Stimulation of transcription \textit{in vitro} by binding sites for nuclear factor I

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ABSTRACT

Nuclear factor I (NFI) is a site-specific DNA binding protein required for the replication of adenovirus DNA \textit{in vitro} and \textit{in vivo}. We have examined the effect of natural and synthetic binding sites for NFI (FIB sites) on RNA synthesis in HeLa whole cell extracts. The natural binding site used is the 26bp FIB-2 site previously isolated from the human genome. When present upstream of the TATA box of the adenovirus major late promoter, the FIB-2 site stimulates RNA synthesis 3 to 5-fold. This stimulation occurs with either orientation of the FIB-2 site. A point mutation in FIB-2 that decreases NFI binding at least 100-fold reduces, but does not completely abolish, the stimulation of transcription. A number of synthetic binding sites for NFI were tested for the ability to increase RNA synthesis. The strongest binding sites stimulated transcription the most, while the weakest sites had the least effect. These studies strongly suggest a role for NFI and cellular FIB sites in the control of RNA synthesis.

INTRODUCTION

The expression of cellular genes is regulated by a large number of developmental and environmental signals. This complex scheme of regulation is mediated in part by site-specific DNA binding proteins that interact with promoter regions and other elements of transcription units (1-6). We have been examining the role of one such protein, nuclear factor I (NFI) in DNA and RNA synthesis.

NFI was first described as a protein isolated from the nuclei of HeLa cells that is required for the efficient initiation of adenovirus (Ad) DNA synthesis \textit{in vitro} (7). NFI binds to a specific site within the origin of replication of adenovirus type 5 DNA (8, 9), and this binding is apparently required for the stimulation of DNA synthesis \textit{in vitro} and \textit{in vivo} (10-15).

The mechanism by which NFI stimulates the initiation of Ad DNA synthesis is unknown, however it has been suggested that NFI may promote the assembly of a multi-enzyme complex at the origin of replication (16). This putative complex, comprised of NFI, other cellular factors (12, 17) and the 3 viral proteins required for replication, would then efficiently replicate viral...
DNA. Binding sites with a wide range of affinities for NFI have been isolated from a number of cellular (18-21) and viral genomes (22-24). By the direct selection of binding sites from HeLa cell DNA, the number of strong FIB sites in human cells has been estimated at ~60,000 (18). Analysis of several viral and cellular FIB sites indicates that the consensus sequence TGGN$_6$-GCCAA is important for the strong binding of NFI to DNA (25-26). Either mutations in the conserved residues (13, 27-29), or alterations in the length and composition of the degenerate N$_6$-7 spacer region (28, 30), can decrease the binding of NFI to a site.

One line of evidence supporting a role for NFI in RNA synthesis is the presence of FIB sites within a number of well defined transcription units. A strong FIB site is present upstream of the hepatitis B virus S antigen gene, and deletion of this site reduces transcription from the gene in vivo 5 to 10-fold (31). Binding sites have also been identified upstream of a number of other cellular (19-21) and viral genes (22-24), but the functional role for most of these sites has not been demonstrated. In addition, recent studies have indicated that NFI is similar or identical to the "CAAT"-box Transcription Factor (CTF), a protein implicated in the expression of a wide variety of genes (32). However, at least three different protein factors appear to interact in vitro with CAAT box-like motifs (32-35) and further characterization of these factors will be needed to assess their roles in RNA synthesis.

We have been examining the role of NFI and FIB sites in DNA and RNA metabolism. To determine whether isolated FIB sites can function in RNA synthesis, we have cloned both natural and synthetic FIB sites upstream of the well characterized Ad major late promoter (MLP). In this report we demonstrate the ability of these sites to stimulate transcription in vitro.

MATERIALS AND METHODS

Cell Lines and Extraction Methods

HeLa S3 cells were obtained from the ATCC and were cultured in suspension in Joklik's medium containing 10% bovine serum. Cells were grown to a density of 0.5-1.0x10$^6$/ml at 37°C, harvested by centrifugation at 800X g for 20 min, washed once in 1/10 volume of PBS and resuspended at 10$^8$ cells/ml in MS30 buffer (50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 10 mM thioglycerol, 0.1 mM DTT, 30% glycerol). Cells were used either immediately for preparation of extracts or were frozen at -20°C for 2 hr and then stored at -70°C until use. Whole cell transcription extracts were prepared as described (36).
Vectors

The plasmid AdSV contains sequences of the adenovirus major late promoter (MLP) from the region -54 to +33 cloned between the Smal and BamHI sites of pUC12 (Fig. 1). The Smal site in the MLP fragment of AdSV was created by mutagenesis of the A residue at position -54 in the wild type MLP to a C residue (see Fig. 2 of reference 37). The promoter fragment present in Ad97 is described in detail elsewhere (37) and this vector is used primarily as a positive control for high transcriptional activity. All of the vectors used are described in greater detail in the legend of Fig. 1. For use in transcription reactions, the vectors were made linear by digestion with NdeI which cleaves at +278 relative to the transcription start site. The concentration of the DNA templates was determined by absorption at 260 nm and the extent of digestion was measured by electrophoresis on 0.8% agarose gels.

Transcription Reactions

In vitro transcription reactions were performed as described (37) using a 15 min preincubation of template DNA with cell extract and a 30-60 min incorporation time. All incubations were at 30°C. Several different preparations of whole cell extract were used in these studies and consistent results were obtained. Products of the transcription reactions were analysed on denaturing 5% acrylamide gels and detected by autoradiography of the dried gels.

Nitrocellulose Filter Binding Assay

Competition binding assays were performed as described previously (18, 30) in 50 µl reactions containing 50 mM NaHepes, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 4 mM DTT, 5 fmol of ³²P-labelled FIB-2 oligonucleotide (~ 1500 cpm/fmol), 5 units of NFI, and the amounts of competitor DNA indicated in the legend of Fig. 4. NFI was isolated as described previously (7) and material purified through the denatured DNA cellulose step was used throughout the study. One unit of NFI is defined as the amount of protein required to bind one fmol of labeled DNA in the filter binding assay. The FIB-2 oligonucleotide is a 26bp site used previously to measure the binding of NFI to DNA (28, 30). It was labeled as described (30) and the sequence is shown in Fig. 1.

RESULTS

Binding sites for NFI have been identified within several cellular and viral transcription units (19-23). To determine if the presence of FIB sites could stimulate RNA synthesis in a simple chimeric promoter system, we cloned a number of natural and synthetic sites upstream of a mutant adenovirus major
Fig. 1. Structure of Transcription Vectors.

Shown above are the vectors used as substrates for in vitro transcription. Ad97 contains the sequences from -677 to -363 and -97 to +33 of the adenovirus major late promoter (MLP) cloned between the EcoRI and BamHI sites of pUC12. AdSV contains the sequence from -54 to +33 of the MLP cloned between the Smal and BamHI sites of pUC12. The A at position -54 in the MLP has been converted into a C in this construction. Shown in expansion is the DNA sequence of AdSV from the EcoRI site at -67 to the mRNA start site at +1. The TATA box at residues -31 to -25 is underlined. Vectors AdFA and AdFB contain a 26bp oligonucleotide homologous to the human FIB-2 site (25) cloned in opposite orientations into the Smal site of AdSV. AdFMA contains a 26bp oligonucleotide with a point mutation in the FIB-2 site cloned into the Smal of AdSV in the same orientation as in AdFA. The bracket and arrow connecting the sequences indicate the insertion of these oligonucleotides between the central C and G residues of the Smal site. Dashes in the sequences indicate residues that are identical to those in AdSV. The AdFN series of vectors (N = 21,17,8,9,23,24) was constructed such that the sequences denoted by the bracket replace the CCC of the Smal site of AdSV. The dashes in the sequences indicate residues that are identical to the sequence directly above. The TGG and GCCAA motifs (and their complements) of each insert are underlined. For use in transcription reactions, each vector was made linear by digestion with NdeI which cleaves once at position +278 as indicated in the schematic diagram of Ad97 DNA.

late promoter (MLP). The MLP mutant used, AdSV, contains only residues -54 to +33 of the promoter which includes the "TATA" box (residues -31 to -25) and the mRNA start site (Fig. 1). This construction lacks the natural upstream element found in the wild type MLP that is required for maximal activity of the promoter in vivo and in vitro (37-41). This upstream element
Fig. 2. Stimulation of Transcription by FIB-2 Sequences.

50 or 100 ng of the DNA's indicated were transcribed in vitro as outlined in Materials and Methods. The transcription products were analysed on a denaturing 5% acrylamide/50% urea gel, the gel was dried, and the labelled RNA was detected by autoradiography. Each lane indicates RNA made under the following conditions: lane 1, in the absence of added DNA; lanes 2 and 3, in the presence of 50 and 100 ng, respectively of Ad97 DNA; lane 4, in the presence of 100 ng Ad97 DNA and 0.5 μg/ml α-amanitin; lanes 5 and 6, in the presence of 50 and 100 ng, respectively, of AdSV DNA; lanes 7 and 8, in the presence of 50 and 100 ng, respectively, of AdFA DNA; lanes 9 and 10, in the presence of 50 and 100 ng, respectively, of AdFB DNA. The arrow on the left shows the position of the 278 nucleotide specific transcription product.

is present in the vector Ad97 which was used as a positive control for high in vitro transcriptional efficiency.

Transcription of these plasmids was assessed by measuring the amount of run-off transcript produced from DNA's linearized at the NdeI site at position +278. In vitro transcription of the Ad97 plasmid shows the clear presence of this 278 nucleotide product (Fig. 2, lanes 2 and 3). No transcript is seen in the absence of added DNA or in the presence of 0.5 μg/ml α-amanitin, a specific inhibitor of RNA polymerase II (Fig. 2, lanes 1 and 4, respectively). As expected, the amount of transcript produced from the AdSV vector, which lacks the MLP upstream element, was 3 to 5-fold lower than the amount seen with the Ad97 vector (Fig. 2, lanes 5 and 6 vs lanes 2 and 3). When a 26bp oligonucleotide containing the previously characterized human FIB-2 site was cloned upstream of the AdSV TATA box, the transcription
Fig. 3. Effect of the AdFMA Mutation on Transcription.

Transcription reactions were analysed as described in the legend to Fig. 1. Each lane represents RNA made under the following conditions: lanes 1 and 2, in the presence of 50 and 100 ng, respectively, of AdSV DNA; lanes 3 and 4, in the presence of 50 and 100 ng, respectively, of AdFA DNA; lanes 5 and 6, in the presence of 50 and 100 ng of AdFMA DNA. The arrow on the left shows the position of the 278 nucleotide specific transcription product.

efficiency of the promoter increased several fold (Fig. 2, lanes 7-10 vs lanes 5 and 6). This stimulation was independent of the orientation of the site (Fig. 2, AdFA vs AdFB), and was seen at both low (50 ng) and high (100 ng) concentrations of DNA.

To assess the role of NFI binding in this stimulation, a similar construction was made using a FIB-2 oligonucleotide containing a point mutation in the TGG motif of the site (Fig. 1, AdFMA). This mutation was shown previously to decrease the binding of NFI to the site by ~100-fold (30). The presence of this point mutation clearly decreased synthesis from the promoter, but did not reduce transcription back to the level seen with AdSV (Fig. 3, lanes 5 and 6 vs 1 and 2). This failure of the mutation to totally prevent the stimulation can be most easily explained by one of two models; 1) only very weak binding of NFI is required for the stimulation or,
Fig. 4. Quantitation of the Relative Affinities of FIB Sites by a Competition Binding Assay.

Each DNA indicated above and in Fig. 1 was tested for its ability to compete with radiolabelled FIB-2 oligonucleotide for binding to NFI. Binding assays were performed as described in Materials and Methods in reaction mixtures containing 5 fmol of $^{32}$P-labelled FIB-2 oligonucleotide, 5 units of NFI and either 25 or 75 ng of competitor DNA. The reactions were filtered and the amount of NFI-$^{32}$P-oligonucleotide complex retained on the filters was quantitated by liquid scintillation counting. The data are presented as a histogram where the first and second bars of each group show the percent of labelled DNA retained in the presence of 25 and 75 ng, respectively, of competitor DNA. The brackets above the figure indicate the grouping of AdFN21 and AdFN17 as strong competitors, AdFN8 and AdFN9 as weak competitors and AdFN23 and AdFN24 as non-competitors. The data are plotted relative to the amount of DNA retained in the absence of any competitor DNA (100%).

2) other stimulatory proteins are interacting with the 26bp site and these interactions are not affected by the point mutation. To help distinguish between these models, we examined the ability of several synthetic NFI binding sites to stimulate transcription from AdSV.

The AdFN series of plasmids ($N = 21, 17, 8, 9, 23$ and $24$) contain different synthetic FIB sites cloned upstream of the TATA box of AdSV (Fig. 1). The FIB sites were isolated from a library of partially degenerate molecules each of which contained the TGG and GCCAA motifs required for NFI binding (28, 30). To quantitate the relative affinities of these synthetic binding sites, the plasmids and their parent vectors were tested for the ability to compete with a $^{32}$P-labelled 26bp FIB-2 fragment for binding to NFI using a nitrocellulose filter binding assay (Fig. 4). As expected, neither AdSV nor Ad97 competed for binding to NFI while AdFA competed strongly. The point
Fig. 5. Transcription of FIB-Site Containing DNA's.

100 ng of each DNA shown was transcribed in vitro and the RNA products were analysed as described in the legend of Fig. 2. Lane 1, AdSV; lane 2, AdFA; lane 3, AdFMA; lane 4, AdFN21; lane 5, AdFN17; lane 6, AdFN8; lane 7, AdFN9; lane 8, AdFN23; lane 9, AdFN24. The specific RNA transcripts from each DNA are resolved into two closely spaced bands, presumably due to premature termination ~10bp from the end of the DNA template. This apparent termination effect was seen in several independent experiments and does not influence the assessment of transcriptional activity. Similar relative levels of transcription of the template DNA's were seen in three separate experiments.

mutant of AdFA, AdFMA, exhibited no ability to compete for binding, suggesting that the binding site was inactivated. These data agree completely with our previous direct-binding studies which showed essentially complete inactivation of the binding site by the point mutation (TGG→TCG) present in AdFMA (30). The AdFN constructs were grouped into three categories; 1) strong-binders/competitors such as AdFN21 and AdFN17, 2) weak-binders/competitors including AdFN8 and AdFN9, and 3) apparent non-binders/competitors such as AdFN23 and AdFN24. One of the non-binding plasmids, AdFN23, contains a point mutation in the TGG motif (TGG→TGC) that may account for its lack of binding. A TGG→TGC mutation at the same position of the FIB site present in the adenovirus type 5 origin of replication decreases the binding of NFI 250 to 400-fold (29).

These plasmids were then tested for transcriptional activity in HeLa whole cell extracts (Fig. 5). As seen previously, AdSV had weak activity, AdFA had ~3-fold higher activity, while AdFMA had intermediate activity (Fig. 5, lanes 1-3, respectively). Among the AdFN vectors, a strong direct
correlation of transcriptional activity with binding affinity was seen. The non-binders (AdFN23 and AdFN24) had the least transcriptional activity, while the strong-binders (AdFN21 and AdFN17) had the most activity. One of the weak-binders, AdFN9, had slightly higher transcriptional activity than the other weak-binder, AdFN8 (Fig. 5, lanes 6 and 7). This difference may represent a non-linearity between NFI-binding and the activation of transcription, although the analysis of a number of other sites of differing binding strength will be required to test this effect. Of interest is the fact that the non-binders gave levels of transcription very similar to that seen with the mutated site AdFMA (Fig. 5, lanes 3, 8 and 9). These findings support the observation that a mutated FIB-site which lacks any apparent strong binding to NFI can still produce a weak but measurable stimulation of transcription in this in vitro system (i.e. AdFMA transcripts in Figs. 3 and 5). One trivial explanation for this weak stimulation by DNA fragments that do not interact strongly with NFI is that such insertions inactivate or remove sequences that inhibit transcription in vitro. We feel that this is unlikely, since we have inserted several different control DNA fragments into the AdSV plasmid which do not affect transcription from this vector (N.G.M., unpublished observations).

DISCUSSION

These studies demonstrate that binding sites for NFI can increase transcription in vitro in a chimeric promoter system. The correlation between NFI binding and stimulatory activity strongly suggests that NFI is the protein responsible for this effect. The centers of the FIB sites analysed here were located 62-65bp from the mRNA start site (Fig. 1). Thus, in these chimeric constructs the FIB sites are present in the same position, relative to the TATA box, as the natural upstream element (UE) required for maximal expression of the wild type MLP in vitro and in vivo (37-41). This apparent replacement of the UE function by the FIB sites confirms that the MLP is composed of distinct modules which can be changed independently of one another to analyse the overall function of the promoter. Very similar conclusions were obtained in previous studies that examined both in vitro and in vivo the separate functions of the upstream elements and TATA boxes of the Ad MLP (37), the SV40 early transcription unit (37), the Herpes virus TK gene (42), and the human β-globin gene (42).

These findings are of particular interest with regard to the location and orientation of FIB sites or FIB-site like sequences present in well
characterized eukaryotic promoters. Although the FIB sites studied here were placed quite near the mRNA start site, FIB sites have also been identified much further upstream of the initiation sites of several cellular and viral genes. In the hepatitis B virus S antigen gene (HBV Sag), a FIB site is present ~190bp upstream of the mRNA start site (31). Deletion of the FIB site reduces transcription of the gene in vivo 5 to 10-fold. The HBV Sag gene differs significantly from the Ad MLP in that the former contains no canonical TATA box element. It would be of interest to determine if the FIB sites used in our study can still function effectively if the distance from FIB site to the MLP TATA box is increased. Such an analysis would help indicate whether FIB sites function by the same mechanism in promoters which either contain or lack TATA boxes.

The orientation independence of the stimulation of transcription by the FIB site suggests a symmetric interaction of NFI with the site. These results are consistent with previously published structural analyses of NFI binding which indicated that the protein binds symmetrically at a FIB site and protects a single side of the DNA helix (25, 26). In addition, when the same FIB-2 site used in our studies was used to replace the FIB site present in the Ad origin of replication, stimulation of replication occurred with either orientation of the site (16). The orientation independence of the activity of the FIB site is reminiscent of the function of transcriptional enhancers (43, 44), and studies are in progress to determine if FIB sites can act, in general, as enhancer elements for cellular and viral genes.

FIB sites were initially characterized as containing TGG and GCCAA motifs separated by 6 or 7bp degenerate spacer regions (19, 21, 25, 30). Recent studies have indicated that the canonical CAAT box motif present in many promoters can be a binding site for NFI and thus may represent one class of FIB sites (32). Interestingly, several DNA fragments containing CAAT boxes that function well in vivo and in vitro exhibit only weak binding to purified NFI (32). These data agree with our observation that a mutation in the FIB-2 site which reduces NFI binding at least 100-fold (28) does not completely abolish the observed stimulation of transcription (Fig. 3). Three possible explanations for these findings are: 1) only very weak binding of NFI is needed to stimulate transcription, 2) the binding of NFI to such weak sites can be increased by the interaction of other proteins at the site, and that these additional interactions are also required to stimulate transcription, or 3) several distinct proteins, including NFI, can interact with CAAT box motifs and affect transcription. Further studies with synthetic FIB sites.
may be useful in determining the mechanism of this residual stimulation of transcription seen with sites that interact only very weakly with NFI.

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