Identification of a protein that interacts with the nuclear factor-1 (NF-1) binding site in cells that do not express NF-1: comparison to NF-1, cellular distribution, and effect on transcription

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ABSTRACT

We examined expression of nuclear factor-1 (NF-1) in different cell lines. Expression was low or undetectable in T and B lymphocyte cell lines, whereas fibroblasts and other adherent cell lines generally had a relatively high level of NF-1 mRNA. In cell lines that did not express NF-1, gel retardation assays, nevertheless, indicated complexes between a protein or proteins and the NF-1 site. These complexes were less abundant than those formed with NF-1, they migrated more slowly, and they appeared as single species instead of the multiple species observed with NF-1. NF-1 site-binding proteins were compared in the fibrosarcoma cell line HT-1080 (expressed the highest level of NF-1 in our study) and the B cell line Raji (does not express NF-1). UV-crosslinking studies indicated that the NF-1 site-binding proteins in both cell lines were similar in size. Proteolytic clipping band shift assays suggested that the Raji protein and NF-1 share structural similarity in their DNA binding domains, but are distinct proteins. The NF-1 site mediated transcriptional stimulation in cell lines where NF-1 is expressed; however, this element did not affect transcription in cell lines that do not express NF-1, suggesting that the NF-1 site-binding protein in these cells is functionally distinct from NF-1.

INTRODUCTION

The sequence (G)CCAA(T) is an important regulatory element for polymerase II-mediated transcription of a number of eukaryotic genes (1-10). Several different promoter elements, which interact with distinct nuclear proteins, share this sequence. CP1 binds the adenovirus major late promoter and the human α -globin promoter and CP2 binds the rat γ -fibrinogen promoter (11). The CCAAT-enhancer binding protein (C/EBP), which appears to promote terminal differentiation of cells, binds elements in several different genes including serum albumin (12).

Another CCAAT binding protein is nuclear factor-1 (NF-1), a cellular protein required for adenovirus replication that binds the consensus sequence TGGN₆-GCCAA found in the adenovirus origin of replication (13-15). How the binding of NF-1 stimulates adenovirus replication has not been elucidated; however, it is thought that NF-1 may promote the formation of a multi-enzyme complex at origins of replication (16). It is estimated that 60,000 NF-1 binding sites exist in the human genome (17). Based on its role in adenovirus replication, it is inferred that these NF-1 binding sites play a role in replication of cellular DNA.

NF-1 is also important for transcription of a number of cellular genes — it stimulates the activity of some promoters (18-21) and represses the activity of others (22, and McQuillan *et al.*, submitted for publication). An NF-1 site in the 5'-flanking sequence of the α_2 (I) collagen gene mediates transcriptional stimulation by transforming growth factor- β 1 (23)—how transforming growth factor- β 1 affects NF-1 to stimulate transcription is not known.

Attempts to purify NF-1 showed that it corresponds to a series of proteins ranging in size from 52 to 56 kDa (18). Some NF-1 heterogeneity appears to result from alternative splicing of the NF-1 gene transcript—three different NF-1 mRNAs, CTF1, C-TF2, and CTF3, have been described (20). Proteins encoded by each of these mRNAs have been expressed in bacteria and they are functionally similar—they each bind the NF-1 site, stimulate adenovirus replication, and affect transcription. Recent evidence suggests that a family of nuclear proteins interacts with NF-1 sites. Several different NF-1-like cDNAs have been identified that encode proteins that bind the NF-1 site. For example, one of these appears to be liver specific and mediates expression of genes such as albumin, fibrinogen, α -1-antitrypsin, α -fetoprotein, and transthyretin (24), whereas others bind the promoter of the 3-hydroxy-3-methylglutaryl coenzyme A reductase gene (25).

We examined the expression and binding activity of NF-1 in different cell lines. Several cell lines were identified that did not

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express NF-1; instead, these cells make another protein that binds the NF-1 site. This protein is similar in size and appears to share at least some structural relatedness with NF-1; however, it is functionally distinct from NF-1.

METHODS

Cell culture and DNA transfection

HeLa, HT-1080, JEG-3, PC-12, NIH 3T3, dermal fibroblasts, baby hamster kidney (BHK), and MG-63 cell lines were grown in Dulbecco's Modified Eagle's high glucose medium in 10% bovine serum. F9 cells were grown in the same fashion, but on collagen coated plates. F9 cells were stimulated to differentiate with cAMP and retinoic acid (26). K562, Raji, Molt-4, Jurkat and U937 cell lines were grown in Roswell Memorial Park Institute (RPMI)-1640 medium supplemented with 10% bovine serum. All cell lines were grown in the presence of 5% CO₂.

The HT-1080 cell line was transfected using the calcium phosphate technique (27) and Raji and Jurkat cell lines were transfected by electroporation using a BTX Transfecter (BTX inc., San Diego, CA) as follows. Approximately 1×10^7 cells were suspended in 0.1 ml of RPMI-1640 media containing HeBS (10 mM Hepes, 70 mM NaCl, 2.5 mM KCl, 0.35 mM Na₂HPO₄ and 3.0 mM Dextrose), salmon sperm DNA at 1.25 mg/ml, and 30 μ g of plasmid DNA and subjected to electroporation at 200V and 950 μ F. Chloramphenicol acetyl transferase (CAT) assays were done 36 hr after transfection (27).

Gel retardation assays

Protein extracts for gel retardation assays were prepared as described (22). A synthetic double-stranded oligonucleotide containing the adenovirus NF-1 binding site (5'-AGCTTGGT-CTGGCTTTGGGCCAAGAGCCGA-3') (15) or the fibronectin NF-1 binding site (5'-GCTTACCCGGAGCCCGGGCCAAT-CGATCGAA-3') (35) was used in gel retardation assays. Competition assays were done with a CP1 site from the adenovirus major late promoter (5'-CTACACCTATAAACC-AATCACCT-3') (11) and a CP2 site from the γ -fibrinogen gene promoter (5'-TGACCAGTTCAGCCACTCTTTA-3') (11). Underlined portions of the sequences correspond to protein binding sites. Gel retardation assays were done as described previously (27).

Proteolytic clipping band shift assays were done as described (27) using a standard gel retardation assay, except, samples were brought to a total of 75 μ g of protein by addition of BSA.

Figure 1. Expression of NF-1 mRNAs in different cell lines. Northern blots of 10 μ g of poly (A⁺) RNA from the indicated cell lines were hybridized to a probe specific for the CTF-1 form of NF-1 mRNA. Numbers indicate the approximate size (kb) of the different NF-1 mRNAs. 'Fib.' denotes fibroblast.

Following incubation with the probe, protein-DNA complexes were digested for 2 min with the indicated amount of trypsin or chymotrypsin and subjected to electrophoresis.

UV-crosslinking of proteins was done essentially as described previously (28). The coding strand of the adenoviral NF-1 site 5'-AGCTTGGTCTGGCTTTGGGCCAAGAGCCGA-3' was hybridized to the complementary primer 5'-TCGGCTC-3' and the primer was extended with the Klenow fragment of DNA polymerase I in a reaction where BrdU was substituted for dTTP and 0.25 mCi of [³²P]-dCTP (3000 Ci/mMol) was substituted for dCTP. Extension of the primer was monitored by denaturing electrophoresis of the product on a sequencing gel. Ten ng of the double-stranded product (approximately 20,000 cpm) was allowed to bind nuclear protein. Then, proteins were UV crosslinked to the DNA, the complexes were digested with a combination of DNase I and micrococcal nuclease, and the products were subjected to SDS-polyacrylamide gel electrophoresis as described (28). The resulting gel was dried and exposed to X-ray film.

Oligonucleotides were synthesized on an Applied Biosystems 391 DNA Synthesizer and were gel purified before use.

Plasmid construction

pTA-NF-1-CAT contains a single copy of the adenoviral NF-1 site cloned into the *Hind*III site immediately upstream of the SV40 early gene TATA box in pTA-CAT (contains the SV40 early gene TATA box driving the CAT gene, 22) in the same orientation found in adenovirus. Complementary oligonucleotides containing the adenoviral NF-1 site with a *Hind*III site on either end (see above) were annealed and ligated into pTA-CAT that had been digested with *Hind*III. The number of copies and the orientation of the NF-1 site was determined by DNA sequencing.

RNA isolation and Northern blotting

RNA was isolated by the guanidinium isothiocyanate/CsCl method (29) and polyadenylated RNA was selected by oligo dT affinity chromatography (30). RNA was separated on formaldehyde-agarose gels and electrophoretically transferred to nylon hybridization membrane. Equal amounts of RNA were loaded based on ethidium bromide staining and no degradation of either rRNA subunit was apparent. NF-1 mRNA was detected with synthetic oligonucleotides derived from the sequence of the

Figure 2. Gel retardation assays examining the binding of proteins to the NF-1 site. The binding site is the adenovirus NF-1 site. The cell line from which nuclear extracts were obtained is indicated above the lanes. F9-D refers to F9 cells that have been stimulated to differentiate with retinoic acid and cAMP. 'Fib.' is fibroblasts and 'BHK' is baby hamster kidney cells.

three different NF-1 mRNAs described previously (20). Northern blots were initially probed with a synthetic oligonucleotide complementary to the 3' end of the CTF1 clone (5'-TTGCTA-TCCCAGATACCAGGACTGTGCCTGATAAATGCCCCGCA-GGATCCCT-3') which is the largest NF-1 cDNA (20). This probe does not hybridize to the other two NF-1 cDNA clones CTF2 or CTF3. Northern blots were reprobed with an oligonucleotide complementary to sequences at the 5' end of CTF3 (5'-GATGCGCCGCATCTTGCCCTTCTGGTCGGGGTTGG-AGAGCACGCAGCCCCGG-3'). This probe should hybridize to each of the three different NF-1 mRNA species. For Northern blot hybridization (29), oligonucleotides were labeled on their 5' ends with ^{32}P from [^{32}P]-ATP using polynucleotide kinase.

RESULTS

Expression of NF-1 mRNAs

NF-1 mRNA levels in different cell lines were analyzed by Northern blot hybridization (figure 1). Three different NF-1 cDNAs that arise from alternative splicing of a common transcript have been characterized (20). Northern blots were initially probed with a synthetic oligonucleotide specific for the largest NF-1 cDNA denoted CTF1. Two different mRNAs corresponding in size to 8.6 and 4.5 kb were observed with the CTF1 specific probe, suggesting that alternate forms of CTF1 are present. These two NF-1 mRNAs are similar in size to those described previously in HeLa cells (20). The ratio of the two mRNAs varied in the different cell lines. In most of the cell lines that contained NF-1 mRNA, similar amounts of the two mRNAs were observed. However in dermal fibroblasts, the larger mRNA was predominant. Generally, CTF1 mRNA was either undetectable or expressed at a low level in lymphocytes, whereas fibroblasts and other adherent cell types expressed relatively high levels (figure 1 and data not shown). No CTF1 was detected in Raji (B cell), Jurkat (T cell), JEG-3 (choriocarcinoma), or F9 (teratocarcinoma) (we are not certain that our human probes will detect mouse NF-1 mRNAs in the F9 cells) (figure 1 and data not shown).

Two additional NF-1 mRNAs, CTF2 and CTF3, have been described that would not be detected with the CTF1 probe (20). Therefore, the Northern blots were rehybridized to probes that would detect CTF2 and CTF3 in addition to CTF1 (see 'Materials

and Methods'). There was no change in the pattern of bands on the Northern blots, suggesting that CTF2 and CTF3 are either not expressed in these cell lines or they are indistinguishable in size from CTF1 (data not shown). Cells that did not express CTF1 also did not express CTF2 or CTF3, indicating that they do not make a known form of NF-1.

NF-1 binding activity

It was of interest to compare nuclear protein binding to the NF-1 site in cells that express NF-1 and in cells where no NF-1 mRNA was detected. Gel retardation assays with nuclear protein extracts from the different cell lines are shown in figure 2. In cells expressing NF-1 mRNA, multiple NF-1-DNA complexes were observed resulting in a smear on the gel. Nuclear extracts from cell lines where no NF-1 mRNA was detected also exhibited binding to the NF-1 site; however, these extracts did not form the typical pattern of complexes observed with NF-1—the complexes were less abundant, they migrated more slowly, and they appeared as a single species. Unlike other cells that do not express NF-1, extracts from F9 cells gave a unique pattern in gel retardation assays. Whereas complexes with extracts from this cell line also appeared as a single species, they migrated more rapidly than complexes with extracts from other cells that do not express NF-1. These results were all obtained with the NF-1 site from adenovirus. Essentially identical results were seen with the NF-1 site from fibronectin (data not shown).

It is unlikely that the protein, which binds the NF-1 site in cell lines that do not express NF-1, could be C/EBP or HNF-1 because of their limited tissue distribution—C/EBP is found in liver, brain, and adipose tissue, and HNF-1 is found in liver (31). The protein also is not CP1 or CP2—the CP1 site from the adenovirus major late promoter and the CP2 site from the γ -fibrinogen gene promoter did not compete for binding to the NF-1 site (figure 3) nor did the NF-1 site compete for binding of CP1 or CP2 to their respective elements (data not shown). Additionally, binding of proteins to the NF-1 site was not competed with the adenovirus NF-III binding site or with a cAMP response element (data not shown). These results suggest that

Figure 3. Binding to the NF-1 site is specific. Fifteen μg of nuclear extract was used in gel retardation assays as in figure 2. However, a 100 fold molar excess of competitor element was added as indicated. See 'Materials and Methods' for the sequence of the different competitors. 'NS', indicates a nonspecific complex.

Figure 4. Proteolytic clipping band shift assays comparing NF-1 site binding proteins in the HT-1080 and Raji cell lines. Gel retardation assays were done as in figure 2, however, protein-DNA complexes were incubated with the indicated amount of either trypsin or chymotrypsin prior to electrophoresis. The radiolabeled probe used in each assay is indicated at the top of the figure. The CRE probe was described previously (35). Arrows indicate complexes with extract from the HT-1080 cell line that appear to comigrate with complexes formed with the Raji cell line extract.

cells that do not express NF-1 make another, as yet, uncharacterized protein that binds specifically to the NF-1 site.

Proteolytic mapping of proteins bound to the NF-1 site

Many DNA binding transcription factors appear to be bifunctional, one portion of the protein is required for DNA binding, whereas another portion of the protein stimulates transcription by interacting with other transcription factors. The DNA binding and transcriptionally active fragments of different transcription factors can be substituted for each other indicating that the two active sites of the proteins function independently [such is the case with NF-1 (32)]. Therefore, portions of the proteins not involved in DNA binding can be removed by proteolysis without disrupting binding to DNA. A combination of limited proteolysis of proteins bound to DNA followed by gel retardation assay—referred to previously as a proteolytic clipping band shift assay (33)—has been used to determine the relationship between different DNA binding proteins.

It was of interest to use the proteolytic clipping band shift assay to examine the relationship between NF-1 binding proteins in cells that express NF-1 and in cells where no NF-1 is expressed. We compared NF-1 site binding proteins in the fibrosarcoma cell line HT-1080, which had the highest level of NF-1 expression in our study (figure 1), and the B cell line Raji, which does not express NF-1 (figure 1). Nuclear extracts from Raji and HT-1080 cell lines were incubated with the NF-1 binding site as described above for gel retardation assays, but DNA-protein complexes were treated with different concentrations of trypsin or chymotrypsin prior to electrophoresis. Digestion with high concentrations of either enzyme (figure 4, left and middle panels) or for extended periods of time (data not shown) resulted in complexes of similar mobility from each cell line that were relatively resistant to further digestion. However, at lower enzyme concentrations, different proteolytic intermediates were seen with extracts from HT-1080 and Raji, suggesting that the NF-1 site binding proteins in the two cells are distinct. When high concentrations of HT-1080 cell extract were used in the

assays with chymotrypsin, intermediates similar in mobility to those seen in Raji cells were apparent (figure 4, middle panel, complexes denoted by arrows).

As a control, a proteolytic clipping band shift assay was done with an unrelated DNA sequence—the cAMP response element (CRE). The CRE gave a different pattern of digestion products than the NF-1 site in this assay (figure 4, right panel), suggesting that the patterns observed with the NF-1 site are specific.

UV-crosslinking of proteins to the NF-1 site

Proteins from HT-1080 and Raji cell lines were UV-crosslinked to the NF-1 site and subjected to SDS-polyacrylamide gel electrophoresis to determine if the proteins that bind the NF-1 site in these two cell lines are distinguishable in size (figure 5). Several closely spaced bands in the 60 kDa size range were observed using proteins from each of the cell lines, indicating that the NF-1 site binding proteins in Raji are similar in size to NF-1 in HT-1080.

The NF-1 site is not a transcriptional activator in cell lines that do not express NF-1

The plasmid pTA-NF-1-CAT, which contains the adenoviral NF-1 site immediately upstream of the SV40 early gene TATA box, was transfected into cell lines that either express or do not express NF-1. The activity of this construct was compared to that of the parent construct pTA-CAT that lacks the NF-1 site. In the HT-1080 cell line, the NF-1 site acted as a transcriptional enhancer; however, in Raji and Jurkat cell lines, which do not express NF-1, the element was inactive (figure 6). Multiple copies of the NF-1 site were still unable to cause activation in Raji and Jurkat cell lines (data not shown).

DISCUSSION

We have identified several cell lines that do not express a known form of NF-1. In these studies, probes from different regions of NF-1 mRNA, which hybridize to all known forms of NF-1

Figure 5. UV crosslinking of proteins to the NF-1 site. The UV crosslinking procedure is described in 'Materials and Methods'. The source of the protein extract is indicated above the lanes. 'Comp.' indicates that a 100 fold molar excess of cold NF-1 site was included in the assay. The points of migration of molecular size standards (in kDa) are shown.

Figure 6. Activity of the NF-1 site in transfection assays. A. Transfection of pTA-CAT and pTA-NF-1-CAT into the HT-1080 cell line. Five μ g of pTA-CAT (contains the SV40 early gene TATA box, a very weak promoter, driving the CAT gene) or pTA-NF-1-CAT (contains the adenovirus NF-1 site immediately upstream of the SV40 early gene TATA box) (both plasmids have been described previously, 22) were transiently transfected into the HT-1080 cell line and CAT activity was determined 36 hr after transfection using thin layer chromatography—radioactivity was quantified by scintillation counting. B. Transfection of pTA-CAT and pTA-NF-1-CAT into the Raji cell line. Thirty μ g of plasmid was transfected into 1×10^7 cells by electroporation and CAT activity was determined as in panel A. C. Transfection of pTA-CAT and pTA-NF-1-CAT into the Jurkat cell line. Jurkat cells were transfected as in panel B. The results are an average of duplicate assays and are representative of at least two separate experiments, each done in duplicate. Percent conversion of chloramphenicol to its acetylated forms was determined by dividing the cpm of acetylated chloramphenicol by the total cpm of chloramphenicol in each assay.

mRNA, were unable to detect NF-1 mRNA in poly (A) selected mRNA from these cell lines. Yet, these cell lines contain proteins that are able to bind the NF-1 site. These proteins interact specifically with the NF-1 site and do not appear to correspond to other well studied CCAAT binding proteins such as C/EBP, HNF-1, CP1, or CP2.

Different intermediates were observed when NF-1 site binding proteins from HT-1080 (the highest level of NF-1 in our study) and Raji (do not express NF-1) cell lines were compared by proteolytic clipping band shift assays, suggesting that the proteins in the two cell lines are distinct.

High concentrations of either trypsin or chymotrypsin or extended periods of digestion produced a relatively stable proteolytic product that exhibited similar mobility in proteolytic clipping band shift assays with extracts from both HT-1080 and Raji cells. These products apparently correspond to minimal protein sequences required for DNA binding and may be relatively resistant to proteolysis because of their close association with DNA. The results suggest that NF-1 in HT-1080 and the NF-1 site-binding protein in Raji share a similar DNA binding domain, which is not unexpected since they each recognize the same DNA sequence.

NF-1 site-binding proteins from each of the cell lines that we have examined are similar in size. Nevertheless, the mobility of complexes formed with different NF-1 site-binding proteins varies greatly, indicating that the different NF-1 site-binding proteins form distinct homotypic (associations between different NF-1 site binding proteins) and/or heterotypic [association between NF-1 site binding proteins and other transcription factors such as occurs with fos and jun to form AP-1 (34)] associations. Furthermore, cells that express predominantly NF-1 exhibit polydisperse complexes in gel retardation assays that vary greatly in their mobility. This complexity must arise at least in part from heterotypic associations of NF-1 with other transcription factors. Along these lines, NF-1 has been shown to dimerize (32) and to associate with heterologous proteins (11); however, other studies have suggested that purified NF-1 is functionally active (20).

Finally, the absence of NF-1 expression in some cell types indicates that it is not absolutely required for DNA replication and cell viability. The other NF-1 site-binding protein that we have identified in the Raji cell line, which is the only detectable factor that interacts with the NF-1 site in these cells, is unable to substitute functionally for NF-1 to cause transcriptional stimulation—its effect on DNA replication is unknown. The presence of functionally distinct NF-1 site-binding proteins may allow the NF-1 site to mediate several different effects.

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