The *Xenopus laevis* ribosomal gene promoter contains a binding site for Nuclear Factor-1

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ABSTRACT

Nuclear Factor I (NF1) is a DNA binding protein that is known to function in the replication of Adeno virus and also binds to many promoters recognized by RNA polymerase II. We have found that there is also an NF1 binding site within the ribosomal gene promoter from *Xenopus laevis* as well as in several other promoters recognized by RNA polymerase I. The function of a binding site for a polymerase II transcription factor within a promoter recognized by polymerase I is not known. However, its presence suggests interesting regulatory possibilities.

INTRODUCTION

Nuclear Factor 1 (NF1) was first isolated on the basis of its ability to stimulate replication in vitro at the Adeno virus origin of replication and was subsequently purified through the use of specific oligonucleotide affinity chromatography (Rosenfeld and Kelly, 1986). It has been proposed that this affinity purified NF1 is closely related to a transcription factor for RNA polymerase II that was independently isolated on the basis of its binding to the CCAAT box homology as well as its ability to stimulate transcription in reconstituted in vitro systems (Jones *et al.*, 1987). A more recent report (Chodosh *et al.*, 1988) indicates that there exists a family of proteins that recognize variants of the CCAAT motif and it will probably require cloning and sequencing of the relevant genes and cDNAs before their precise relationships can be sorted out. Binding sites for the NF1 family have been reported in many transcriptional control regions including the MMTV LTR (Nowock *et al.*, 1987), the human alpha globin gene promoter (Jones *et al.*, 1987), a collagen gene promoter (Rossi *et al.*, 1988) and the mouse albumin gene promoter (Lichtsteiner *et al.*, 1987). Although in many cases the function of NF1 binding has not been determined, it would appear that the precise role of NF1 can change from promoter to promoter depending upon the exact context in which it finds itself.

We report here that, in addition to its ability to function as a factor

both for replication and for RNA polymerase II transcription, NF1 can also bind to a promoter for RNA polymerase I.

MATERIALS AND METHODS

Preparation of nuclear extracts

Nuclear extracts from *X. laevis* livers were prepared as previously described (Reeder *et al.*, 1987). Nuclear extracts from an *X. laevis* kidney cell line (Xlk) were prepared as follows: Cells were grown in roller bottles in 50% L-15 medium (Flow labs), 10% fetal calf serum, 2 mM glutamine, 10 mM HEPES pH 7.6 until they reached about 80% confluency. All the following manipulations were performed at 4°C. Cells were harvested by scraping, washed twice with phosphate buffered saline, resuspended in 10 packed cell volumes of homogenization buffer (0.3M sucrose, 2 mM Mg acetate, 3 mM CaCl₂, 0.1% NP-40, 0.5 mM DTT, 10 mM Tris (HCl) pH 8.0), and homogenized with an all glass Dounce homogenizer. The homogenate was mixed with one volume of centrifugation buffer [1.85 M sucrose, 5 mM Mg acetate, 10 mM Tris (HCl) pH 8.0] layered in 30 ml aliquots over 4 ml cushions of centrifugation buffer, and was spun at 27,000 RPM (Sorvall AH627 rotor) for 45 min. The nuclear pellets were resuspended using a Dounce homogenizer in 1.2 packed cell volumes of extraction buffer (25% glycerol, 0.4M KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 20 mM HEPES pH 7.9) and incubated on ice for 30 min with intermittent swirling. The insoluble material was removed by centrifugation at 12,000 RPM (Sorvall HB-4 rotor) for 15 min. The supernatant was dialyzed for 4 hrs against 50 volumes of dialysis buffer (20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 1mM DTT, 20 mM HEPES pH 7.9). A precipitate that formed during dialysis was removed by spinning for 15 min at 10,000 RPM (Sorvall HB-4 rotor). The extracts were frozen in aliquots and stored at -80°C. All buffers used to prepare extracts were freshly supplemented with the following mixture of protease inhibitors: 0.1 mM PMSF, 1 mM Na metabisulfite, 1 mM benzamidine, 2 ug/ml Aprotinin (Sigma), 2.5 ug/ml leupeptin (Sigma).

Partial purification of the *X. laevis* NF1-like activity

An 11 ml column of DEAE Sepharose CL6B (Pharmacia) was equilibrated with dialysis buffer. 20 ml (100 mg protein) of nuclear extract was passed over the column, the flowthrough (containing the NF1-like activity) was collected and loaded onto a 2 ml Biorex-70 column (Biorad). The Biorex column was step eluted with dialysis buffer containing increasing amounts of KCl. The NF1-like activity was eluted at 0.3M and 0.4 M KCl.

Plasmids and probes

The *X. laevis* ribosomal gene promoter and various linker scanner mutants

of it have been described previously (Reeder et al., 1987). To examine footprinting over the gene promoter (footprints shown in Figures 1, 2, 3, and 5) plasmids were cut with Bam HI (cuts at + 50 relative to transcription initiation), and labeled with kinase on the extended 5' terminus.

To examine footprinting on the Adeno virus origin of replication (Figure 4) we used pEA (gift from J. Lewis), a plasmid which contains nucleotides from 1 to 1193 of Adeno virus type 2 inserted into the Pst I site of pUC 19. A 220 bp probe was prepared by kinase labeling at the Hind III site within the pUC19 polylinker, recutting with Acc I (at Adenovirus position 192) and isolating the relevant fragment. The plasmid pEBH10 (gift from E. Bateman) contains the ribosomal gene promoter from *Acanthamoeba castellanii* (Bateman, et al, 1985) and was used for the footprints in Figure 7. It was digested with Hind III, kinase labeled, recut with Eco RI, and the appropriate 250 bp fragment was isolated for footprinting.

DNase I footprinting

The footprinting method was adapted from the procedure of Galas and Schmitz (1978). About 1 ng (10,000 CPM) of an end-labelled fragment was added to a 50 ul reaction containing 10% glycerol, 50 mM KCl, 0.1 mM EDTA, 10 ug/ml poly(dIdC), 0.5 mM DTT, 10 mM HEPES pH 7.9, 2% (w/v) polyvinyl alcohol and various amounts of protein extract. After 15 min on ice, one volume of 10 mM MgCl₂, 5mM CaCl₂ was added at room temperature. DNase I, freshly diluted in cold water, was added to final concentrations of 4 ng/ml to 40 ug/ml depending on the protein concentration of the extract. After 1 min at room temperature the reaction was stopped with one volume of 1% SDS, 0.2 M NaCl, 250 ug/ml tRNA, 20 mM EDTA pH 8.0. The nucleic acids were then extracted with phenol/chloroform, precipitated with ethanol, dissolved in formamide-dye mix, boiled for 3 mins and loaded on a DNA sequencing gel.

RESULTS

Characterization of an NF1-like activity in *X. laevis* nuclear extracts

We have published a preliminary footprint analysis of the *X. laevis* ribosomal gene promoter using a crude extract made from *Xenopus* liver nuclei (Reeder et al., 1987). This extract produces a number of changes in the DNase I digestion pattern, both protections and enhancements, over the entire promoter region. However, the major protection was seen to extend from about -5 to -30. We have since reproduced these observations with nuclear extracts from a line of cultured *X. laevis* kidney cells and have partially purified the activity responsible for the -5 to -30 footprint. The footprinting activity eluted in the 0.1 M KCl flow-through of a DEAE Sepharose column and was then

Figure 1. Chromatography of an *X. laevis* NF1-like DNA binding activity on Biorex-70. An extract made from nuclei of cultured *X. laevis* kidney cells was applied to DEAE Sepharose as described in the Materials and Methods. The flow-through fraction was then applied to Biorex-70 in 0.1M KCl and the column was step eluted as shown. The fractions from each step were pooled and assayed for binding to the ribosomal gene promoter by DNase I footprinting.

step eluted from Biorex-70 between 0.2M and 0.4 M KCl (as shown in Figure 1). In the experiments that follow we have used either the crude nuclear extract or the Biorex-70 eluate as indicated in each case. We determined the binding site for the factor producing the -5 to -30 footprint through use of a series of linker scanner mutants whose structure and transcriptional activity have been previously reported (Reeder *et al.*, 1987). Figure 2B shows the sequence of the linker scanner mutants we tested and Figure 2A shows the footprints obtained with each. We found that LS -29/-21 destroyed the footprint while mutations on either side had no effect. This implies that the binding site is within a

Figure 2. DNase I footprinting of the *X. laevis* NF1-like activity on various linker scanner mutants of the *X. laevis* ribosomal gene promoter.

A. DNase I digestion patterns are shown for a series of wild type and linker scanner mutations of the ribosomal gene promoter in the absence and in the presence of *X. laevis* liver nuclear extract. On the wild type sequence the NF1-like footprint extends from about -5 to -30. The position of each linker scanner mutation is indicated by a solid vertical bar. Note that only LS -29/-21 destroys the footprint.

B. Sequences of the wild type promoter and various linker scanner mutations of the ribosomal gene promoter in the region of NF1-like footprint. The region covered by the footprint (-5 to -30) is indicated by a black bar under the wild type sequence. The relative transcription activity of each mutant promoter, assayed either by oocyte injection or in an in vitro transcription reaction (Reeder et al., 1987), is shown to the right. At the bottom is shown the sequence of a wild type oligonucleotide that will compete away the NF1-like footprint (see Figure 3) and a mutant oligonucleotide (bearing the LS -29/-21 mutation) that will not compete for the footprint.

Figure 3. The WT oligonucleotide (sequence shown in Figure 2) contains the binding site for the NF1-like activity. The *X. laevis* liver nuclear extract was used to footprint over the -5 to -30 region of the *X. laevis* ribosomal gene promoter. In the left hand series of footprints the footprint was competed with an increasing amount of the wild type oligonucleotide. In the right hand series the same increasing amount of phage lambda was added as a non-specific competitor. Note that only the WT oligonucleotide competes for the footprint and therefore the oligonucleotide must contain the binding site for the NF1-like activity.

13 bp region between -30 and -17. To verify this conclusion we synthesized a double-stranded oligonucleotide which contained this sequence (shown in Figure 2B as the wild type-oligo) and showed that a 100-fold molar excess of this oligonucleotide could effectively compete away the footprint (Figure 3). A similar excess of a non-specific competitor had no effect. At this point we realized that the LS -29/-21 mutant altered a sequence (TTGGCAT) that resembles half of a binding site for human NF1 (Borgmeyer *et al.*, 1984; Jones *et al.*, 1987), a protein that has been shown to interact with the Adeno virus origin of replication as well as with a wide variety of promoters transcribed by RNA polymerase II. This raised the possibility that our footprinting activity was the frog equivalent of NF1. To test this notion we first tried footprinting the *Xenopus* factor on several well-characterized NF1 binding sites. We found that the *Xenopus* factor binds to two known NF1 sites on the Herpes Simplex Virus thymidine kinase gene promoter (data not shown) as well as to the Adeno

Figure 4. The *X. laevis* NF1-like activity binds to the NF1 site on the Adeno virus origin of replication. An end labeled probe containing the Adeno virus origin of replication was footprinted with the 0.4 M KCl eluate from a Biorex-70 column. Footprinting reactions were then competed with increasing amounts of either the polymerized wild type oligonucleotide (left hand series) or Hind III-digested phage lambda DNA (right hand series). The sequence of the wild type oligonucleotide is shown in Figure 2. Only the WT oligonucleotide affects the footprint and only over the known NF1 binding site. Note that the *Xenopus* extract also has activities that bind over the NF3 (octamer) site as well as several other uncharacterized sites.

virus origin of replication (shown in Figure 4). The partially purified *Xenopus* material clearly contains several DNA binding activities. This is shown by the fact that it not only protects the NF1 site but also the adjacent NF3 site as well as some other unidentified sites (Figure 4 A). However, only the footprint over the NF1 site was competed away by the specific wild-type oligonucleotide (Figure 4B). We then tested the ability of affinity-purified human NF1 (a generous gift from Tom Kelly and Ed O'Neill) to bind to the *X. laevis* ribosomal gene promoter. Figure 5 shows that the human NF1 produces a

Figure 5. Affinity purified human NF1 produces the same -5 to -30 footprint on the *X. laevis* ribosomal gene promoter as does the Xenopus NF1-like activity. Affinity purified human NF1 (gift of Ed O'Neill and Tom Kelly) and the Xenopus Biorex 0.4M KCl eluate were compared for their ability to footprint on the *X. laevis* ribosomal gene promoter. The footprints are essentially identical.

footprint on the gene promoter that is identical to that produced by the partially purified Xenopus activity. We conclude from these results that the Xenopus extract contains a DNA binding activity that is closely related to NF1. Furthermore, the ribosomal gene promoter contains an authentic NF1 binding site.

NF1 sites are present in other ribosomal gene promoters

In a computer search of other ribosomal DNAs we found several in lower eukaryotes that have apparent NF1 binding sites (listed in Figure 6). In *Tetrahymena* and *Glaucoma* the homology lies within a short sequence (the "type

<i>Xenopus laevis</i>	-31	ACGCTTTTTTGGCAT
<i>Acanthamoeba</i>	-47	<u>GC</u> ACTTTT <u>T</u> TGGCAC
<i>Glaucoma</i>	-52	<u>IC</u> GATTT <u>T</u> TGGCA <u>A</u>
<i>Tetrahymena</i> <i>pyriformis</i>	-52	AAATTTTT <u>T</u> TGGCA <u>A</u>
<i>thermophila</i>	-52	AAAATT <u>T</u> TTTGGCA <u>A</u>
<i>Dictyostellium</i>	+150	<u>GAC</u> ATTTTTTGGCA <u>G</u>
NF-1 consensus (Borgmeyer, et al., 1984)		TGGCANNMTGGCA

Figure 6. NF1 sites in other rDNAs. The NF1 site in the *X. laevis* ribosomal gene promoter is compared with other potential sites in the ribosomal DNAs of *Acanthamoeba*, *Glaucoma*, *Tetrahymena* (both *pyriformis* and *thermophila*) and *Dictyostellium*. Nucleotides which differ from the *X. laevis* sequence are underlined. Shown at the bottom is the NF1 consensus sequence derived by Borgmeyer et al. (1984). The numbers indicate the distance from the most leftward nucleotide to the site of transcription initiation.

I" sequence) that is present in four copies (*T. thermophila*) or three copies (*T. pyriformis* and *G. chattoni*) in the 5' non-transcribed spacer (Challoner et al., 1985). The type I repeats have been implicated in both replication and transcription of the rDNA (Larson et al., 1986).

We also found an apparent binding site within the promoter for the ribosomal genes of *Acanthamoeba* (Figure 6). The NF1-like activity in the *Xenopus* extract protects this site. The specificity of the binding is demonstrated by the fact that the footprint is competed away by the specific WT-oligo but not by an oligonucleotide bearing the LS -29/-21 mutation (Figure 7). The NF1 binding site in the *Acanthamoeba* promoter is within motif B, a domain that is also protected by TIF, the (apparently single) transcription factor required in addition to polymerase to assemble the stable preinitiation complex in vitro (Iida et al., 1985; Bateman et al., 1985; Kownin et al., 1987). Motif B appears to be required for stable complex formation although it is not absolutely required for transcription (Iida et al., 1985).

We have tried footprinting the *Xenopus* NF1-like activity on the mouse and human ribosomal gene promoters but so far have failed to detect any footprints that are specifically competed by the WT oligo (data not shown).

Figure 7. The *Xenopus* NF1-like activity also binds to the *Acanthamoeba* ribosomal gene promoter. An *Xenopus* nuclear extract was used to footprint on the *Acanthamoeba* ribosomal gene promoter. The footprint is competed away by the WT oligo but not by the mutant oligo. The extent of the footprint produced by the *Acanthamoeba* transcription factor, TIF, is indicated as well as the two domains, A and B, which influence its binding.

DISCUSSION

We conclude that there is an authentic NF1 binding site within the *X. laevis* ribosomal gene promoter and there is a DNA binding activity in frog nuclei that fractionates and binds like authentic NF1. Does this NF1 site have a function and, if so, what is it? We have previously reported the construction and testing of a series of linker scanner mutants that cover the *X. laevis* ribosomal gene promoter (Reeder *et al.*, 1987). The LS -29/-21 mutant which destroys NF1 binding decreases transcription five-fold as assayed by oocyte injection and about two-fold as assayed in vitro (summarized in Figure 2). This is consistent with NF1 playing an important role in ribosomal

gene transcription. However, we have also noted (C. Pikaard and P. Walker, unpublished) that there is at least one other DNA binding protein, chromatographically distinct from NF1, that footprints in this general region. The binding site for this other protein has not yet been precisely defined and it remains to be seen which protein is responsible for the loss of transcription seen with LS -29/-21.

On the *Acanthamoeba* promoter the NF1 site is located in a region with a clear function in transcription. However, there is as yet no direct evidence that NF1 itself influences transcription in that system.

Possible functions for NF1 binding to a polymerase I promoter could be grouped in two broad categories. Perhaps the most interesting class of possibilities center around the idea that NF1 is part of a mechanism for regulating ribosomal gene transcription in concert with that of other genes in the cell, possibly for coordinating ribosomal gene transcription with growth regulation. It has been reported, for example, that an NF1 site in a collagen gene promoter is required for transcription of the gene to respond to the growth factor, TGF beta (Rossi *et al.*, 1988). In another version of this idea, it has been reported that transcription of the U6 and 7SK genes is effected by RNA polymerase III in conjunction with at least two polymerase II transcription factors, the octamer binding protein and probably a TATA binding factor (Carbon *et al.*, 1987; Murphy *et al.*, 1987). Sharing of factors between different polymerase classes suggests obvious models for coordinate regulation.

Another class of model is that in which NF1 binding is important for some unknown function which is indirectly related to ribosomal gene transcription. For example, Smale and Tjian (1985) have shown that there is a cryptic polymerase II promoter overlapping the human polymerase I promoter. Under conditions where the polymerase I promoter is damaged by mutation, transcription from the polymerase II promoter can be detected. Lopata *et al.* (1985) have reported a similar situation in the mouse ribosomal DNA. If *Xenopus* has a similar cryptic polymerase II promoter we might imagine that the NF1 site is related to that (although at present there is no idea what purpose a polymerase II promoter might have overlapping a polymerase I promoter). A possibly similar puzzle exists on the human c-myc gene where it has been shown that a cryptic polymerase III promoter overlaps the polymerase II promoter that is normally used for transcription (Chung *et al.*, 1987; Bentley and Groudine, 1988).

It is also possible that the NF1 binding site is involved in replication and not in transcription. A recent review by DePamphilis (1988) contained the interesting speculation that origins of replication containing NF1 sites might

be utilized for gene amplification, an event which is well-known to occur on the *Xenopus* ribosomal genes.

As mentioned earlier, the NF1 and CCAAT binding proteins probably comprise a family of distinct proteins (Chodosh, 1988). The precise member of this family that recognizes the *Xenopus* polymerase I promoter is unknown at present. We should remain alert to the possibility that there may even be a nucleolus-specific member of the NF1 family.

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