NFI-B3, a Novel Transcriptional Repressor of the Nuclear Factor I Family, Is Generated by Alternative RNA Processing*

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Nuclear factor I (NFI) proteins constitute a family of sequence-specific transcription factors whose functional diversity is generated through transcription from four different genes (NFI-A, NFI-B, NFI-C, and NFI-X), alternative RNA splicing, and protein heterodimerization. Here we describe a naturally truncated isoform, NFI-B3, which is derived from the human NFI-B gene, in addition to characterizing further human NFI-B1 and NFI-B2, two differentially spliced variants previously isolated from hamster and chicken. Although NFI-B1 and NFI-B2 proteins are translated from an 8.7-kilobase message, the mRNA for NFI-B3 has a size of only 1.8 kilobases. The NFI-B3 message originates from the failure to excise the first intron downstream of the exons encoding the DNA binding domain and subsequent processing of this transcript at an intron-internal polyadenylation signal. The translation product includes the proposed DNA binding and dimerization domain and terminates after translation of two additional “intron” encoded codons. In SL-2 cells, which are void of endogenous NFI, NFI-B3 by itself had no effect on transcriptional regulation and failed to bind DNA. Coexpression of NFI-B3 with other isoforms of the NFI-B, -C, and -X family, however, led to a strong reduction of transcriptional activation compared with the expression of these factors alone. Gel shift analysis indicated that NFI-B3 disrupts the function of other NFI proteins by reducing their DNA binding activity by heterodimer formation. The efficiency of NFI-B3 heterodimers to bind to DNA correlated with the degree of transcriptional repression. The abundance of NFI-B transcripts varied significantly between different human cell lines and tissues, suggesting a potential involvement of these factors in the complex mechanisms that generate cell type specificity.

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Nuclear factor I (NFI)³ comprises a family of sequence-specific DNA-binding proteins that bind to the palindromic sequence TGGC/A(N)₅GCCA or with lower affinity to the half-palindrome. NFI proteins bind their recognition sequence as homo- or heterodimers that are already formed in solution (1, 2). Initially, NFI was identified as a factor required for the replication of adenovirus DNA (3–5) but has since been recognized as a potent transcriptional regulator of many viral (6–9) and cellular genes (10, 11). Molecular cloning and sequence analysis of cDNAs from different animal species (12–18) led to the identification of four different genes: NFI-A, NFI-B, NFI-C, also referred to as NFI/CTF, and NFI-X. The diversity of NFI proteins is increased further by differential RNA splicing (12, 16). The highly conserved 189-amino-acid NH₂-terminal region of NFI is required for DNA binding and dimerization (1, 2). The COOH-terminal transactivation domain diverges extensively, both among factors derived from different genes as well as between different spliced isoforms derived from the same gene. A high proportion of proline residues is common to all of them, suggesting that transcriptional activation occurs through proline-rich sequences (1). The proline-rich region of NFI/CTF seems dispensable for transcriptional activation in yeast, however (19).

The existence of a number of structurally different NFI proteins and their differential expression as well as the involvement of NFI binding sites in cell type-specific gene expression (20–24) suggests that the individual isoforms may have distinct functions. NFI/CTF isoforms, first isolated from human HeLa epithelial cells and later from pig liver, function as transcriptional activators with a broad range of transactivation potential in different cellular environments (1, 19, 25). We and others have shown recently that differentially spliced isoforms from the human (24) and mouse (26) NFI-X genes can function as transcriptional repressors, and some of these isoforms are expressed differentially in different human tissues (24, 27). A novel repressor domain, which inhibits DNA binding in vitro, was also characterized for Xenopus laevis NFI-X isoforms (28). The underlying mechanism for the varying function of the different isoforms is not clearly understood. It has been demonstrated that the transcriptional activation of NFI/CTF-1 in yeast depends on a sequence motif related to the heptapeptide repeat of the COOH-terminal domain of RNA polymerase II (29, 30). Another member of the NFI-C family which lacks this domain was found to be an even more potent activator, however (25). The activity of individual NFI isoforms may also be modulated by other factors as observed with the transforming growth factor-β responsive region, located in the proline-rich activation domain of NFI/CTF-1 (31).

Our earlier studies indicated that NFI plays a crucial role in the epithelial cell type-specific transcription of human papillomaviruses (9, 32, 33). Differences in the gel shift pattern of NFI proteins between human epithelial cells, where the viral enhancer is active, and human fibroblast cells, which do not support viral transcription, suggested that fibroblast cells express a different subset of NFI proteins than do epithelial cells, which express mainly factors of the NFI-C family (9, 12). The screening of a human fibroblast library led to the isolation of NFI-XI, an isoform that represses the activity of NFI/CTF by the formation of heterodimers (24). In addition to different spliced isoforms of the NFI-X family, we also isolated a novel clone of the NFI-B family from human fibroblast cells. Two

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³ The abbreviations used are: NFI, nuclear factor I; kb, kilobases(s); CAT, chloramphenicol acetyltransferase; Ad, adenovirus; PCR, polymerase chain reaction; bp, base pairs.

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spliced isoforms of the NFI-B gene, NFI-B1 and NFI-B2, have been isolated before from chicken and hamster liver cells (13, 16), but no human homologs have been characterized so far.

Here we describe a novel NFI-B form, NFI-B3, and its expression pattern and functional characteristics. We show that NFI-B3 is encoded on a 1.8-kb mRNA, which is generated by use of a premature polyadenylation site located in an intron. In contrast, NFI-B1 and NFI-B2 are generated by alternative splicing, leading to an 8.7-kb message. NFI-B3 is a truncated protein that lacks the transcriptional activation domain. It cannot bind to DNA as a homodimer or as a heterodimer with NFI-B2, but it binds in the form of a heterodimer with isoforms of the NFI-C and NFI-X family. For functional analysis we also cloned the human homolog of chicken NFI-B2 and show that the truncated NFI-B3 protein can repress the transcriptional activity of NFI-B1, -C, and -X isoforms containing full-length activation domains.

MATERIALS AND METHODS

Plasmid Constructs—The chloramphenicol acetyltransferase (CAT)-reporter construct pCAT3xAd contains the wild-type α-globin promoter from chicken NFI-B1 cDNA (clone YL11). The 5' and 3' flanking DNA sequences are homologous to the cDNA clones NFI-B1 and NFI-B2 (9, 24). To construct the expression vectors pXJ-NFI-B3 and pXJ-NFI-B2, the cDNA inserts were isolated from the respective phage clones by polymerase chain reactions (PCRs) using cDNA insert-flanking primers (cDNA insert-screening amplifiers, Clontech) and cloned into the EcoRI site of a cytomegalovirus promoter-driven expression plasmid pXC40 (34). The control plasmids pBCAT2 and pPacSp1 are described elsewhere (35).

Library Screening and DNA Sequencing—A commercially available human skin fibroblast cDNA library (Clontech HL 1052a) was screened with a previously isolated 1.2-kb NFI-X cDNA clone, FN6, under hybridization conditions as described (9). From a total of 5 × 10^6 individual plaques, eight positive clones were purified. The cDNA inserts were amplified by PCR and subcloned into the EcoRI site of pUC19, and both strands of the cDNAs were sequenced.

Reverse Transcriptase-PCR Analysis and Direct DNA Sequencing—Genomic DNA (SiHa cells) and total RNA (HeLa, MRHF, H4 and HISM cells) for PCRs was prepared using TRIzol Reagent according to the manufacture's instructions (Life Technologies, Inc.). First-strand cDNA synthesis and subsequent PCRs were performed as described (9). Primer pairs YB14–YB15, YB14–YB31, and Do108–Do109 were used for PCR amplification and subcloning into the respective phage clones by polymerase chain reactions (PCRs) using cDNA insert-flanking primers (cDNA insert-screening amplifiers, Clontech) and cloned into the EcoRI site of a cytomegalovirus promoter-driven expression plasmid pXC40 (34). The control plasmids pBCAT2 and pPacSp1 are described elsewhere (35).

Northern Blot Analysis—Poly(A)^+ RNA was isolated from total RNA using the Poly/Tract mRNA isolation system (Promega). Approximately 1.5 μg of mRNA was separated on a 1% agarose gel and blotted to a nylon membrane (Hybond-N, Amershams Corp.). The multiple tissue Northern blot containing 2 μg of poly(A)^+ RNA of each of various human tissues was obtained from Clontech. DNA probes containing the 5'-untranslated region (position 76–440 bp) and the novel 3' sequence of NFI-B3 (position 1009–1270 bp) were created by PCR with NFI-B3 cDNA template. The probes were probed in a hybridization solution containing 0.5 M sodium phosphate, pH 7.9, 7% sodium dodecyl sulfate, 15% formamide, and about 10^6 cpm/ml of the random primed labeled probe. The filters were exposed under the same conditions as described before (33). The human smooth muscle cell line H1S3 (ATCC, CRL-92) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The H4 human neuroglioma line (ATCC, HTB-148) was grown in Dulbecco's modified Eagle's medium containing 4.5 g glucose/liter and 10% fetal calf serum. Drosophila Schneider SL-2 cells were grown in Schneider's insect medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum at 25 °C. Twenty-four h before transfection, the cells were seeded at a density of 10^6 cells/ml and then transfected by calcium phosphate coprecipitation as described (36). Three μg of an NF1 expression vector (pXJ-NFI-B2, -B2, -X2, or pCGND-CTF-1) was cotransfected with 3 μg of the respective expression construct pSA-CAT3xAd, and pUC19 plasmid DNA was added as a carrier to a total of 10 μg of DNA. Cells were harvested 36–38 h after transfection, and 50 μg of protein was used for each CAT assay.

Nuclear Extracts, Gel Shift Assays, and In Vitro Translation—The correct expression of NFI-B3 and NFI-B2 from the pXJ40 vector construct, which contains a T7 promoter upstream of the cloning site, was tested by in vitro transcription and translation. The plasmids (0.5 μg) were added separately to 50 μl of a reaction mix containing T7 RNA polymerase and rabbit reticulocyte lysate (TNT Coupled Reticulocyte Lysate System, Promega). The electrophoretically separated protein products, which had incorporated [35S]methionine, were observed by autoradiography. Nuclear extracts of Drosophila SL-2 cells transfected with 5 μg of pXJ-NFI-B3, pXJ-NFI-B2, pXJ-NFI-X2, and pCGND-CTF-1 were prepared according to Schreiber et al. (37). Gel shift assays were performed with an oligonucleotide representing an Ad-NFI consensus binding site as described previously (9). The DNA-protein complexes were separated on a 4.5% polyacrylamide, 0.25 × TBE gel.

RESULTS

Cloning of a Novel cDNA from the NFI-B Gene That Encodes a Truncated Protein—We have reported previously the isolation of a human NFI-X isoform in a screen of a human fibroblast cDNA library with an NFI-C probe and the detection of further NFI-X isoforms by rescreening the same library using this NFI-X clone as a probe (24). The same study led to the detection of a novel NFI-B cDNA. The 5' region to this cDNA was homologous to the cDNA clones NFI-B1 and NFI-B2 originally isolated from hamster and chicken (13, 16); however, sequences downstream of the DNA binding domain diverged from those of all known NFI sequences. This clone was termed YL11.

Clone YL11 (GenBank™ accession number U70862) is 1,299 bp long, starting 457 bp upstream of an ATG initiation codon and terminating in a poly(A) tail downstream of a putative AATAAA consensus polyadenylation signal. The 3' untranslated region of YL11 is 267 bp long. The sequence of 502 bp of the DNA binding domain is 99.8% similar to a human NFI-B segment amplified by reverse transcriptase-PCR (18). No homology with other known sequences could be found downsteam of nucleotide 1015. Translation of the cDNA sequence reveals an open reading frame of 564 bp, coding for a 188-amino acid polypeptide chain. The N1 terminally 186 amino acids are conserved among all known members of the NFI family and comprise the DNA binding domain. The remaining two amino acids, an alanine and arginine, replace the whole COOH-terminal transactivation domains observed in hamster NFI-B1 and chicken NFI-B1 and NFI-B2. Since the sequence similarity indicated that the COOH-terminal truncated YL11 clone belonged to the NFI-B family, it was designated NFI-B3.

To analyze the novel human NFI-B3 isoform and to compare it with other proteins and cDNAs derived from the NFI-B gene, we attempted to clone the human homologs of the hamster and chicken NFI-B1 and NFI-B2 isoforms. Reverse transcriptase-PCR was performed with RNA from MRHF fibroblast cells using a pair of primers, Do108 and Do109, flanking the full-length coding frames of human NFI-B1 and NFI-B2 cDNAs. No PCR product could be isolated and characterized from MRHF fibroblasts. We then performed similar PCR analyses with cDNAs generated from epithelial cells (HeLa and C33A)

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The NFI-B3 mRNA arises from splicing the 5' region downstream of nucleotide 1015 bp of the NFI-B2 clone. No product of 1.68 kb, which would account for NFI-B1, could be amplified from the cDNAs used. To determine if the lack of a NFI-B1 PCR product is the result of an inefficient amplification of the larger product, PCR primers overlap- ping shorter segments were used. Using primer Do108 and YB11, which are located at the 5' homologous region of the NFI-B cDNAs, a single band of the predicted size of 505 bp could be amplified from HeLa cDNA. Primer pair YB14 and Do109 led to the predicted fragment of 1,252 bp for NFI-B1 and 941 bp for NFI-B2. The identity of the fragments was verified by sequence analysis. A schematic diagram of the NFI-B isoforms and the location of the PCR primers are given in Fig. 1A.

**A**

![Schematic diagram of human NFI-B cDNAs and location of PCR primers](image)

Panel A, schematic representation of human NFI-B cDNAs and location of the PCR primers used to characterize the RNA processing mechanisms. All three isoforms share the 5' region (white bars) encoding the DNA binding and dimerization domains but differ in the 3' terminal portion, coding for the activation domain. Panel B, PCR analysis with cDNAs from HeLa (H) and MRHF (M) cells and genomic DNA from SiHa cells (S) and with different sets of PCR primers flanking the splice junctions in NFI-B3 (lanes 1–3) and NFI-B1/B2 (lanes 4–6). X represents a nonspecific PCR product amplified from HeLa cDNA. Marker VI, Boehringer Mannheim, was used as a size marker (lane 7). The NFI-B3 mRNA arises from splicing the 5' exon to a new exon with an internal polyadenylation site. Alternatively, a polyadenylation signal in the adjacent intron may have been used to terminate the NFI-B3 transcript. To distinguish between these two hypotheses we performed PCR analysis on cDNA and genomic DNA with PCR primers flanking the potential splice junction site. If the novel sequence were derived from an intron contiguous to the sequence encoding the DNA binding domain, the PCR should generate identical fragments in cDNA and genomic DNA; however, if alternative splicing is employed, the PCR product from genomic DNA should be larger than the cDNA product, or, in case of very large intron, undetectable. PCR primer YB14 was derived from the known exon sequence, and primer YB15 from the novel sequence as indicated in Fig. 1A. A PCR product of 267 bp was amplified from HeLa cDNA (Fig. 1B, lane 1), MRHF cDNA (lane 2), and SiHa genomic DNA (lane 3). Sequencing analysis showed that the amplified fragment was identical in both genomic DNA and cDNAs. The specificity of the reaction was tested by a PCR performed with the reaction mix, including the primers, but substituting water for the DNA template. No product was amplified (data not shown). In control experiments PCRs on the same genomic DNA and cDNA preparations, primer YB14 was paired with primer YB31, derived from the known exon sequence of NFI-B1 and NFI-B2 (Fig. 1A). A PCR product of the expected size of 163 bp could be amplified from the cDNA preparations (lanes 4 and 5) but not from genomic DNA (lane 6), confirming the presence of an intronic sequence between these two exons. These PCR results show that the NFI-B3 mRNA is generated by using a polyadenylation site present in the intron adjacent to the intermediary coding sequence, a mechanism thus far not described for any other NFI isoform.

The NFI-B3 mRNA Differs from the mRNA for the NFI-B1 and NFI-B2 Spliced Isoforms—The expression of human NFI-B isoforms in epithelial and fibroblast cell lines as well as in different human tissues was analyzed by Northern blot hybridization. Filters containing poly(A)+ RNA from HeLa epithelial cells (Fig. 2A, lane 1), MRHF fibroblasts (lane 2), and different human tissue samples (lanes 3–10) were hybridized with the novel 3' sequence of the NFI-B3 cDNA. One mRNA species of about 1.8 kb was detected in both cell lines in similar amounts and with significant quantitative variations in different human tissues.

To confirm the identity of the 1.8-kb message the filters were reprobed with a 5' fragment of NFI-B3. Since the first 550 bp of the coding region are highly similar among all members of the NFI family, we used the 5'-untranslated region of NFI-B3, which is conserved within the NFI-B family but which has no known homology to the NFI-A, -C, and -X cDNAs. A major mRNA species of about 8.7 kb was detected in HeLa cells and different tissues (Fig. 2B, lanes 1 and 3–10) but not in MRHF fibroblast cells (Fig. 2B, lane 2). This 8.7-kb mRNA was also detected by reprobing the filter with the full-length NFI-B2 cDNA (data not shown), and a message of similar length has been described previously for NFI-B1 in hamster liver cells (13). The 1.8-kb mRNA was observed with the 5’ probe in all lanes only after longer exposure of the filter because of the lower expression of this message. In several human tissues and HeLa cells NFI-B3 mRNA is expressed about 6-fold lower than the 8.7-kb NFI-B1 and NFI-B2 mRNA, whereas it is the only detectable NFI-B mRNA in MRHF fibroblasts. The absence of the 8.7-kb message in fibroblast cells correlates with our observation from PCR analysis that NFI-B1 and NFI-B2 are not expressed or only at extremely low levels in human fibroblast cells. Fig. 2C shows the same filter probed with β-actin as control.

DNA Binding and Dimerization Properties of NFI-B3—The truncated NFI-B3 protein lacks a transcriptional activation domain but contains all four cysteine residues essential for DNA binding of full-length NFI proteins (39). To confirm whether the truncated NFI-B3 is able to bind DNA and to
NFI-B3 and NFI-B1/B2 mRNA abundance in human cell lines and human tissues. Northern blot filters contained poly(A)^+ RNA from HeLa epithelial (lane 1) and MRHF fibroblast (lane 2) cell lines or different human tissues: heart (lane 3), brain (lane 4), placenta (lane 5), lung (lane 6), liver (lane 7), skeletal muscle (lane 8), kidney (lane 9), and pancreas (lane 10). Filters were probed under highly stringent hybridization conditions with a labeled 3' fragment of the NFI-B3 cDNA (panel A), a labeled fragment of the 5'-untranslated region common to all NFI-B isoforms (panel B), or a β-actin probe as a control (panel C).

![Figure 2](image1.png)

**Fig. 2.** NFI-B3 and NFI-B1/B2 mRNA abundance in human cell lines and human tissues. Northern blot filters contained poly(A)^+ RNA from HeLa epithelial (lane 1) and MRHF fibroblast (lane 2) cell lines or different human tissues: heart (lane 3), brain (lane 4), placenta (lane 5), lung (lane 6), liver (lane 7), skeletal muscle (lane 8), kidney (lane 9), and pancreas (lane 10). Filters were probed under highly stringent hybridization conditions with a labeled 3' fragment of the NFI-B3 cDNA (panel A), a labeled fragment of the 5'-untranslated region common to all NFI-B isoforms (panel B), or a β-actin probe as a control (panel C).

NFI-B3 (lanes 6 and 7). In separate cotransfection assays, we observed that NFI-B3 could also form a complex of intermediate mobility with other NFI family members such as NFI-X1, NFI/CTF-2, and NFI/CTF-3 (data not shown). Although the DNA binding capacity of NFI-B, NFI-C, and NFI-X homodimers was decreased to a similar extent by NFI-B3, the amount of newly formed DNA binding complexes varied significantly (compare lanes 3, 5, and 7). The migration pattern of the newly formed complexes correlates with the considered sizes of heterodimeric protein moieties as assumed NFI-X2-NFI-B3 complexes migrate with faster mobility than NFI/CTF-1-NFI-B3 complexes (compare lane 5 and lane 7). The specificity of the newly formed complexes was confirmed by competition studies with unlabeled wild-type and mutant NFI binding sites (data not shown).

These experiments show that NFI-B3, which lacks the transcriptional activation domain of other NFI-B family members, cannot bind DNA on its own; however, it can heterodimerize with other NFI proteins, containing a transcriptional activation domain.

**Fig. 3.** DNA binding analysis of different NFI homo- and heterodimers. Panel A, in vitro transcription/translation showing the expression of proteins with molecular weights as expected from the size of the open reading frame of the newly isolated human NFI-B3 and NFI-B2 cDNAs. Panel B, gel shift analysis with a labeled Ad-NFI binding site oligonucleotide and nuclear extracts from Drosophila SL-2 cells transfected with expression vectors for NFI-B3 (lane 1), NFI-B2 (lane 2), NFI-X2 (lane 4), and NFI/CTF-1 (lane 6). The binding capacity of potential heterodimers was examined by cotransfection of NFI-B3 with NFI-B2 (lane 3), NFI-X2 (lane 5), or NFI/CTF-1 (lane 7), respectively. X, free [35S]methionine.

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sites, was cotransfected with equal amounts of different NFI expression vectors into SL-2 cells. NFI-B3 alone did not affect the activity of the reporter construct (Fig. 4A, first two columns). Another NFI-B family member with a full-length activation domain, NFI-B2, stimulated CAT activity 23-fold, whereas coexpression of NFI-B2 and NFI-B3 led only to a 13-fold stimulation, a 1.7-fold reduction of the activation potential of NFI-B2 homodimers (Fig. 4A, second pair of columns). The activity of the NFI-X2 homodimers and NFI/CTF-1 homodimers was reduced by the interference of NFI-B3 homodimers (Fig. 4A, second pair and fourth pair of columns). All CAT activities given in Fig. 4 were averaged from at least three independent experiments.

Interestingly, the level of transcriptional reduction by the different heterodimers (NFI-B3-NFI-X2 > NFI-B3-NFI/CTF-1 > NFI-B3-NFI-B2) correlated with the amount of protein complexes bound to DNA (compare Figs. 3B and 4A).

The specificity of the transcriptional repression by NFI-B3 was determined by cotransfection of the transcription factor Sp1 with NFI-B3 and a reporter construct, pBCAT2, which contains two Sp1 binding sites. The activity of Sp1 was not affected by NFI-B3 (Fig. 4A), indicating that NFI-B3 specifically inhibited the transactivation potential of NFI proteins.

**NFI-B3 Inhibits the Activation Potential of Full-length NFI Proteins in a Concentration-dependent Manner**—To study the function of heterodimers between the truncated NFI-B3 and full-length NFI we cotransfected equal amounts of the expression vectors (see above), which gave rise to similar amounts of the DNA-bound proteins (see Fig. 3). Northern blot data shown in Fig. 2 had indicated, however, that NFI-B3 is expressed in vivo in lower amounts than the other NFI proteins in most human tissues. To examine whether NFI-B3 can repress the activity of other NFI proteins at low concentrations we performed a titration experiment. A constant amount of NFI/CTF-1 expression vector (1.5 μg) and increasing amounts (0.1–1.5 μg) of NFI-B3 expression vector were cotransfected with 2 μg of the poCAT3xAd construct into SL-2 cells, followed by the determination of CAT activity. Fig. 4B, first column, shows the background activity of the CAT reporter construct alone, the effect of cotransfected NFI-B3 (second column), and NFI/CTF-1 alone (third column) or increasing amounts of NFI-B3 with constant amounts of NFI/CTF-1 (fourth through eighth columns). These data show that although 1.5 μg of cotransfected NFI-B3 plasmid reduced the CAT activity almost to background levels, there was still a significant suppression of the NFI-C-stimulated CAT activity with 15-fold less (0.1 μg) NFI-B3 vector.

**NFI-B3 Represses NFI-dependent Transcription in Human Epithelial and Fibroblast Cell Lines**—Our previous studies had shown that human epithelial cells express NFI proteins of the NFI-C family, whereas human fibroblasts express NFI-C and NFX isoforms. To confirm that NFI-B3 also interferes with the endogenous NFI proteins in their natural environment, we overexpressed NFI-B3 in HeLa epithelial and MRHF fibroblast cell. The CAT activity of the reporter construct poCAT3xAd in HeLa cells (Fig. 5, lane 1) was down-regulated by exogenous NFI-B3 expression 2.4-fold (lane 2), and in MRHF cells 3.3-fold (lanes 3 and 4). The expression of high levels of NFI-X proteins in MRHF cells (24) may account for the stronger effect of
NFI-B3 in these cells since NFI-X2-NFI-B3 heterodimers also showed the strongest level of repression in SL-2 cells.

**DISCUSSION**

Transcriptional control plays a central role in determining the level of gene expression in various tissues during development and differentiation. Fundamental to such intricately controlled processes are the interactions of protein factors with specific sequence motifs in the promoter region and the interplay of these factors with components of the general transcriptional machinery. Specific sequence motifs often may be bound by related proteins with varying activation and repressing potentials, increasing the flexibility of transcriptional regulation to disparate cellular cues. Several mechanisms have been described which generate proteins with similar DNA binding specificity but different functions. Discrete isoforms can be transcribed from different, but related genes. Alternatively, a single gene can give rise to several distinct proteins by alternative RNA splicing or differential use of initiation codons (for review, see Ref. 40).

NFI constitutes such a family of transcription factors comprised of multiple subtypes. The variation among all NFI proteins isolated so far is the result of transcription from four different genes as well as alternative RNA splicing within the subfamilies. All differentially spliced RNA products characterized so far were produced by alternative exon usage affecting the transcriptional activation domain. We have identified a new member of the NFI family, designated NFI-B3, which is generated by an alternative mechanism of RNA processing. The first intron after the proposed DNA binding domain, which is conserved among all NFI proteins, is maintained, and a polyadenylation site in the internal intron is used to terminate the transcript. This mechanism has not been described for NFI proteins before but is also used to generate functionally different isoforms of the hepatocyte nuclear factor I family (41). Transcripts generated by this mechanism have not been described so far from the NFI family. The nucleotide sequence of the exon/intron junction in NFI-B3 which leads to the alternative transcripts is not conserved among the different NFI genes. A splice junction has, however, also been documented for the NFI-C (15) and NFI-A (42) genes in a similar position separating the conserved DNA binding domain from the divergent transactivation domains. A truncated human NFI-C cDNA (hCTF4), previously isolated from HeLa cells, contains 10 amino acids divergent from all known exon sequences of the NFI-C gene (15) in a position comparable to the novel sequences of NFI-B3, which possibly represents a NFI-B3 equivalent in the NFI-C family.

NFI-B3 contains the sequences thought to be sufficient for DNA binding and dimerization but lacks the proline-rich activation domain. Proteins with similar characteristics from other transcription factor families have been found to function as transcriptional repressors either by competing with activators for a common binding site or by forming heterodimers with activators and thereby reducing their binding activity or their ability to activate transcription (43, 44). Expression of NFI-B3 in SL-2 cells, which are void of endogenous NFI proteins, had no effect on the activity of a NFI binding site-containing reporter construct. NFI-B3 repressed, however, the activity of other NFI transcriptional activators when coexpressed in SL-2 cells, as well as the activity of endogenous NFI proteins when overexpressed in HeLa and MRHF cells.

Gel shift analysis showed that the interference with the activity of the transcriptional activators occurs in two different ways. NFI-B3 reduces the binding activity of an activator protein from the NFI-B subgroup, possibly because of the formation of heterodimers that cannot bind DNA. The activation potential of NFI-C and NFI-X proteins, however, is apparently reduced by the formation of heterodimers with NFI-B3 which retain the capacity to bind DNA. The potential for heterodimer formation among different NFI family members has been demonstrated recently in vitro (45). NFI-B3 contains 186 amino acids of the highly conserved NH₂-terminal portion of NFI as well as two novel residues. Studies with deletion mutants of the NFI-C and NFI-A gene (1, 2) have demonstrated earlier that the regions essential for DNA binding and dimerization lie within the NH₂-terminal 220 amino acids. The minimum region may have to be redefined, however, because amino acids 178–202 can be spliced to yield NFI/CTF-3, which can still form dimers and bind to DNA (1). A protein from a deletion mutant of the NFI-A gene which encodes only the first 186 amino acids of NFI, similar to NFI-B3, could neither bind to DNA nor form heterodimers with the wild-type protein containing the activation domain (4), suggesting that such a truncated protein has no functional role at all. The naturally occurring NFI-B3, which contains only two additional amino acids, similarly cannot bind to DNA probably because of a failure to form homodimers. In contrast to the NFI-A deletion mutant, NFI-B3 apparently forms heterodimers with other NFI proteins thereby interfering with their function. Our observation that the heterodimers of NFI-B3 with other proteins derived from the NFI-B, NFI-C, and NFI-X genes have different DNA binding affinities, indicates a potential contribution of the COOH-terminal region to DNA binding. Functional data furthermore suggest that a single activation domain in a heterodimer may not be sufficient for transcriptional activation.

The expression of all NFI-B isoforms, as examined by Northern blot analysis, varies significantly among different human tissues, with the highest levels of both the 8.7-kb message for NFI-B1 and NFI-B2 and the 1.8-kb message for NFI-B3 being observed in heart and skeletal muscle. No 8.7-kb mRNA could be detected in human fibroblast cells. This differential expression pattern suggests NFI-B factors to be one out of several sources for cell type-specific transcriptional differences observed with promoters and enhancers with NFI binding sites. It has been shown recently that NFI-B and NFI-C genes are up-regulated by thyroid hormones during metamorphic transition in X. laevis development (46), suggesting that these factors are important for postembryonic organ development. As both genes can potentially give rise to truncated proteins with repressing functions (see above) it would be interesting to examine the possible contribution of NFI-B3 and any potential counterpart of the NFI-C family in organ development.

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**REFERENCES**

1. Mermod, N., O’Neill, E. A., Kelly, T. J., and Tian, R. (1989) *Cell* 58, 741–753
2. Gounari, F., De Francesco, R., Schmitt, J., van der Vleit, P. C., Cortese, R., and Stunnenberg, H. (1990) *EMBO J.* 9, 559–566
3. Nagata, K., Guggenheimer, R. A., and Hurwit, J. (1983) *Proc. Natl. Acad. U. S. A.* 80, 6177–6180
4. Mull, Y. M., and Van der Vleit, P. C. *EMBO J.* 11, 751–760
5. Armentero, M. T., Hurwit, M., and Mermod, N. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 11537–11541
6. Plumb, M., Fulton, R., Breimer, L., Stewart, M., Willison, K., and Neil, J. C. (1991) *J. Virology* 65, 1991–1999
7. Mink, S., Hartig, E., Jennewein, D., Duppler, W., and Cato, A. C. (1992) *Mol. Cell. Biol.* 12, 4986–4990
8. Kumar, K. U., Pater, A., and Pater, M. M. (1993) *J. Virology* 67, 572–576
9. Apt, D., Chong, T., Liu, Y., and Bernard, H. U. (1993) *J. Virology* 67, 4455–4463
10. Cereghini, S., Raymondjean, M., Carranca, A. G., Herbomel, P., and Yaniv, M. (1987) *Cell* 50, 627–638
11. Rossi, P., Karsenty, G., Roberta, A. B., Roche, N. S., Sporn, M. B., and de Crombrugghe, V. (1988) *Cell* 52, 405–414
12. Santoro, C., Mermod, N., Andrews, P. C., and Tian, R. (1988) *Nature* 334, 218–224
13. Gil, G., Smith, J. R., Goldstein, J. L., Slaughter, C. A., Orth, K., Brown, M. S., and Osborne, T. P. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 8963–8967
Transcriptional Repressor, NFI-B3

14. Paonessa, G., Gounari, F., Frank, R., and Cortese, R. (1988) *EMBO J.* 7, 3115–3123
15. Meisterernst, M., Rogge, L., Foeckler, R., Karaghiosoff, M., and Winnacker, E. L. (1989) *Biochemistry* 28, 8191–8200
16. Kruse, U., Qian, F., and Sippel, A. E. (1990) *Nucleic Acids Res.* 18, 2607–2616
17. Kruse, U., Qian, F., and Sippel, A. E. (1991) *Genomics* 10, 567–571
18. Qian, F., Kruse, U., Lichter, P., and Sippel, A. E. (1995) *Genomics* 28, 66–73
19. Altmann, H., Wendler, W., and Winnacker, E. L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 3901–3905
20. Tamura, T., Aoyama, A., Inoue, T., Misawa, M., Okano, H., and Mikoshiba, K. (1988) *Mol. Cell. Biol.* 9, 3122–3126
21. Inoue, T., Tamura, T., Furuichi, T., and Mikoshiba, K. (1990) *J. Biol. Chem.* 265, 19065–19070
22. Graves, R. A., Tontonoz, P., Ross, S. R., and Spiegelmann, B. M. (1991) *Genes Dev.* 5, 428–437
23. Jackson, A. D., Rowerder, K. E., Stevens, K., Jiing, C., Milos, P., and Zaret, K. S. (1993) *Mol. Cell. Biol.* 13, 2401–2410
24. Apt, D., Liu, Y., and Bernard, H. U. (1994) *Nucleic Acids Res.* 22, 3825–3833
25. Wenas, S., Altmann, H., Wendler, W., and Winnacker, E. L. (1996) *Nucleic Acids Res.* 24, 2416–2421
26. Nebl, G., and Cato, A. C. (1995) *Cell. Mol. Biol. Res.* 41, 85–95
27. Sumner, C., Shinohara, T., Durham, L., Major, E. O., and Amemiya, K. (1996) *J. Neurosci.* 2, 87–100
28. Roulet, E., Armentero, M. T., Krey, G., Corthesy, B., Dryer, C., Mermod, N., and Wahl, W. (1995) *Mol. Cell. Biol.* 15, 5552–5562
29. Xiao, H., Lis, J. T., Xiao, H., Greenblatt, J., and Friesen, J. D. (1994) *Nucleic Acids Res.* 22, 1966–1973
30. Wendler, W., Altmann, H., and Winnacker, E. L. (1994) *Nucleic Acids Res.* 22, 2601–2603
31. Alevizopoulos, A., Dusserre, Y., Tsai-Pflugfelder, M., von der Weid, T., Wahl, W., and Mermod, N. (1995) *Mol. Cell. Biol.* 65, 3122–3126
32. Qian, F., Kruse, U., Lichter, P., and Sippel, A. E. (1995) *Genomics* 10, 567–571
33. Kruse, U., Dusserre, Y., Tsai-Pflugfelder, M., von der Weid, T., Wahl, W., and Mermod, N. (1995) *Mol. Cell. Biol.* 65, 3122–3126
34. Xiao, J. H., Davidson, I., Matthes, H., Garnier, J. M., and Champon, P. (1991) *Cell.* 65, 551–568
35. Hagen, G., Müller, S., Beato, M., and Suske, G. (1994) *EMBO J.* 13, 3843–3851
36. Chan, W. K., Klock, G., and Bernard, H. U. (1989) *J. Virol.* 63, 3261–3269
37. Schreiber, E., Matthis, P., Müller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* 17, 6419
38. Jackson, I. J. (1991) *Nucleic Acids Res.* 19, 3795–3798
39. Novak, A., Guyal, N., and Gronostajski, R. M. (1992) *J. Biol. Chem.* 267, 12986–12990
40. Feulner, N. S., and Sassone-Corsi, P. (1992) *Cell.* 68, 411–414
41. Bach, I., and Yaniv, M. (1993) *EMBO J.* 12, 4229–4242
42. Kruse, U., and Sippel, A. E. (1994) *J. Mol. Biol.* 238, 860–865
43. Jones, N. (1990) *Cell.* 61, 9–11
44. Jones, N. (1991) *Curr. Biol.* 1, 224–226
45. Kruse, U., and Sippel, A. E. (1994) *FEBS Lett.* 348, 46–50
46. Puzianowska-Kuznicka, M., and Shi, Y.-B. (1996) *J. Biol. Chem.* 271, 6273–6282
