Targeting of Sp1 to a non-Sp1 site in the human neurofilament (H) promoter via an intermediary DNA-binding protein

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Received August 27, 1992; Revised and Accepted October 19, 1992

ABSTRACT

The human neurofilament (H) promoter contains multiple binding sites for nuclear proteins including a Proximal (Prox) site centered around the sequence GGTGGACC and an adjacent pyrimidine (Pyr) tract site centered around the sequence CCCTCCTCCCC. Surprisingly binding to a probe containing the Prox/Pyr region of the NF(H) promoter was competed in gel shifts by an oligonucleotide containing only an Sp1 binding site (GGGGCGGGG). Supershift assays with a polyclonal anti-Sp1 antisera confirmed that Sp1 was part of the complex formed with the Prox/Pyr probe. However neither bacterially expressed Sp1 516C or vaccinia virus expressed full-length Sp1 778C bound to the Prox or Pyr sequences in DNase I footprints or gel shift assays. Gel shift competitions and supershift assays with probes containing either Prox or Pyr tract sites alone demonstrated targeting of Sp1 to the Prox binding site and identified a non-Sp1 containing complex which contains a Prox binding protein. Adding exogenous Sp1 to a HeLa nuclear extract enhanced the Sp1-containing complex but had no effect on the Prox complex. These studies show that Sp1 can be targeted to a non-Sp1 site in the human NF(H) promoter through protein/protein interactions with a distinct sequence specific DNA-binding protein.

INTRODUCTION

Transcription initiation by RNA polymerase II requires an array of cellular factors. Many factors are sequence specific DNA-binding proteins which enter the transcription complex through their DNA-binding activity (1). However other factors lack DNA-binding activity and instead enter the transcription complex through protein/protein interactions. Except for the TATA box binding factor (TBP/TFIID) all of the general initiation factors utilize this latter mechanism (2). Yet components of the transcription complex other than the general initiation factors may be recruited by protein/protein interactions as well. The adenovirus E1a protein activates viral gene transcription even though it lacks DNA-binding activity. One mechanism of

targeting E1a to viral promoters is through E1a's interaction with a cellular DNA-binding protein ATF-2 (3). Similarly the Herpes virus transcription factor VP-16 interacts with VP-16 responsive promoters through protein/protein interactions with cellular factors rather than by DNA-binding (4). How frequently cellular promoters utilize this mechanism to recruit transcription factors other than the general initiation factors is not known. Additionally it is not known how frequently sequence specific DNA-binding proteins are recruited through protein/protein interactions rather than DNA-binding. Sp1 is one of the best studied cellular transcription factors (5). Sp1 binds DNA in a sequence specific manner interacting with sequences such as GGGGCGGGG (GC boxes) and some closely related sequences found in many viral and cellular promoters (6). Transcriptional activation by Sp1 also requires non-DNA binding co-activators (7, 8). Currently Sp1 is believed to enter transcription complexes exclusively through its DNA-binding activity. Here we show an alternative mechanism which targets Sp1 to the human Neurofilament (H) promoter via a protein/protein interaction with a sequence specific DNA-binding protein.

MATERIALS AND METHODS

HeLa cell nuclear extracts (9) and bacterially expressed Sp1-516C (10) were prepared as previously described. Vaccinia virus expressed full length Sp1 778C was purchased from Promega.

Gel shift assays and DNase I protection assays were performed as previously described (9). The following oligodeoxynucleotides were used:

Proximal binding site (Prox): 5'-AATCACTGCCAAGGGGTTGGACC-CGGATC-3'

Pyrimidine tract (Pyr): 5'-GATCCCCTCCTCCCCGAATT-3'

Prox/Pyr: 5'-AGCTTGGGCTGCAGGTCGACTCTCCCCACCTCTCA-CTGCCAAGGGGTTGGACCCGGCCGC-3'

Sp1: 5'-GATCCATCGATCGGGGCGGGCGATGAATT-3'

AP-1: 5'-CTAGTGATGAGTCAGCCGGATC-3'

NF-1: 5'-TCGACCCTGGCCCGGACCAGCGCCGC-3'

In gel shift competition assays, unlabeled competitor oligodeoxynucleotides were incubated with the nuclear extract for 10 min prior to addition of labeled probe. For supershift

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assays binding reactions were incubated with polyclonal rabbit anti-Sp1 (obtained from Stephen Jackson) or Pre-immune serum for 30 min at room temperature before radiolabeled probe was added to the binding reaction for 30 min.

RESULTS

Previously we identified multiple binding sites for nuclear proteins within the human NF(H) promoter (9) (Figure 1). Two typical G/C boxes were presumed to bind Sp1. However two other sequences appeared unrelated to any known consensus sequence. One of these sites we called the Prox binding site due to its location closest to the NF(H) TATA box. The Prox binding site was centered around the sequence GGTTGGACC. Two sites more distally were centered around a pyrimidine (Pyr) tract (CCCTCCTCCCC). These Pyr sites formed an interrupted palindrome around one of the Sp1 sites. Pyr tract binding sites were subsequently found in the human NF(M) promoter (10) and

Figure 1. DNA-Protein Binding sites in the human NF(H) promoter. The nucleotide sequence of the human NF(H) gene promoter region is presented. Numbering is relative to the start site of transcription (9). A TATA homology at -29 and binding sites for nuclear factors which are discussed in the text are underlined. 'Pyr' refers to a pyrimidine tract which is present in two copies forming a palindrome as indicated by the arrows. 'Prox' refers to proximal binding site.

Figure 2. Gel mobility shift assays with a probe containing the Pyr and Prox binding sites of the NF(H) promoter. A 62 bp probe (see Materials and Methods) including NF(H) sequence from -38 to -80 was used in a gel shift assay. This probe contains all of the Prox binding site as well as enough of the 3' Pyr tract to serve as a functional binding site (9). Binding reactions contained labeled probe incubated without extract (lane 1) or 5 μ g of HeLa nuclear extract (lanes 2-6). Binding reactions were pre-incubated without competitor in lane 2, or 25 ng of an Sp1 competitor in lane 3, a proximal binding site competitor in lane 4, a pyrimidine tract competitor in lane 5, or an AP-1 competitor in lane 6.

a related pyrimidine tract in the mouse NF(M) gene (CCCCTTCCCC) likely binds the same protein(s) (unpublished observations). Nuclear extracts from both neural and non-neural cell lines and mouse brain contain proteins that bind to the Prox and Pyr sites suggesting that these proteins may be ubiquitously distributed (9).

Gel shifts utilizing probes containing the Prox and the first Pyr tract sites produce one major and two minor complexes as seen in Figure 2. Since neither the Prox or Pyr sequences looked like Sp1 sites (6), we were surprised that an Sp1 competitor could block binding to a probe containing only the Prox and 3' Pyr tract sites without Sp1 sites (Figure 2). Surprisingly the Sp1 competitor blocked binding more effectively than either a Prox competitor which produced only a slight reduction in the major complex or a Pyr competitor which had no more effect on complex formation than non-specific competitors (Figure 2).

Is Sp1 part of the complex?

Since in crude nuclear extracts a non-Sp1 G/C box binding protein(s) might be binding to the Prox and Pyr sites, it was necessary to confirm that Sp1 was contained in the complex. Therefore we performed gel shift (supershift) assays with a polyclonal anti-Sp1 antisera (gift of Dr S.Jackson). In gel shifts with HeLa nuclear extract and an Sp1 probe this antisera reduces probe binding and produces a supershifted complex (data not shown). As shown in Figure 3, in a binding reaction pre-incubated with the Sp1 antibody before addition of probe, binding to the two upper complexes was reduced or eliminated and a supershifted complex appeared. This result confirmed that HeLa Sp1 or an immunologically very closely related protein was part of the complex. However the lack of effect of the Sp1 antibody

Figure 3. Supershift assay with a probe containing the Pyr and Prox binding sites of the NF(H) promoter and anti-Sp1 antibody. Supershift assays were performed with the same probe as in Figure 2. Binding reactions contained no extract (lane 1) or 10 μ g of HeLa nuclear extract (lanes 2-4). Binding reactions were pre-incubated without antibody (lanes 1 and 2), with 3 μ l of rabbit polyclonal anti-Sp1 antibody (lane 3) or 3 μ l of pre-immune serum (lane 4).

Figure 4. DNase I footprints with cloned Sp1 and the human NF(H) promoter region. DNase I footprinting reactions were performed with probes containing NF(H) sequence from -205 to +37 labeled on the coding strand as previously described (3). The binding reactions contained no extract (lane 3), HeLa nuclear extract 52 μ g (lane 4), bacterially expressed Sp1 8 μ g (lane 5), 16 μ g (lane 6), or 32 μ g (lane 7) and 37 μ g of extract from an *E. coli* culture transformed with pBluescript (lane 8). G (lane 1) and G+A (lane 2) are the corresponding chemical sequencing reactions. Boxes alongside the autoradiograph indicate the footprinted regions which are discussed in the text. A hypersensitive site separating the Prox and Sp1/Pyr footprints is indicated by an arrow.

on the faster migrating complex suggested that a non-Sp1 protein also formed part of the complex.

Can Sp1 bind to the Pyr and/or Prox sequences?

To investigate this question we determined if Sp1 could bind to the Prox and/or Pyr tract sequences in DNase I footprints. Both HeLa and PC12 nuclear extracts protect three major footprints on the human NF(H) promoter (9). A proximal region containing the Prox binding site is separated by a prominent DNase I hypersensitive site (Figure 4) from a more distal region which includes the first Pyr and adjacent Sp1 site. A second Sp1 site is protected more distally. Bacterially expressed Sp1 516C contains the 516 C-terminal amino acids of Sp1 including the DNA binding domain and produces footprints with probes of the SV40 early region which are identical to those given by full-length Sp1 (5). As shown in Figure 4, bacterially expressed Sp1 516C did not protect the proximal region and had at most a minimal effect over the Pyr site even at levels in several fold excess to those needed to protect the two G/C box containing Sp1 sites. Similar results were subsequently obtained in DNase I footprints with full-length vaccinia virus expressed Sp1 778C (data not shown). Sp1 778C also showed little if any detectable binding to probes containing the Pyr or Prox sites in gel shifts although both probes produce distinct complexes with HeLa extracts (See Figures 5 and 6). We have also found that neither Sp1 516C or 778C binds to the pyrimidine tract in the human NF(M) promoter (10).

Sp1 is targeted to the Prox binding site

Since Sp1 alone does not bind to the Prox or Pyr sites it seemed likely that Sp1 was being targeted to either the Prox or Pyr sites through an intermediary protein. To investigate this possibility we performed gel shift competitions and supershift assays with probes containing either Prox or Pyr sites alone.

In gel shifts with HeLa extracts, a Prox site probe produces three complexes (Figure 5). A fast migrating complex (labeled

Figure 5. Competition Gel Shifts and Supershifts with a Prox site containing probe. In (A) an end-labeled Prox site probe was used in a gel shift assay. Binding reactions contained no extract (lane 1) or 10 μ g of HeLa nuclear extract (lanes 2-6). Binding reactions were pre-incubated without competitor (lane 2), or with 100 ng of Prox (lane 3), Sp1 (lane 4), Pyr (lane 5) or NF-1 (lane 6) competitors. Complexes C1, C2 and C3 are discussed in the text. In (B) supershift assays were performed as described in Figure 2 with the probe utilized in Figure 5A. Binding reactions contained no extract (lane 1), or 10 μ g of HeLa nuclear extract (lanes 2-4) and were pre-incubated without antibody (lanes 1 and 2), with 2 μ l of Sp1 antibody (lane 3) or 2 μ l of pre-immune serum (lane 4). In lane 5, the binding reaction contained probe and 15 ng of vaccinia virus expressed Sp1 778C. In (C) gel shifts were performed as in Figures 5A and 5B. Binding reactions contained no extract (lane 1), 5 μ g of HeLa extract (lanes 2 and 3) and 15 ng of Sp1 778C (lanes 3 and 4).

Figure 6. Competition Gel Shifts and Supershifts with a Pyr site containing probe. In (A) a Pyr site containing probe was end-labeled and utilized in a gel shift assay. Binding reactions contained no extract (lane 1) or 10 μ g of HeLa nuclear extract (lanes 2–6). Binding reactions were pre-incubated without competitor (lane 2), or with 100 ng of Pyr (lane 3), Sp1 (lane 4), Prox (lane 5) or NF-1 (lane 6) competitors. In (B) supershifts were performed with the same probe as in Figure 6A. Binding reactions contained no extract (lane 1) or 10 μ g of HeLa nuclear extract (lanes 2–4) and were pre-incubated without antibody (lanes 1 and 2), with 1 μ l of Sp1 antibody (lane 3) or 1 μ l of pre-immune serum (lane 4). In lane 5, the binding reaction contained probe and 15 ng of Sp1 778C.

C1 in Figure 5A) appeared to be non-specific since it was unaffected by cold Prox competitor. However Prox competitor blocked binding to two other specific complexes (C2 and C3). An Sp1 competitor reduced the upper C3 complex but had no effect on the lower C2 complex. Other competitors including NF-1 and Pyr tract competitors had no effect on either complex. These results implied that Sp1 was part of the C3 complex.

We next performed supershift assays and found that the C3 but not the C2 complex was reduced and slightly supershifted by Sp1 antibody (Figure 5B). This effect occurred whether antibody was added before (as in Figure 5B) or after (data not shown) protein binding to probe. In a complementary study we tested whether adding exogenous Sp1 to a HeLa extract would enhance the C3 complex. At sub-saturating levels of nuclear extract, adding exogenous Sp1 to the binding reaction enhanced the C3 but had no effect on the C2 complex (Figure 5C). Thus a non-Sp1 protein (Prox) must form the C2 complex while likely a combination of Sp1 and Prox form C3. Since Sp1 alone cannot bind to the Prox site (see Figure 5C and above) these data argue that Sp1 is targeted to the Prox site through protein/protein interactions with the Prox site binding protein.

Gel shifts performed with a Pyr site probe produced one major complex that was unaffected by an Sp1 competitor (Figure 6A). In supershift assays, a non-specifically shifted band was seen with both the anti-Sp1 and pre-immune serum, but no effect was seen on the major specifically shifted band (Figure 6B). As noted above purified HeLa Sp1 778C showed little if any affinity for a Pyr site probe (Figure 6B). Thus Sp1 does not bind to the Pyr tract or interact with the protein(s) that bind there.

DISCUSSION

These experiments show that Sp1 can be targeted to a region of the human NF(H) promoter which lacks an Sp1 binding site. Targeting likely occurs through a separate protein (Prox) which

binds around the sequence GGTGGACC. Supershift data with an Sp1 antibody demonstrated that Sp1 is part of the Prox binding complex. However Sp1 does not bind to the Prox site in gel shifts or DNase I footprints. In gel shifts a distinct 'Prox' complex can be competed by a Prox but not by an Sp1 competitor. This complex does not supershift with Sp1 antibody. Thus the Prox binding protein is not a cofactor which allows Sp1 to recognize an atypical binding site but rather a separable DNA binding protein.

Sp1 contains a zinc finger DNA binding domain as well as a glutamine rich transcriptional activation domain (11, 12) and is phosphorylated upon DNA binding (13). It must also contain regions for protein/protein interactions with transcriptional co-activators (7, 8). Sp1 must as well contain a region for protein/protein interactions with the NF(H) Prox binding protein. Sp1 has been shown to complex with the E2 transcription factor of bovine papillomavirus, itself a sequence specific DNA-binding protein (14). This interaction can target E2 to a promoter lacking E2-binding sites. However to our knowledge it has not been previously shown that Sp1 can be targeted to a promoter lacking a functional Sp1 binding site.

The Prox/Sp1 interaction could serve as a mechanism for recruiting a strong transcriptional activator such as Sp1 into a promoter which lacks Sp1 binding sites. Possibly recruitment of other sequence specific DNA-binding proteins into the transcription complex occurs through intermediary proteins as well. Such interactions could add an additional level of complexity to the combinatorial regulation of transcription. However it is unclear why this mechanism would be used in the NF(H) promoter which contains functional Sp1 binding sites just upstream of the Prox site (see Figure 1).

Prior studies showed that in transfected cells the Prox site alone linked to the NF(H) TATA box activates transcription poorly being possibly no more active than the TATA box alone (9). Thus targeting Sp1 to the Prox site may not by itself have great

functional significance. However linking the adjacent Pyr site to the Prox site produced a strong synergistic activation of transcription which was 5–10 fold greater than the activation seen with either site alone (9). Possibly only when all three proteins, ie. Prox, Sp1 and the Pyr tract binding protein, interact does full transcriptional activation occur. The exact role each protein plays in such a complex is not known. Further studies on these possible interactions will require cloned Prox and Pyr binding proteins.

ACKNOWLEDGEMENTS

We would like to thank Stephen Jackson for the gift of Sp1 antibody. This research was performed while G.E. was a Pfizer Scholar and was supported by a National Institutes of Health grant (AGO 5138-07) to R.A.L. This is publication # 115 from the Brookdale Center for Molecular Biology.

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