the linearized DNA template with purified viral proteins (DBP, pTP-pol) in the presence of the four deoxynucleoside triphosphates, leading to replication of the total 1343 bp fragment. Replication can be visualized by electrophoresis of the reaction mixture in a SDS containing agarose gel followed by autoradiography. In this way the replicated fragments, to which a pTP molecule is covalently attached, can be separated from the 1343 bp input fragment that also incorporates label due to repair synthesis. Addition of nuclear factor I to the mixture was shown to stimulate the *in vitro* elongation reaction, with cloned DNA fragments as template, approximately 4-fold (41). In Fig. 4 evidence is presented that the magnitude of stimulation by nuclear factor I depends on the conditions that are used. Adding more DBP to the reaction leads to an increase of the stimulatory effect of nuclear factor I on the reaction. At the highest DBP concentration tested, the stimulation by nuclear factor I was 28-fold. In the absence of nuclear factor I DNA replication decreased by addition of increasing amounts of DBP.

Effect of pointmutations on DNA replication

We used the conditions at which a 28-fold stimulation by nuclear factor I is obtained for a sensitive test of the effect of the pointmutations (Fig. 5). All mutants were tested in the presence and the absence of nuclear factor I. Xpm46G (lane g) was stimulated to the same level as XD-7 (lane a), while Xpm2426 (lane c) showed no significant stimulation and Xpm3637 (lane f) only a slight stimulation by nuclear factor I. These results agree well with the results of the filter binding experiments. However, Xpm34A and Xpm34C (lanes d, e) were stimulated to almost the same level as XD-7 though their binding affinity for nuclear factor I is markedly decreased. On the contrary Xpm2223, which has a higher binding affinity for nuclear factor than Xpm34A, only shows a very weak, if any, stimulation by nuclear factor I (lane b).

Effect of pointmutations on initiation

By addition of dCTP as the only nucleotide to the *in vitro* replication system, we have studied initiation as measured by formation of a pTP-dCMP complex. The products were analyzed by electrophoresis in a polyacrylamide-SDS gel and autoradiography. Fig. 6 shows that the various pointmutants behave

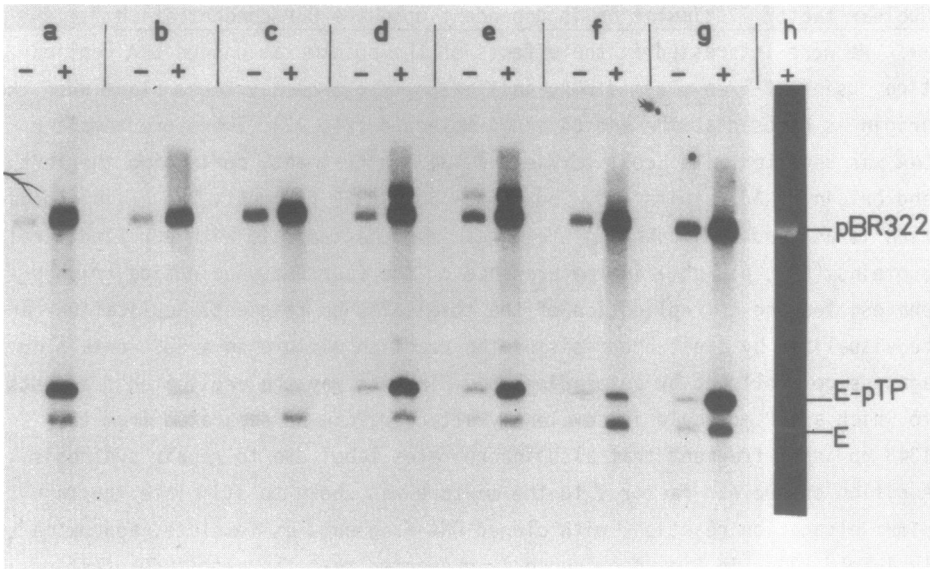


Figure 5 The effect of pointmutations on DNA replication. All plasmids were digested with EcoRI and used as template molecules in the *in vitro* replication reaction (described in materials and methods) in the absence (-) or the presence (+) or nuclear factor I. The stimulation of the replication reaction by nuclear factor I was about 30-fold for XD-7 DNA (lane a). The additional band observed in lanes d and e is caused by partial digestion of part of the plasmid molecules (less than 10 percent). The repair reaction is also increased by addition of nuclear factor I. However, for two reasons we can explain that the enhanced incorporation of label in the replication band (E-pTP) is not due to stimulation of repair. Firstly, only a very small part of the molecules is replicated (no replication band is visible on an ethidium-bromide stained gel, lane h) which means that random repair synthesis can not account for such a large incorporation of label in the E-pTP band. Secondly, mutant DNA molecules which show no stimulation of replication, still show the stimulation of repair synthesis. Therefore, we believe that the stimulation of repair synthesis is due to an impurity in the nuclear factor I preparation. Lane a: XD-7; lane b: Xpm2223; lane c: Xpm2426; lane d: Xpm34A; lane e: Xpm34C; lane f: Xpm3637; lane g: Xpm46G; lane h: XD-7 (ethidiumbromide stained gel).

very similar in initiation and elongation with respect to their response to nuclear factor I. The only exception is that the initiation reaction of Xpm3637 is weakly stimulated and that the stimulation with Xpm34A is somewhat weaker than with Xpm34C, Xpm46, and XD-7. This indicates that the primary effect of nuclear factor I is on initiation.

Taking the results of the binding experiments and the *in vitro* replication experiments together, we conclude that binding of nuclear factor I to the

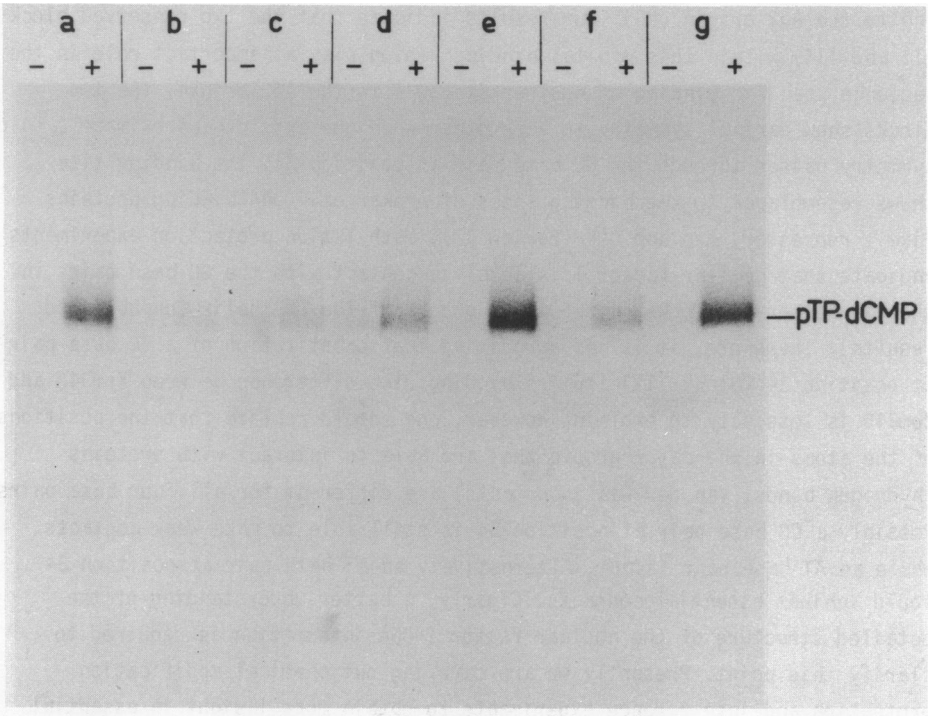


Figure 6 The effect of pointmutations on the pTP-dCMP complex formation. The reaction is carried out under the same conditions as the elongation reaction except that dCTP is the only nucleotide present and the incubation was carried out at 30° C. Lane a: XD-7; lane b: Xpm2223; lane c: Xpm2426; lane d: Xpm34A; lane e: Xpm34C; lane f: Xpm3637; lane g: Xpm46G.

Ad2 origin is necessary for its enhancing effect on the replication reaction. However, a decreased binding affinity for nuclear factor I, as with Xpm34A and Xpm34C, does not necessarily lead to a corresponding decrease of the replication reaction in the presence of nuclear factor I. Furthermore, the results obtained with Xpm2223 reveal that binding of nuclear factor I to the origin is not always sufficient for carrying out its function.

DISCUSSION

Binding of nuclear factor I to mutants

Nuclear factor I, a sequence specific DNA binding protein isolated from uninfected HeLa cells (16), stimulates the *in vitro* replication reaction by binding to the origin region (19, 27). Recently a minimal region of 15 or 16 nucleotides (25-39 or 40) was shown to be required for nuclear factor I bind-

ing to the Ad2 origin (20). Our results indicate that the two conserved blocks (II and III) within this minimal binding region play an important role in the sequence specific binding of nuclear factor I to the Ad2 origin. The two blocks show partial symmetry to each other when the axis of approximate 2-fold symmetry passes through the GC base pair at position 31. The binding site shows resemblance to the binding sites of prokaryotic DNA binding proteins like λ repressor, cro and CAP (review 42). Methylation protection experiments indicate that nuclear factor I is in close contact with the GC base pairs in the two conserved blocks in the major groove of the DNA helix (unpublished results). Therefore, it is not surprising that substitution of a GC base pair at position 34 (block III) impairs binding. The difference between Xpm34A and Xpm34C is less easy to explain. However, one should realize that the positions of the atoms in the major groove that are able to interact with proteins (hydrogen bonds, Van der Waals contacts) are different for all four base pairs. Possibly a CG base pair at position 34 is still able to make weak contacts, while an AT base pair is not. Alternatively an AT base pair at position 34 could inhibit essential contacts. Clearly, a better understanding of the detailed structure of the nuclear factor I-DNA interaction is required to clarify this point. Presently we are carrying out chemical modification protection and interference experiments to obtain more insight in essential contact points.

A potential hairpin structure that might be formed in the region of 17-51 has been proposed as a recognition sign for nuclear factor I (17, 27). Considering the results obtained with Xpm2223 and Xpm46G, in which this hairpin would be seriously destabilized, this hypothesis seems less likely. The results furthermore agree with the recent observation that the first 40 base pairs of Ad2 are sufficient for efficient binding of nuclear factor I (20). Therefore, we conclude that conserved block IV plays no role in nuclear factor I binding under the conditions used.

It has been suggested that base pairs just outside the minimal binding sequence influence the binding affinity for nuclear factor I (20). The effects of Xpm2223, which has mutations 2 and 3 base pairs outside the minimal binding sequence, confirm this notion. Whether these influences are due to direct protein-DNA interactions at these positions or to stereochemical changes in the DNA structure that effect binding ot the protein remains to be tested.

Correlation between binding and DNA replication

Thus far only deletion mutants have been obtained that bind to nuclear factor I like wildtype DNA or do not bind at all. These mutants demonstrate

that binding of nuclear factor I to the Ad2 origin is essential for its enhancing effect on replication and define, more or less accurate, a minimal origin of replication (19, 20, 27). The results obtained with Xpm2426 and Xpm3637 confirm this and indicate that conserved blocks II and III are essential template requirements for maximal replication *in vitro*. However, mutants with a markedly decreased binding affinity for nuclear factor I within block III (Xpm34A, Xpm34C), still show a stimulation of replication by nuclear factor I like wildtype DNA. This means that a decreased binding affinity for nuclear factor I still might result in an efficient template. On the other hand it is possible that *in vivo* a limiting amount of nuclear factor I exists. Therefore, adenovirus DNA may have to compete with a, perhaps, large number of nuclear factor I binding sites on the host DNA. In that case a mutation that decreases the binding affinity might severely impair the functional origin of the virus. *In vitro* replication competition experiments might eludidate this.

A mutation in conserved block IV (Xpm46G) shows no effect on the stimulation of replication by nuclear factor I. This agrees with the observation that the first 40 base pairs are sufficient for efficient stimulation by nuclear factor I (20). At present we can not exclude that there are still other factors required for optimal replication that could possibly interact with block IV.

Xpm2223 is a mutant with a comparable binding affinity for nuclear factor I as Xpm34C and a DNase I footprinting pattern like wildtype DNA. From experiments with this mutant we conclude that, though binding of nuclear factor I to the Ad2 origin is a prerequisite for its stimulation of replication, binding alone does not guarantee an efficient stimulation. A priori, the mutations in Xpm2223 might disturb the interaction with replication proteins other than nuclear factor I, e.g. pTP. However, comparison of XD7 and Xpm2223 at higher DNA concentrations (results not shown) revealed no difference in efficiency of replication in the absence of nuclear factor I. To explain how the substitution of the TA base pairs at positions 22 and 23 by GC base pairs impairs the stimulation by nuclear factor I, the mechanism by which nuclear factor I acts should be known. It has been proposed that nuclear factor I stimulates the initiation of replication by unwinding the DNA at the origin (25). Alternatively it might increase the accessability to the origin for other replication proteins, either by direct protein-protein interactions or by the induction of stereochemical changes in the DNA structure. In theory, both of these hypotheses are compatible with the results obtained with Xpm2223.

With respect to the mechanism of nuclear factor I action, our observation that the level of stimulation depends on the DBP concentration is interesting. One hypothesis that could explain the results is that nuclear factor I must interact with DBP to carry out its function, for example in the unwinding of the DNA. On the other hand it has been reported that the processivity of the Ad pol is greatly enhanced by DBP with poly(dT) as template (43). This together with the fact that the initiation reaction is inhibited by DBP in the absence of nuclear factor I and stimulated in its presence (16), may also explain the effect of the DBP concentration. When comparing the *in vitro* conditions with the concentration of DBP *in vivo*, it is clear that the intranuclear DBP concentration is considerably higher. Assuming that all DBP is inside the nucleus and that the diameter of nuclei varies between 3 and 10 μm, a concentration of 100 mg/ml to 4 mg/ml can be calculated. This high DBP concentration may well explain the absolute requirement of a nuclear factor I binding region *in vivo* (44).

When considering these studies using plasmid DNA one should realize that the function of the terminal protein, which is covalently linked to the DNA *in vivo*, is neglected. Therefore, it will be of interest to study the effects of the pointmutations in an *in vivo* system. Recently reported experiments, in which origin containing plasmid constructions were co-transfected with Ad2 DNA, indicate that the nuclear factor I binding region is also essential *in vivo* (44). These results are in agreement with the results obtained *in vitro* with DNA fragments devoid of the terminal protein.

Recently a number of nuclear factor I binding sites have been discovered in cellular DNA (45). Sites have been located upstream the human c-myc gene, the human IgH locus (46), and the chicken lysozyme gene (47). Some of these sites are close to DNase I hypersensitive sites and may function in gene activation. Nuclear factor I-like proteins can be isolated from chicken oviduct nuclei (47), mouse cells, and hamster cells (19). A possible conservation in evolution of the protein might indicate an important role for nuclear factor I in the regulation of cellular replication or transcription.

In contrast to the cellular nuclear factor I binding sites, the Ad2 site can be submitted to a functional test in the adenovirus *in vitro* replication system in a simple way. The importance of this is stressed by the results obtained with Xpm2223. Therefore, *in vitro* site-directed mutagenesis is an efficient technique to investigate the requirements for a functional nuclear factor I binding site. We notice that this does not necessarily mean that a

nuclear factor I binding site which is not functional in the *in vitro* replication assay (like Xpm2223), is also not functional when present in host cell DNA.

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