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A sensitive and rapid gel retention assay for nuclear factor I and other DNA-binding proteins in crude nuclear extracts

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ABSTRACT

The paper describes a rapid and sensitive assay for DNA binding proteins which interact with specific and defined binding sites. It exploits the observation that complexes of proteins and small synthetic DNA fragments (40 bp) containing the protein/DNA binding site can enter native polyacrylamide gels and remain stably associated during electrophoresis under non-denaturing conditions. The assay was applied to nuclear factor I, to its identification and purification from porcine liver, to an analysis of its binding site on adenovirus type 5 DNA and to an exploration of other potential binding sites for DNA binding proteins within the inverted terminal repetition of adenovirus DNA. The extreme sensitivity of the assay which surpasses that of conventional footprint assays by at least two orders of magnitude permitted the identification of nuclear factor I-like activities in Saccharomyces cerevisiae.

INTRODUCTION

Protein/DNA interactions have in the past been analyzed by sucrose gradient centrifugation and/or nitrocellulose filtration. Fried and Crothers (2, 3) have shown that DNA/protein complexes as exemplified by lac operator and lac repressor can jointly enter native polyacrylamide gels and remain stably associated during electrophoresis. The protein, the lac repressor in this case, can be identified on the gels through autoradiography if the DNA fragment is appropriately labelled. The unexpected stability of such complexes is not entirely understood but is attributed, at least in part, to a caging effect mediated by the gel matrix. We have exploited this observation in order to develop a rapid assay for nuclear factor I which can be performed on crude, unfractionated nuclear extracts and which has been employed not only to follow nuclear factor I activity during its purification from porcine liver but also to perform a mutational analysis of its binding site on adenovirus type 5 DNA and to identifiy nuclear factor I-like activities in S. cerevisiae. The assay can be applied to any other sequence-specific DNA binding protein as long as its binding site is known.

MATERIALS AND METHODS

Preparation of oligonucleotides.

Oligonucleotides were synthesized on an Applied Biosystems Model 308A DNA synthesizer using the chemistry and the purification methods described by Dörper and Winnacker (17). The identity of the desired sequences was confirmed by plasmid DNA sequencing (1) after cloning of appropriate oligonucleotide pairs into linearized pBR327 DNA.

Gel retention assay.

Oligonucleotides were 5'-labelled with ^{32}P- ATP by T4-polynucleotide kinase (Boehringer Mannheim) to a specific activity of 50-100 bcq/fmole DNA (1-2 nCi/fmole DNA). Six pmoles of oligonucleotide (single-stranded) were added to 10 ul doubly-distilled water together with 18 pmole ^{32}P- ATP (50 uCi or 2,5 x 10^6 bcq), 2 ul (10 units) of polynucleotide kinase and 1 ul of kinase buffer (10). After 10 minutes at 37 C, the reaction was stopped by addition of 5 ul 0,5 M EDTA and the reaction mixture was purified by chromatography on DE-52. Oligonucleotides were always labelled separately and only mixed later in appropriate pairs by heating for 30 seconds to 100 C followed by a cooling step for 15 minutes at room temperature.

Between 10-20 fmole of a DNA fragment were incubated for 15 minutes at room temperature together with 2-5 ug protein (e.g. from a crude nuclear extract) in 25 mM HEPES, pH 7,5, 1 mM EDTA, 5 mM DTT, 10 % glycerol, 150 mM sodium chloride and 0,5 mM PMSF (phenyl methyl sulfonyl fluoride, Serva, Heidelberg). The total volume per assay was 20 ul. Nuclear extract was added last. The reaction mixture was subsequently subjected to gel electrophoresis on 11 % polyacrylamide gels (acrylamide/bisacrylamide = 44/0,8) in 0,375 M Tris-glycine, pH 8,8. Gels were electrophoresed at 15-25 mA with 40 mM Tris-glycine, pH 8,5 in the buffer chambers. Samples were applied in 5 % glycerol, 10 mM DTT and 0,05 % bromophenolblue. Following electrophoresis, gels were soaked in 5 % glycerol, dried and autoradiographed.

Preparation of nuclear extracts from porcine liver.

All steps were performed at 4 C. A fresh liver from the slaughter house (1,3 kg) was broken up in a Waring blender. The cell homogenate was taken up in 3 ml per g of liver of a solution containing 50 mM Tris-HCl, pH 7,5, 340 mM sucrose and 4 mM calcium chloride. Following homogenization in a "magic stick"-homogenizer (ESGE AG, Mettlen, Switzerland) and a centrifugation step for 10 minutes at 600xg, the pellet was resuspended in 50 mM Tris-HCl, pH 7,5, 250 mM sucrose, 3 mM calcium chloride and underlayered with an identical volume of 50 mM Tris-HCl, pH 7,5 340 mM sucrose, 4 mM calcium chloride.

Subsequent centrifugation in a SS34 Sorvall rotor for 1 hour at 20.000 rounds/min yielded a pellet which was washed three times by resuspension in 10 volumes of 50 mM Tris HCl, pH 7,5, 240 mM sucrose, 3 mM calcium chloride and centrifugation for 10 minutes at 1500xg. A total of 5 x 10^{10} nuclei were obtained per liver. Nuclear extracts were prepared by resuspension of the nuclei pellet in 10 volumes of 50 mM Tris-HCl, pH 7,5, 240 mM sucrose, 3 mM calcium chloride, 2 mM dithiothreitol, 200 mM sodium chloride and 0,5 mM PMSF. After incubation for 30 minutes at 4 C, the suspension was centrifuged for 20 minutes at 12.000xg. The sodium chloride concentration in the supernatant was raised to 300 mM. The extract which could be kept frozen at this stage, was either used directly for binding studies or for the purification of nuclear factor I.

Purification of nuclear factor I.

In order to purify nuclear factor I, the nuclear extract (20,2 mg protein/ml) was subjected to DEAE-cellulose chromatography (Whatman DE-52) in 50 mM Tris-HCl, pH 7,5, 1 mM EDTA, 2 mM dithiothreitol, 10 % glycerol, 0,5 mM PMSF, 300 mM sodium chloride. All nuclear factor I activity which was assayed fraction by fraction with the electrophoretic assay described above, was found in the flow-through. In a second purification step, this material was loaded onto a phosphocellulose column in the same buffer describe above and eluted with one column volume of 600 mM sodium chloride in this buffer. The nuclear factor I activity eluted at 450 mM sodium chloride. This material was estimated to be enriched by a factor of approximately 100 from the crude nuclear extract but is still only 0.01 % pure. It was used as such in most of the binding studies described in this paper. The purification protocol is based on the procedure of Nagata et al. (12) with some modifications required due to the different source of starting material.

Preparation of nuclear extracts from yeast.

S. cerevisiae strain IJ103 was grown in YPD-medium containing 5g Bacto-yeast extract, 10g Bacto-Pepton and 50 ml of a 20% solution of glucose in distilled water per 500 ml. One liter cultures were harvested at approriate OD_{600} values (preferably 0,6 to one) and centrifuged for 15 minutes at 4 C and 4000 rounds/min. The pellet was resuspended in 6 ml sorbitol buffer (1 M sorbitol, 50 mM Tris-HCl, pH 7,8 and 10 mM magnesium chloride) containing 30 mM DTT, left for 15 minutes at room temperature and centrifuged again as described above. After resuspension in sorbitol containing 3 mM DTT, zymolase (100.000 unit) was added to the suspension and left for 40-60 minutes at 37 C. After centrifugation at 4 C for 5 minutes and 4000 rounds/min, the cell pellet was

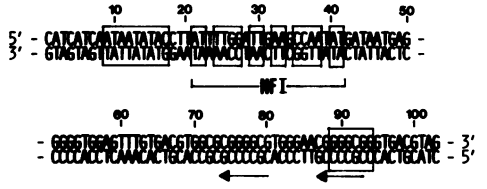

Fig. 1. Structure of the inverted terminal repetition of adenovirus type 5 DNA. A highly conserved region is located between positions 9 and 18 ("9-18 box"). The nuclear factor I binding site consists of a region with dyad-symmetry centered around bp 31 and extending from positions 21 to 41. A "GGGCGG"-box between positions 89 to 94 may represent a binding site for the transcription factor Spl (7). A six base pair long repetition is indicated by two arrows (4). DNA fragments A1/B1 and K1/K2 contain base pairs 17 to 51 or 60 to 94, respectively. They were synthesized with protruding EcoRI and HindIII termini (see Fig. 2).

resuspended in 3 ml of hypotonic buffer (15 mM sodium chloride, 10 mM Hepes, pH 7,8, 5 mM magnesium chloride, 0,1 mM EDTA and 3 mM DTT). The suspension was dounced five times in a tight-fitting Dounce homogenizer, left for 20 minutes at 4 C and dounced again five times. After centrifugation at 4 C and 10.000 rounds/min, the cytoplasmic supernatant was discarded and the nuclear pellet resuspended in 0,5 ml hypotonic buffer. Following addition of ice-cold 5 M sodium chloride to a concentration of 0,4 M, the mixture was centrifuged at 40.000 rounds/min in a SW41Ti rotor. Aliquots of 100 ul of this S100 extract were kept frozen at -80 C. Even at this temperature they loose activity with a halflife of 30 days.

RESULTS

A sensitive and rapid gel retention assay for nuclear factor I.

Nuclear factor I was originally characterized as a protein from HeLa cell nuclei required for *in vitro* replication of adenovirus type 5 DNA (12). Footprint analyses (13, 14) have subsequently established that adenovirus DNA contains a binding site for this protein between positions 17-49 or 19-42 within the 102 bp long inverted terminal repetition (ITR) of the adenovirus genome (Fig. 1). This binding site displays a region of dyad-symmetry centered around basepair 31. Similar sequences have been identified in other viral DNA molecules as well as in human DNA (8). In order to purify nuclear factor I and to identify nuclear factor I-like activities on other systems a rapid and sensitive assay was required which would also work in crude nuclear extracts in the presence of many non-specific DNA-binding activities. The improved gel retention assay which was eventually developed and which works

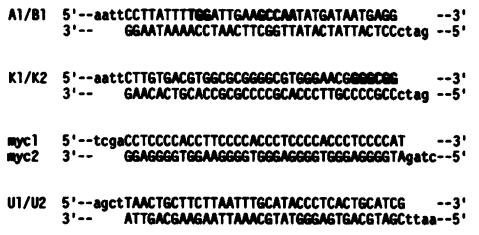

Fig. 2. Structure of oligonucleotides used in the gel retention assay. The pair A1/B1 extends from basepairs 17 tp 51 of the inverted terminal repetition of adenovirus DNA; fragment K1/K2 from basepairs 60 to 94 of the adenovirus ITR (4). Fat letters represent nuclear factor I and transcription factor Sp1 binding sites, respectively. Myc1/2 represent base pairs 2175 to 2214 of the human c-myc gene promoter (6) and fragments U1/U2 a sequence from the recombined mouse kappa light chain gene between position 13 to 50 (15). Lower case letters are protruding termini characteristic for various restriction enzyme recognition sites.

in the absence of any carrier DNA employs short synthetic DNA fragments carrying the expected or known binding sites.

The DNA fragment A1/B1, composed of two complementary synthetic oligonucletides, carries the nuclear factor I binding site between positions 17 to 51 of the adenovirus ITR; a control fragment K1/K2 extends from positions 60 to 94 of this sequence (see Fig. 2). Both fragments carry protruding 5'-EcoRI- and BamHI termini, respectively. The 5' ^{32}P-labelled DNA fragments are mixed with protein extracts (either crude or enriched) containing nuclear factor I, incubated for 15 minutes at room temperature and subjected to polyacrylamide gel electrophoresis. The small size of the oligonucleotide derived DNA fragments permits electrophoresis even in high percentage polyacrylamide gels (up to 15%). Under our conditions (see Materials and Methods) the unbound DNA fragments move with the front while the protein-bound fragments are retained and produce a characteristic band (Fig. 3, lane 1, arrow 2) upon autoradiography. This band is missing in lane 2 with the control DNA fragment which lacks the appropriate binding site. The origin of a band specific for the control fragment K1/K2 (Fig. 3, lane 2 arrow 1) will be discussed below. The bands in Fig. 3 disappear when the assay mixtures are supplemented with increasing concentrations of the identical but unlabelled DNA fragment (see below). Addition of control DNA-fragments to the assay mixtures does not interfere with the banding pattern indicating that, indeed, these bands arise through specific interactions of distinct proteins (see below; Figs 4 and 5).

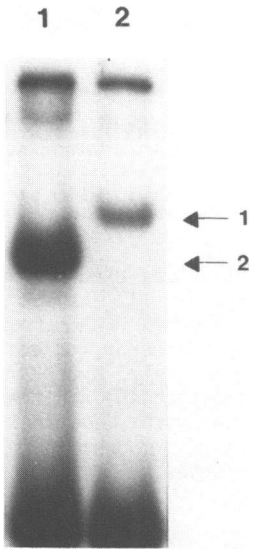

Fig. 3. Assay for nuclear factor I. A crude nuclear extract from porcine liver was incubated for 15 minutes at room temperature with ^{32}P-labelled DNA fragments A1/B1 (lane 1) or K1/K2 (lane 2) and subjected to gel electrophoresis on a native, 11% polyacralamide gel. Radioactive bands were identified by autoradiography. Fragment specific bands are indicated by arrows and labelled 1 or 2, respectively for the control fragment K1/K2 or the nuclear factor I binding site containing fragment A1/B1. The assay was performed in a volume of 20 ul with 10 fmole of the respective fragments (4000 cpm per fmole) and with 10 ul of a crude nuclear extract from porcine liver.

Sensitivity and specificity of the gel assay.

The sensitivity of the gel assay for nuclear factor I as well as the amount of nuclear factor I in protein extracts were determined from competition experiments (Fig. 4). A constant amount of the ^{32}P-labelled fragment A1/B1 was mixed with increasing amounts of the unlabelled fragment (10, 100, 500 and 1000-fold the amount of the labelled fragment) and incubated with nuclear factor I containing crude nuclear extract from porcine liver under conditions of protein excess. Following electrophoresis, the nuclear factor I containing bands could be identified through autoradiography. From the radioactivity present in each band and the known specific radioactivity of the ^{32}P-labelled fragment it was possible to calculate the amount of bound oligonucleotide in each sample. A reciprocal representation of bound fragment versus the total fragment concentration permits an extrapolation to saturation conditions (see legend to Fig. 4). Under the assumption that 2 moles of protein are bound to

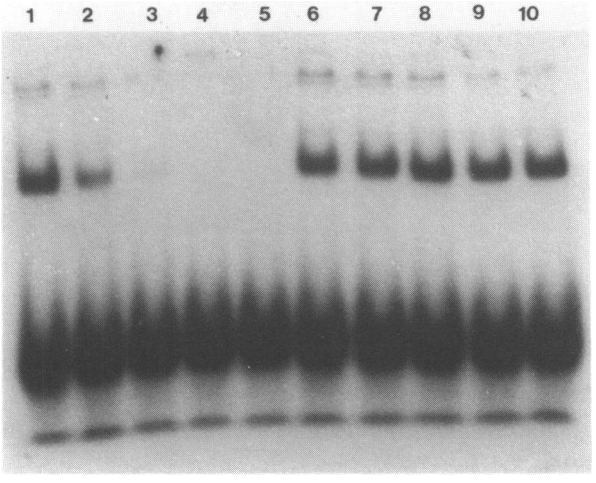

Fig. 4. A competition gel retention assay for nuclear factor I. Each assay was performed with 10 ul of a crude nuclear extract from porcine liver in the presence of 6 fmole of the ^{32}P-labelled DNA fragment A1/B1 with a specific activity of 4000 cpm per fmole. Lanes 1 and 6 did not contain any additional DNA while lanes 2 to 5 represent additions of 60, 600, 3000 and 6000 fmole, respectively, of the unlabelled fragment A1/B1 and lanes 7 to 10 the equivalent amounts of the unlabelled control fragment K1/K2. The amounts of bound (retained) fragment A1/B1 in lanes 1 to 5 were 0,8, 3,9, 19, 34 and 38 fmole, respectively.

every mole of DNA fragment at saturation (see Discussion), we could then calculate that 10 ul of a crude nuclear extract from porcine liver contain approximately 50 fmole of nuclear factor I. This would amount to 2 - 3 nmole of protein (100-150 ug) per liver.

The lower limits of sensitivity of the gel assay can be estimated from these assays as well. In the assay of Fig. 4, lane 1, 0.8 fmole (out of a total of 6 fmole) oligonucleotide were retained in the presence of approximately 50 fmole of nuclear factor I. At a 1:1 molar ratio of nuclear factor I to DNA (see Discussion) this would correspond to approximately 0,8 fmole or 50 pg of protein. With a similar or slightly increased amount of DNA (at a specific activity of 4000 cpm per fmole) and thus at conditions which are closer to saturation it is easy to detect approximately o.1 fmole (5 pg) of protein. Fig. 6, lane 8, for example, displays an assay which identifies 0.06 fmole of nuclear factor I (in an assay containing a total of 5 fmole of this protein). The assay is thus at least two orders of magnitude more sensitive than a typical DNAseI-footprint assay (7, 8) not considering the comparable ease with which it is performed.

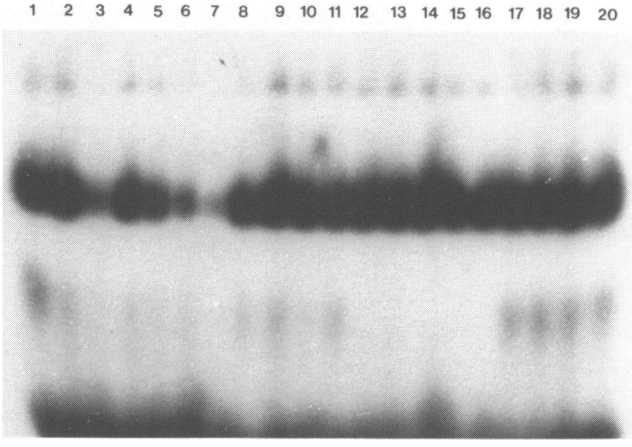

Fig. 5. An extended competition assay for nuclear factor I with various control fragments. The assays were performed under conditions similar to those described in the legend to Fig. 6. Lanes 1 and 11 represent controls containing only the 6 fmoles of the ^{32}P-labelled fragment A1/B1. Lanes 2 to 7 show increasing concentrations (100 fmole, 4 pmole, 500 fmole, 1 pmole, 2 and 8 pmole) of the homologous, unlabelled fragment A1/B1 displaying the expected competition. Lanes 8, 9 and 10 represent additions of 1, 4 and 8 pmoles of fragment U1/U2 (c.f. Fig. 2), lanes 12 to 16 additions of 500 fmoles, 1, 2, 4 and 8 pmoles of fragment mycl/myc2 (c.f. Fig. 2) and lanes 17 to 20 additions of 1, 2, 4 and 8 pmoles of fragment K1/K2. No competition could be observed for these fragments both by autoradiography and scintillation counting (the latter is not shown).

In order to determine the specificity of the assay, competition experiments were performed in the presence of constant amounts of the ^{32}P-labelled fragment A1/B1 and increasing concentrations of DNA fragments with different sequences but of identical size.

The fragments used in the experiment of Fig. 5 are derived from a more internal region of the adenovirus ITR (fragment K1/K2 of Fig. 2), from the human c-myc gene promoter and from the mouse immunoglobuline kappa light chain gene (c.f. Fig. 2). These fragments were employed in excesses of up to 800-fold. No decrease in the binding of the specific, homologous fragment A1/B1 could be detected under these conditions (see also Fig. 4, lanes 6 to 10)., indicating that the assay, indeed, is specific for a DNA fragment carrying the nuclear factor I binding site.

A mutational analysis of the nuclear factor I binding site.

We have exploited the above-mentioned assay to map the nuclear factor I binding site in Ad5 DNA by the introduction of specific mutations. A total of seven pairs of oligonucleotides were synthesized comprising the region

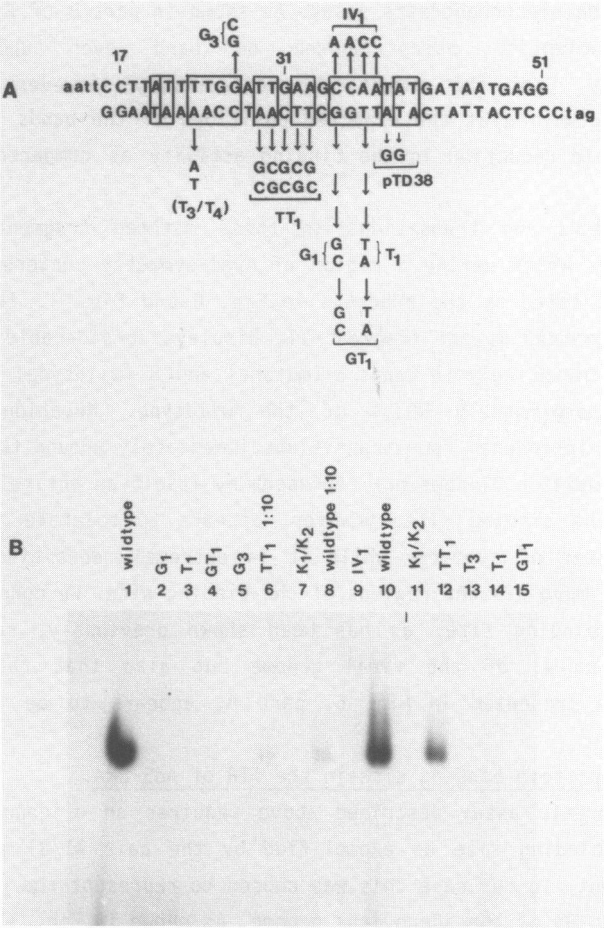

Fig. 6. Electrophoretic analysis of mutations in the nuclear factor I binding site. Mutant fragments were synthesized as oligonucleotide pairs, 5' ^{32}P-labelled and incubated with crude nuclear extracts from porcine liver. The assay conditions are described in Materials and Methods. The label "wildtype" refers to a DNA fragment containing the original Ad5 derived sequence which is outlined in part A of the Figure together with the various mutant fragments. Wildtype and mutant fragments were subjected to electrophoresis on an 11% polyacrylamide gel under native conditions. K1/K2 refers to incubations with the control fragment K1/K2 (c.f. Fig. 1 or Fig. 2); the label 1:10 in lanes 6 and 8 indicates 1:10 dilutions of the nuclear extract. Mutations were always introduced into both strands.

between positions 17 to 51 of the Ad5 genome and containing the transitions and transversions indicated in Fig. 6A. Appropriate pairs were 5'-endlabelled, incubated with a purified nuclear factor I preparation and

subjected to the electrophoresis assay. As shown in part B of Fig. 6, six of these oligonucleotide pairs showed no band even upon prolonged autoradiography. From the amount of radioactivity, however, observed by scintillation counting at the expected positions of the bands we estimate a 250- to 400-fold reduction in the binding activity as compared to fragment A1/B1.

The transversions and transitions in these mutated fragments invariably reside in areas which define a region of dyad-symmetry centered around base pair 31 as indicated by the brackets in Fig. 6 and Fig. 1. In contrast to these six fragments, mutant fragment TT_1 displays considerable activity. As demonstrated convincingly in lanes 6 (mutant) and 8 (wildtype) this activity amounts to approximately 50 % of the wildtype. Mutation TT_1, which simultaneously introduces four transitions immediately around the central G/C base pair at position 31 does not represent by itself an entirely symmetrical arrangement. The binding site, however, appears to tolerate these changes around the center of symmetry while it is extremely sensitive towards any changes in the more distant regions of the binding site. We conclude not only that the NFI binding site, as has been shown previously, resides around positions 17 to 41 of the viral genome but also that the symmetrical arrangement as presented in Fig. 6, part A, appears to be of particular significance.

An additional protein binding site in the ITR of Ad5 DNA.

The electrophoretic assay described above requires an oligonucleotide pair containing a binding site as exemplified by the pair A1/B1 as well as a control fragment. In our case this was chosen to represent the region between positions 60 to 94 of the adenovirus genome. As shown in Fig. 3, lane 2, this DNA fragment creates a different band with a slower mobility as compared to the NFI binding site containing fragment when exposed to a purified nuclear factor I preparation. This band is considerably weaker than the NFI specific band, indicating that this protein is either present in comparatively lower concentrations or that it displays only a weak binding interaction with this DNA fragment. The nature of this protein which elutes at a lower sodium chloride concentration from phosphocellulose as compared to NFI (350 versus 450 mM) has not been elucidated unequivocally as yet. However, a sequence comparison of the interior, GC-rich portion of the ITR as represented in the control fragment indicates a characteristic sequence motif, "GGGCGG"", between positions 89 to 94 (Fig. 1; Fütterer and Winnacker (4)). This sequence occurs repeatedly within the early promoter region of SV40 DNA and

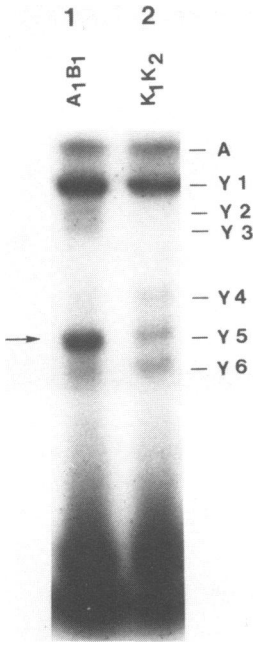

Fig. 7. Gel retention assay with nuclear extracts from S. cerevisiae. 20 ul of a crude nuclear extract from S. cerevisiae were incubated with 20 fmole each of DNA fragments A1/B1 (lane 1) or K1/K2 (lane 2), respectively and subjected to gel electrophoresis on an 11% polyacrylamide gel. The autoradiogram was exposed for 13 hours and shows an intensive band at position Y5 specific for fragment A1/B1 and a weaker band at position Y4 which appears to be specific for fragment K1/K2. The label A indicates the start position.

has been shown to represent a binding site for transcription factor Sp1 (7). Although it is thus tempting to conclude that the protein binding to the DNA fragment K1/K2 represents transcription factor Sp1, a final answer as to the identity of this protein, however, will only be obtained from footprint analyses and from the purification of this factor.

A nuclear factor I-like activity in S. cerevisiae

Although the function of nuclear factor I in human or mammalian cells in general is not known, the possibility was considered that this function might be of considerable importance and may thus have been conserved in evolution. Nuclear extracts from actively growing S. cerevisiae were thus subjected to the gel retention assay described above for nuclear factor I using the DNA fragment A1/B1 carrying the nuclear factor I binding site derived from adenovirus DNA (Fig. 2). As shown in Fig. 7, one band out of a total of six

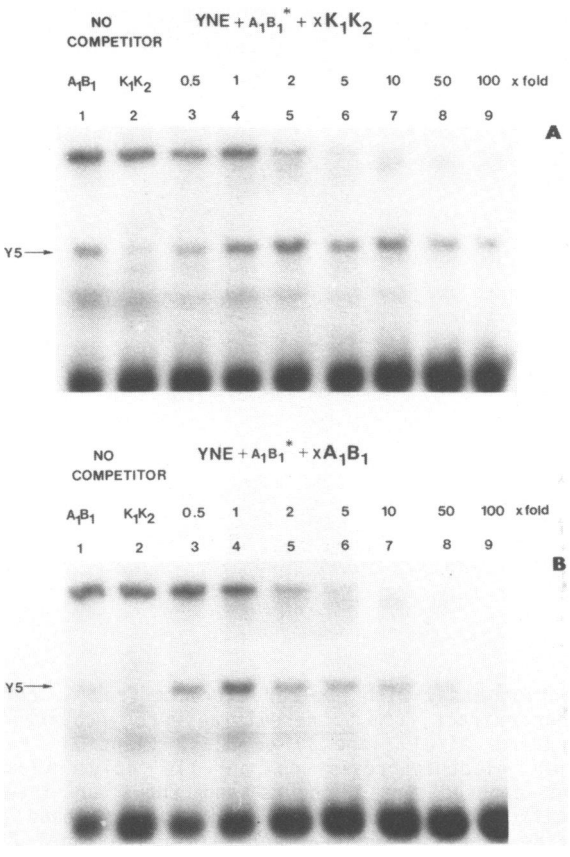

Fig. 8. Competition assay for nuclear factor I like activities in S. cerevisiae nuclear extracts. 10 ul of a crude nuclear extract (YNE) from yeast were incubated with 10 fmole of the homologous fragment A1/B1 and competed with increasing amounts (lanes 3 to 9; 0,5 to 100-fold) of either the unlabelled control fragment K1/K2 (part A) or the unlabelled homologous fragment A1/B1 (part B). Y5 indicates the A1/B1 specific band (Fig. 7) which can be competed much more efficiently with the homologous fragment A1/B1 than with the control fragment.

bands could be identified to display a certain specificity for the fragment carrying the nuclear factor I binding site. As shown in Fig. 8, this band could be competed specifically with the unlabelled, homologous fragment while it was resistant to competition with the control fragment K1/K2 (Fig. 2). From calculations similar to the ones described above we conclude that the amount of nuclear factor I like activity in these extracts is at least 100 times less than that of nuclear factor I in porcine liver extracts. Such a

calculation does not take into account the fact that nuclear factor I-like activity in yeast nuclear extracts was measured with a binding site specific for a mammalian factor. In a homologous system using appropriate yeast DNA sequences, much higher activities may be encountered. The identification of such yeast DNA sequences as well as the purification of nuclear factor I-like activities from yeast are in progress.

DISCUSSION

The gel retention assay described in this paper exploits previous observations (2, 3, 5, 16) according to which a DNA/protein complex can stably enter a polyacrylamide gel matrix upon gel electrophoresis under non-denaturing conditions. The approach by Fried and Crothers requires low percentage gels (3-4%) since they use comparatively long DNA fragments (200 bp). Our conditions with 40 bp long oligonucleotides permit a wide range of polyacrylamide concentrations to be employed. In addition, we recommend buffers with comparatively high-ionic strength in order to reduce unspecific binding and to permit analyses of crude extracts in the absence of unspecific, competing DNA, e.g. E. coli DNA. Our assay thus may appear somewhat similar in its concept but is in fact very different in its actual design and performance as compared to previously known procedures. Salt concentrations, pH-dependance and polyacrylamide crosslinking have been optimized as described in Materials and Methods and should be strictly adhered to.

The sensitivity of the assay is, of course, dependant on the binding constants of complex formation between the protein in question and the oligonucleotide carrying the binding site. In the case of nuclear factor I, a lower limit of sensitivity of between 0,1 to 1 fmole could easily be achieved surpassing the footprinting methods by at least two orders of magnitude.

The absolute amount of nuclear factor I is very different in extracts from different sources. The best source in our hands, nuclear extracts from porcine liver, contain about 250 ng of nuclear factor I per ml at a protein concentration of 20 mg per ml, indicating that it would require an almost 80.000-fold enrichment to purify nuclear factor I to homogeneity. In HeLa cells, the concentration of nuclear factor I is only a fifth of that in porcine liver.

One pertinent questions refers to the number of molecules of nuclear factor I which bind to each binding site. This question can easily be adressed to by

this assay since addition of a second molecule of nuclear factor I would be expected to lead to the formation of a new complex with a higher molecular weight and with greatly reduced mobility. All experiments depicted in this paper were performed far below saturation (see legend to Fig. 4), i.e. at protein/DNA concentrations which only lead to formation of a 1:1 complex. However, upon reduction of the DNA concentrations it is possible to demonstrate that indeed a 2:1 complex can be formed (not shown). The exact concentrations and conditions required for this transition are currently under investigation.

The present assay permitted a mutational analysis of the nuclear factor I binding site. According to our results the binding site displays dyad-symmetry centered around base pair 31 in the Ad5 genome. It extends approximately 10 bp to either site of this central G/C pair and does not tolerate any transitions or transversions in its flanking regions. Using filter-binding assays, Leegwater et al. (11) conclude that DNA fragments containing the first 40 bp are bound normally while the first 38 bp are insufficient to sustain binding. These results confirm our observations and permit us to conclude that the terminal 40 bp of adenovirus type 5 DNA carry the minimal recognition site for nuclear factor I. The significance of a hairpin structure formed by nucleotides 17-51 as a prerequisite for nuclear factor I binding thus appears questionable (9).

In the course of the purification of nuclear factor I, as assayed by the electrophoretic assay, we observed the binding of another protein of a different and reduced mobility to a control fragment covering adenovirus type 5 positions 60 to 94. A sequence comparison with the SV40 early promoter region leads us to suspect that this region contains a binding site for transcription factor Sp1 and that the observed protein may, in fact, be factor Sp1. This is under investigation. In the present context, the identification of a protein binding to the G/C-rich portion of the Ad5 DNA ITR served only the purpose to demonstrate that there may, in fact, be three functional domains in the ITR and that the assay described may be of general significance for the identification of DNA binding proteins.

We thus felt encouraged to assay crude nuclear extracts from S. cerevisiae for nuclear factor I-like activities. Using the DNA fragment A1/B1 carrying the nuclear factor I binding site from adenovirus type 5 DNA and the control fragment K1/K2 we could identify five bands which are retained on 11% polyacrylamide gels. Band Y5 appeard specific for fragment A1/B1 and could indeed be efficiently competed with the unlabelled, homologous fragment. The

competition assays appear less impressive than those within a homologous system, i.e. nuclear factor I from porcine liver and fragment A1/B1, but it should be kept in mind that the experiments were performed with a fragment which may not contain an optimal binding site for a yeast nuclear factor I-like activity. Using the various genetic options offered in the yeast system we now hope to successfully approach the problem of a biological and biochemical function for nuclear factor I.

The assay has in the meantime been used to demonstrate centromere binding proteins from S. cerevisiae (P. Philipson et al., submitted) and to identify enhancer binding proteins specific for the SV40 enhancer and the enhancer of the mouse kappa light chain immunoglobuline gene (our laboratory, unpublished).

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