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Cloning and functional analysis of spliced isoforms of human nuclear factor I-X: interference with transcriptional activation by NFI/CTF in a cell-type specific manner

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ABSTRACT

Previous studies of the epithelial specificity of the human papillomavirus type 16 (HPV-16) enhancer pointed to an important role of nuclear factor I (NFI). In epithelial cells, NFI proteins are derived from the NFI-C gene and referred to as NFI/CTF. In contrast, fibroblasts, where the enhancer is inactive, express high levels of NFI from the NFI-X gene. To compare NFI-C and NFI-X derived transcription factors, we cloned and functionally investigated two differentially spliced forms of NFI-X from human fibroblasts. NFI-X1 has 95% homology with a transcript previously identified in hamster liver cells. NFI-X2, a spliced variant, misses 41 amino acids of the proline-rich activation domain. NFI-X expression, examined by Northern blots, shows strong cell-type specific variation in comparison with NFI/CTF. While the transcriptional activation domain of NFI-X2, functionally tested as GAL4-fusion protein in epithelial and fibroblast cells, activates transcription from promoter as well as enhancer position similar to NFI/CTF-1, the activation domain of NFI-X1 fails to activate transcription from enhancer position. In *Drosophila* cells, void of endogenous NFI proteins, full length NFI/CTF-1 and NFI-X2 activate a reporter construct containing only NFI sites as well as the NFI dependent HPV-16 enhancer. In contrast, NFI-X1 fails to activate the HPV-16 enhancer. Furthermore, overexpression of NFI-X1 in epithelial cells down-regulates the HPV-16 enhancer. Our findings suggest that the family of NFI transcription factors should not be viewed as constitutive activators, but rather, that NFI-C and NFI-X have divergent functions after binding in promoter or enhancer position. This property, combined with the differential expression of NFI-X, can achieve cell-type specificity of NFI dependent promoters and enhancers.

INTRODUCTION

The designation NFI stands for a family of transcription factors essential for the optimal expression of several cellular as well as viral genes (1–3). Originally, NFI was identified because of its importance in directing the initiation of adenovirus DNA synthesis by binding to a consensus recognition sequence 5′-TGG(A/C)N_5GCCAA-3′ (4,5). NFI proteins bind their consensus motif as dimers which are already formed in solution (6). Different NFI proteins were first cloned from human HeLa cells (7) and subsequently from hamster (8), rat (9), pig (10), and chicken (11). Sequence homology led to the identification of four genes, NFI-A, B, C and -X, which are coding for the various NFI proteins described so far. The diversity of NFI proteins is further increased by differential RNA splicing leading to different NFI proteins even in a given family (7,11). The N-terminal DNA binding and dimerization domains of all NFI proteins are highly conserved, with five cysteine residues in specific positions being essential for DNA binding (12). Conversely, the C-terminal proline-rich transactivating domains are heterogeneous, both among factors derived from different genes as well as between different spliced forms. Some reports showed that NFI binding sites play a critical role in the expression of tissue specific genes (13,14) but did not describe any tissue specific variants of NFI. In our research on the enhancers of human papillomaviruses we observed cell-type specificity of NFI dependent functions that correlated with variations of NFI proteins (15).

The long control regions of all human papillomaviruses that have been examined contain epithelial-specific enhancers that correlate with NFI clusters (16,17). In the enhancer of human papillomavirus type 16 (HPV-16), seven half palindromic NFI binding sites of the sequence 5′-TTGGC-3′ make this element the most abundant transcription factor binding site. Epithelial-specificity was found to be even retained by non-overlapping subclones of the HPV-16 enhancer, which have only NFI sites in common. Mutations in any of these NFI sites resulted in loss

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of enhancer activity. Activation, or lack of activation of the HPV-16 enhancer, correlated with cell-specific subsets of NFI proteins formed upon binding to these NFI sites. Bandshift patterns with nuclear extracts from epithelial cells differed from those obtained with fibroblasts, which indicated that different members of the NFI family may be expressed in these two cell lines. Three different spliced forms of the NFI-C family, namely CTF-1, CTF-2 and CTF-3 could be cloned from HeLa cells (7). Exogenous expression of these three isoforms in SL-2 cells, void of endogenous NFI, led to similar DNA-binding profiles observed in epithelial cells (15), which suggests, that CTF-1, CTF-2 and CTF-3 are the predominant forms expressed in epithelial cells. Furthermore, we could show in our previous analysis, that human fibroblasts express, in addition to NFI-C, the NFI-X gene. NFI-X transcripts had originally been cloned from hamster liver cells (8) and from a chicken promacrophage library (18).

To extend our understanding of the function of different transcription factors derived from the NFI gene family, we decided to clone and functionally analyze human NFI-X transcripts. Here we report the isolation of a full length NFI-X cDNA, and an additional spliced form, both cloned from human fibroblast cells, and we compare the expression of NFI-X and NFI/CTF mRNA in epithelial and fibroblast cells and in different human tissues. To test our hypothesis, that NFI-X proteins have functional properties different from NFI/CTF, we studied the function of these proteins in *Drosophila* SL-2 cells, which lack endogenous NFI. Beyond this, we made a series of fusion protein constructs based on the GAL4 DNA binding domain fused to the proline rich activation domain of NFI-X and NFI/CTF-1, which allowed us to study the different activation domains independently of the endogenous NFI background in human cells. Overexpression of NFI-X in HeLa cells, which contain only very low amounts of NFI-X mRNA, demonstrates the interference of NFI-X with the activation potential of NFI/CTF.

MATERIALS AND METHODS

Plasmid constructs

The CAT-reporter constructs pHPV16/91mer, derived from the HPV-16 enhancer, has been described before (15) The plasmid pαCAT3XAd (6), which contains the α-globin promoter and three palindromic NFI sites, and the CTF expression plasmids p113-CTF-1 (19) were a gift from N.Mermod. To construct the expression clone pXJ-NFI-X1, the full length cDNA of NFI-X was isolated by *Eco*RI digestion of the respective phage lambda DNA and subcloned in the *Eco*RI site of a CMV driven expression plasmid pXJ40 (20), a gift from P.Chambon. The plasmid pXJ-NFI-X2 was generated by ligation of a *Eco*RI/*Cla*I fragment from NFI-X1 (spanning 5′ *Eco*RI cloning site and *Cla*I restriction site at position 842 bp), containing the conserved DNA binding domain, and a *Cla*I/*Eco*RI fragment (position 842 to 3′ *Eco*RI cloning site) of NFI-X2, containing the activation domain, into the *Eco*RI site of pXJ40. The OVEC-constructs p1GAL/SV40 and pβ/5GAL, containing 1 or 5 GAL4 binding sites in 'promoter' or 'enhancer' position, respectively, the reference plasmid pOVEC-Ref and pSP6βTS were a generous gift from W.Schaffner and are described elsewhere (21). To generate the expression clone p[GAL4]−NFI-X1*c*, which contains the DNA binding domain of GAL4 and 160 amino acids of the transcription activation domain of NFI-X1, a *Cla*I/*Eco*RI fragment (*Cla*I site at position 842 to 3′ *Eco*RI cloning site) was isolated from cDNA clone NFI-X1, blunt ended using the Klenow fragment of *Pol*I and cloned in frame in the single *Sma*I site of pSCTEVGAL(1-93) (21). The expression clones p[GAL4]−NFI-X1 and p[GAL4]−NFI-X2 were generated by isolating a 626 bp and a 503 bp polymerase chain reaction (PCR) fragment from cDNA clone NFI-X1 and NFI-X2 respectively, using PCR primers Do112 and Do5. The location of the 23 bp long primers (position 723 bp at the 5′ end and position 1349 bp at the 3′ end) is shown in Figure 1B. The construct pSCTEVGAL4(1-93) containing the DNA binding domain of GAL4, and the plasmids pSCTEVGAL-CTF(399-49), and pSCTEVGAL-VP16(80), containing 100 amino acids of the activation domain of NFI/CTF-1 and 80 amino acids of VP16 fused to the DNA binding domain of GAL4, respectively (21), were a gift from W.Schaffner.

Library screening and DNA sequencing

Two commercially available human skin fibroblast cDNA libraries (Clontech H1052a, H1052b) were screened with a previously isolated 1.2 kb cDNA clone FN6, coding for human NFI-X under hybridization conditions as described (15). For high stringency screening 50% formamide (v/v) was used and the filters were finally washed at 65°C. After three rounds of rescreening, cDNA products were isolated by PCR, subcloned in pUC19 and both strands of the DNA were sequenced as described (15).

Reverse transcriptase-coupled PCR analysis of different splice products

First strand cDNA was prepared from total RNA and mRNA of HeLa and MRHF cells as described earlier (15). The following primer pairs were used for PCR analysis: Do110−Do5: position 1−1349 bp, Do110−Do111: position 1−404 bp, Y3−Do5: position 350−1349 bp, Y4−Do5: position 550−1349, Do112−Do5: position 723−1349 bp (Fig. 1B). All primer oligonucleotides were 23 bp in length including *Eco*RI cloning sites. PCR reactions were performed as described (15) and the reaction products were separated on a 1.5% agarose gel. The specificity of the PCR products was verified by Southern blot analysis, and positive fragments were subcloned into the *Eco*RI sites of pUC19 and sequenced as described (15).

Northern blot analysis

Total RNA from different epithelial and fibroblast cell lines was isolated by CsCl/guanidium gradient centrifugation (22) and preparation of poly(A)$^+$ mRNA was carried using PolyATtract mRNA isolation system (Promega). About 1.5 μg poly(A)$^+$ RNA of each cell line were separated in a 1 % agarose gel and blotted on nylon membrane (Hybond-N, Amersham) in 10×SSC (1×SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7). The Multiple Tissue Northern (MNT) Blot (Clontech), contains about 1 μg poly(A)$^+$ RNA for each tissue. The filters were probed with a 631 bp *Bst*XI/*Eco*RI fragment of p113-CTF-1 in a hybridization solution containing 0.5 M sodium phosphate pH 7.9, 7 % SDS, 15 % formamide and 10^6 c.p.m./ml of the random primed labeled (Random primed DNA labeling kit, Boehringer Mannheim) DNA probe. After 16 h hybridization at 65°C, the filters were washed 3×20 min in 50 mM sodium phosphate pH 7.9/0.1% SDS at 65°C. The filters were reprobed with a random labeled 753 bp *Bam*H1/*Eco*RI fragment of NFI-X, after stripping off the NFI/CTF probe for 5 min at 95°C in

10 mM sodium phosphate/0.1% SDS solution. Quantification of the radioactive signals was performed on the PhosphorImager using the integrated volume function.

Cell culture, transfection, CAT assays

All cell lines were cultured using standard procedures (23). To study the effect of NFI-X overexpression in HeLa cells on the HPV-16 enhancer HeLa and MRHF cells were transfected by electroporation with a Bio-Rad Gene Pulser. Ten micrograms of reporter plasmid were cotransfected with 1−5 μg of effector plasmid. CAT assays followed procedures previously described (24). *Drosophila* Schneider cells, SL-2, were grown and transfected as described (15). Prior to transfection the cells were plated at a density of 10^6 cells/ml and a total of 20 μg of DNA, containing 5 μg of each expression vector and/or reporter plasmid and pUC19 plasmid as carrier were used for transfection. Cells were harvested 36−38 h after transfection and 50 μg of protein were used for CAT assays.

RNAse protection analysis

HeLa and MRHF cells were transfected by the calcium phosphate coprecipitation procedure (24) with 10 μg reporter plasmid, 5 μg transactivator plasmid, 1 μg reference plasmid and 4 μg pUC19 carrier DNA. The cells were harvested 45 h after transfection and cytoplasmic RNA was extracted according to Schreiber *et al.* (25). This method allowed also the extraction of nuclear proteins to check the expression of the transcription factor fusion proteins in bandshift analysis. Twenty micrograms of total RNA was used for hybridization to a radioactive complementary strand RNA generated by SP6 polymerase according to the manufacturer's recommendation (Riboprobe Gimini II core system, Promega). Hybridization and RNAse digestion were performed using standard procedures (22). The hybrids were separated on a 6% polyacrylamide/7 M urea gel. The quantification of the radioactive incorporation was performed on the PhosphorImager using the integrated volume function.

Nuclear extracts and DNA binding assay

HeLa cells were transfected with 10 μg of pXJ-NFI-X1 and pXJ-NFI-X2 and nuclear extracts were prepared according to Schreiber *et al.* (25). About 10^6 cells were used for each preparation and nuclear proteins were dissolved in a final volume of 50 μl. For band shift assays, 1 μl of nuclear extracts were incubated with 3 μg of poly(dI-dC) in a buffer containing 4 mM spermidine, 5 mM $MgCl_2$ and 100 mM KCl for 10 min on ice. After adding 5000 c.p.m. of α-^{32}P- labeled AdNFI-oligo (23) the reaction mix was further incubated for 25 min. The complexes were separated on a 5% PAA/0.25×TBE gel.

RESULTS

Analysis of two alternatively spliced variants of NFI-X cDNA clones expressed in human fibroblast cells

We previously reported the cloning of a NFI cDNA clone, FN6, from a human fibroblast cDNA library (15). This clone aligned best to *ha*NFI-X (GenBank J04123) isolated from hamster liver cells. FN6 was used for further screening of 5×10^6 phage lambda clones from human fibroblast cDNAs. Another seven cDNA clones coding for NFI-X proteins were isolated and partially sequenced. Six cDNA clones were homologous on nucleic acid sequence level to cDNA FN6, but of different size.

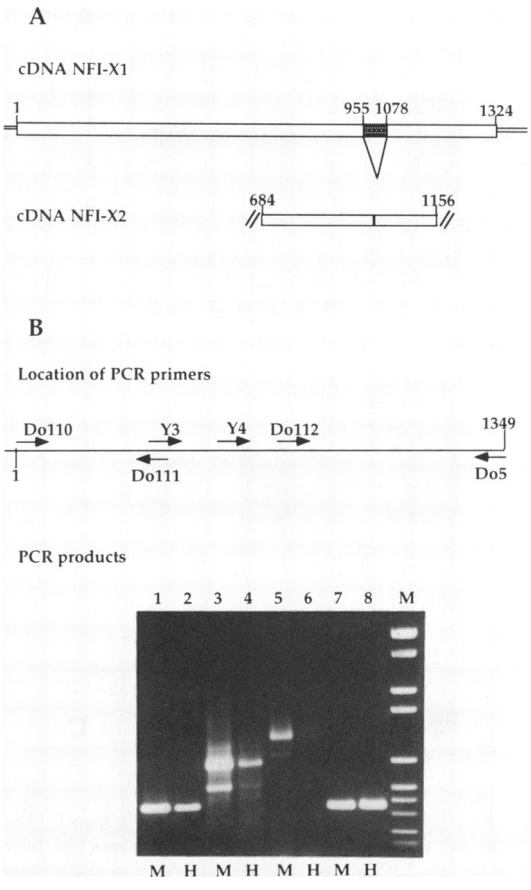

Figure 1. Cloning and PCR analysis of two differentially spliced cDNAs of human NFI-X. Schematic representation of two cDNAs cloned from a human fibroblasts cDNA library (**A**). cDNA NFI-X1 contains a full length open reading frame, while cDNA NFI-X2 contains a partial sequence. The boxed area between position 955 and 1078 shown in cDNA NFI-X1 indicates the exon, which is spliced out in cDNA NFI-X2. (**B**) PCR analysis of MRHF (M) and HeLa (H) cDNA using different sets of PCR primers spanning the open reading frame of NFI-XI. Primers pair Do110 and Do111 (1 and 2), Do112 and Do5 (3 and 4), Y4 and Do5 (5 and 6). Primers spanning the POU domain of octamer transcription factors (lanes 7 and 8) were used as a control. The molecular weight (Marker VI, Boehringer Mannheim) is shown in the first lane.

The nucleic acid sequence of the longest 1.5 kb cDNA, NFI-X1 (GenBank accession no. L31881), shows 95% homology in the coding region to *ha*NFI-X. Most of the variations are conservative changes on the nucleotide sequence level and result in only two amino acid variations between the hamster and the human proteins.

Restriction digestion of another cDNA clone, NFI-X2, indicated a potentially different spliced product. The sequence of NFI-X2 revealed that it lacks a 123 bp segment within the 3′ coding region of NFI-X1, which is part of the proline rich transcription activation domain. The splicing occurred at the sequences AG/GC at position 955 bp and AG/GG at position 1078 bp. In contrast to NFI/CTF, where alternative splicing generates NFI proteins with unrelated amino acids at the C-terminal end (7), NFI-X2 uses the same reading frame as NFI-X1 downstream of the 41 amino acids, which are lost due to splicing. Both cDNAs are presented as a schematic diagram in

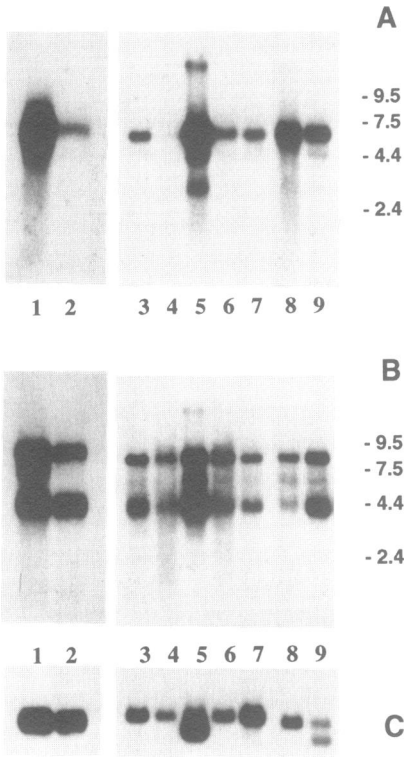

Figure 2. NFI-X and NFI/CTF-1 mRNA abundance in human fibroblast and epithelial cells and in different human tissue. The Northern blot filters contain about 1.5 μg poly(A)$^+$ RNA from MRHF fibroblast and HeLa epithelial cells (lanes 1 and 2) and 1 μg poly(A)$^+$ RNA of different human tissues: pancreas (lane 3), kidney (4), skeletal muscle (5), liver (6), lung (7), brain (8) and heart (9). Filters were probed under stringent hybridization conditions with random labeled fragments of the transcriptional activation domains of NFI-X (**A**) and NFI-CTF-1 (**B**) and β-actin cDNA (**C**). Figures on the right site represent the sizes of the RNA marker in kilobases.

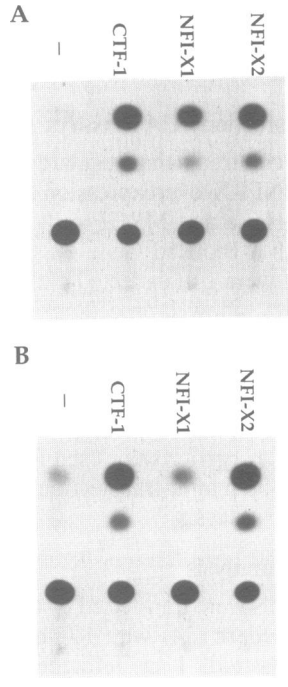

Figure 3. Activation potential of NFI-X proteins in *Drosophila* SL-2 cells, which lack endogenous NFI proteins. CAT assays of mock transfected SL-2 cells (−) and SL-2 cells transfected with the transcriptional activators NFI-X1, NFI-X2 and NFI/CTF-1. (**A**) Cotransfected with the reporter construct pαCAT3XAd, containing the α-globin promoter and three additional palindromic Ad NFI sites. (**B**) Cotransfected with a reporter construct containing the viral HPV-16/91mer enhancer fragment, which contains two half palindromic NFI sites and additional binding sites for AP-1 and TEF-1/TEF-2.

Figure 1A. The dotted box in cDNA NFI-X1 indicates the exon which is spliced out in cDNA NFI-X2.

cDNA clone NFI-X2 encoded only a partial NFI-X sequence. To obtain a full-length NFI-X2 clone, and to examine the possible existence of additional spliced forms not detected during the screen of the phage lambda library, we analyzed RNA preparations from human fibroblasts by reverse transcription and PCR. Several sets of primers spanning the coding sequence of NFI-X1 (Fig. 1B) were used for the PCR reactions with cDNA as template that had been prepared from MRHF fibroblasts. Using primer pair Do110 and Do5, including the ATG start codon and the TGA stop codon, two bands of a size expected for NFI-X1 and NFI-X2 transcripts could be amplified (data not shown). Primers Do110, located at the 5′ end and Do111 at position 404 bp, lead to only one PCR product (Fig. 1B, lane 1), while using primers Y3 at position 350 bp (data not shown), Y4 at position 550 bp (Fig. 1B, lane 5) and Do112 at position 723 bp (Fig. 1B, lane 3) in combination with Do5, located at the 3′ end, resulted in two PCR products. This allowed us to locate the splicing event between positions 723 and 1349 bp. The two resulting PCR products were subcloned and sequenced. The sequences could be aligned with the two original phage lambda cDNA clones and confirmed that human fibroblasts express two forms of NFI-X proteins.

PCR analysis revealed significant quantitative differences of the PCR products specific for NFI-X1 (lanes 3 and 5, upper band) and NFI-X2 (lanes 3 and 5, lower band) and confirms the indication from library screening, namely that NFI-X1 is expressed at higher levels than NFI-X2.

We have previously published that PCR analysis of cDNA preparations from HeLa cells, which we use as a standard for epithelial cells, failed to detect full length transcripts of NFI-X (15). We confirmed this result with primer pair Do110 and Do5, which flank the full length coding region of NFI-X. In contrast, PCR primers flanking shorter segments resulted in reaction products similar to those amplified from MRHF cDNA (Fig. 1B, lanes 2, 4 and 6). While the relative abundance of NFI-X1 to NFI-X2 is similar to the one observed in MRHF cells, the total abundance of of both forms is much lower (compare lanes 1 and 2, 3 and 4, 5 and 6). As a control we used PCR primers specific for the POU domain of octamer transcription factors which gave rise to similar amounts of PCR products in both cell lines (Fig. 1B, lanes 7 and 8). From these observations and from Northern blotting (see below), we conclude that HeLa cells contain NFI-X mRNA, but only at such a low level that standard PCR reactions, which amplify larger DNA segments at lower efficiencies, do not produce detectable amounts of full length cDNA products. The specificity of all PCR products was confirmed by probing Southern blots with a 3′-DNA fragment of NFI-X, which codes for the heterogeneous transactivation domain and does not cross-react with other NFI family members (data not shown).

Distribution of NFI-X and NFI/CTF mRNAs in fibroblast and epithelial cell lines and different human tissues

The expression of NFI-X mRNA in epithelial and fibroblast cell lines as well as in different human tissues was analyzed by Northern blot and compared with the expression pattern of NFI/CTF. To reduce cross-hybridization of the two NFI species, the filters were examined by hybridization to 3′ fragments of NFI/CTF-1 and NFI-X cDNAs encoding the heterogeneous transactivation domains.

When RNA preparations from MRHF fibroblasts, HeLa cells (Fig. 2A, lanes 1 and 2) and tissue samples (lanes 3−9) were hybridized to the NFI-X probe, one RNA species of about 6.5 kb hybridizes specifically in all cases, while RNAs from human muscle (lane 5) and heart tissue (lane 9) show some additional RNA species of lower and higher molecular weights. The qualitative similar expression of the 6.5 kb transcript of NFI-X contrasts with quantitative differences in different cell lines and tissues (see below).

In contrast to the quantitative heterogeneity of NFI-X expression, we observed two RNA species of similar intensity in MRHF and HeLa cells (Fig. 2B, lanes 1 and 2) and in different human tissues (lanes 3−9) with sizes about 8.6 and 4.5 kb with the NFI/CTF probe. These two RNA species have been previously identified in HeLa cells (7). A third, weak band of about 6.5 kb is detected by the NFI/CTF probe in several lanes. The size of this RNA band is identical to the NFI-X transcript, and probably originates from a slight cross-hybridization of the NFI/CTF probe with NFI-X mRNA. For quantity control the filters were reprobed with a cDNA coding for β-actin (Fig. 2C).

While both NFI/CTF and NFI-X are expressed in all human cell lines and tissues that we examined in a qualitatively similar manner, NFI-X expression shows significant quantitative variations. NFI-X mRNA is expressed at a higher level than NFI/CTF in human fibroblast cells, muscle and brain tissue, while the levels of NFI-X are lower than NFI-CTF mRNA in epithelial cells and in kidney tissue. The amount of mRNA for NFI-X and NFI/CTF was quantified for HeLa and MRHF cells and normalized against β-actin levels, because these two cell lines were of special interest for us in that they support, or fail to support, activation of the NFI dependent enhancer of HPV16 (15). While NFI/CTF mRNA levels are of equal abundance in both cell lines, the level of NFI-X mRNA was 13 times higher in MRHF cells than in Hela cells. To confirm that the results obtained from HeLa cells are not an exception, we included in this study mRNA preparations from several cell lines of epithelial origin, namely SiHa, Caski and C4-1 cells, which contain endogenous HPV genomes, the cervical carcinoma cell line C33A, the breast carcinoma MCF-7 and the keratinocyte line HaCaT. All cell lines show a similar ratio of NFI-X versus NFI/CTF mRNA as HeLa cells, with MCF-7 cells expressing the lowest amount of NFI-X (data not shown).

NFI-X1 and NFI-X2 show different functional properties on different reporter constructs in *Drosophila* SL-2 cells

The existence of different members and different spliced variants of NFI proteins, which are very conserved at the N-terminal DNA binding domain and heterogeneous at the C-terminal transactivation domain, raises the possibility that different NFI proteins may possess different functional activation capabilities. *Drosophila* SL-2 cells, which do not express endogenous NFI

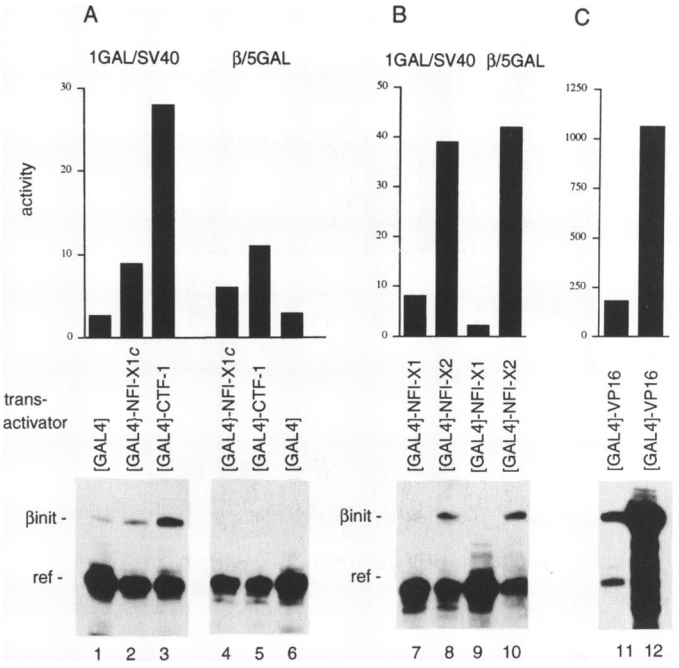

Figure 4. Different functional properties of the proline rich activation domains of NFI-X1, NFI-X2 and NFI/CTF-1 regulating transcription from promoter proximal and remote positions. Quantitative RNAse protection analysis of β-globin RNA isolated from HeLa cells transfected with the OVEC reporter genes 1GAL/SV40 (1 GAL4 binding site in promoter position and SV40 enhancer) and β/5GAL (TATA-box and 5 GAL4 binding sites in enhancer position), and the individual GAL4-fusions as transactivators. Lower panels show the correctly initiated β-globin RNA from the reporter genes (β-init) and the reference signal from OVEC-Ref (ref), which has a deletion of 28 bp around the transcription start site. As size marker (not shown) a sequencing lane was used. Quantification of the signals was performed on the PhosporImager and is shown above the autoradiographs. The activity numbers are arbitrary units of the signals from the reporter genes (β-int) relative to the signals from the reference gene (ref) and are calculated from three independent RNAse protection assays. (**A**) Comparison of activation domains of NFI-X1 ([GAL4]-NFI-X1*c*) and NFI/CTF-1 ([GAL4]-CTF-1) in relation to the GAL4 DNA binding domain alone ([GAL4]). (**B**) Comparison of the activation domains of NFI-X1 ([GAL4-NFI-X1]) and NFI-X2 ([GAL4-NFI-X2]). (**C**) Activation potential of the acidic activation domain of VP16 ([GAL4-VP16]) from promoter position (lane 11) and enhancer position (lane 12).

proteins, were chosen to test the functional properties of the newly isolated full length NFI-X proteins. For CAT-assay analysis, we used a plasmid pαCAT3XAd, which contains three additional palindromic NFI binding sites cloned into the α-globin promoter (6) and a 91 bp subclone of the HPV-16 enhancer which contains two half palindromic NFI sites. Both fragments are known to be activated by NFI/CTF-1 in SL-2 cells (15).

Figure 3A shows the effect of NFI/CTF-1 compared with NFI-X1 and NFI-X2 proteins on CAT expression from pαCAT3XAd. While NFI/CTF-1 and NFI-X2 could stimulate transcription about seven- to 10-fold, NFI-X1 stimulated transcription only about three- to four-fold. The HPV-16 enhancer construct pHPV-16/91mer (Fig. 3B), which already has a relatively high activity in SL-2 cells due to an additional AP-1 site (15), could be further stimulated by NFI/CTF-1 and NFI-X2 about two- to threefold, while NFI-X1 failed to activate this reporter construct. The relative stimulation values where calculated from at least four different independent experiments.

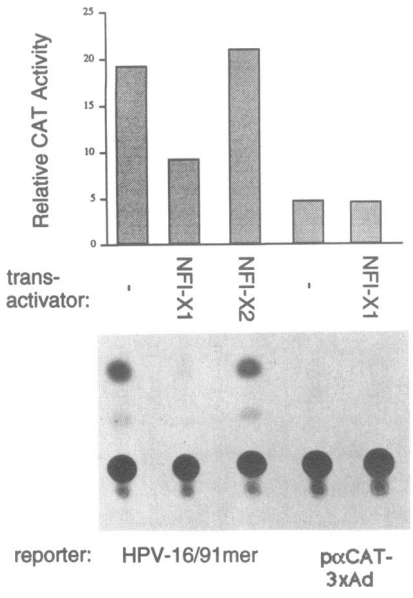

Figure 5. Overexpression of NFI-X1 in HeLa cells leads to a downregulation of the HPV-16 enhancer construct. CAT assays from HeLa cells transfected with the NFI dependent reporter constructs pHPV-16/91mer and pαCAT3XAd alone (−) or cotransfected with the transactivators plasmids pXJ-NFI-X1 and pXJ-NFI-X2 as indicated. Upper panel shows the relative CAT activities, given as picomoles per minute per milligram of protein. The values represent a mean of five independent transfections and CAT assays involving different DNA preparations.

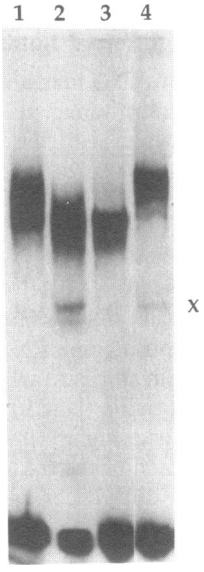

Figure 6. DNA binding profile of NFI proteins from HeLa cells transfected with NFI-X. Bandshift experiment using an α-^{32}P labeled AdNFI binding site oligonucleotide with nuclear extracts from HeLa cells (lane 1), and HeLa cells transfected with NFI-X1 (lane 2), NFI-X2 (lane 3) and NFI/CTF-1 (lane 4). Band X results from nonspecific binding.

The activation domains of NFI-X1 and NFI-X2 exert different function from proximal 'promoter' and distal 'enhancer' positions

To test the transcriptional activation domains of NFI-X proteins in human cell lines independent of the endogenous background, we made fusion protein constructs, based on the first 93 amino acids of the GAL4 DNA binding domain fused to the activation domains of NFI-X1 and NFI-X2. The transcriptional active domain of NFI/CTF-1 is located within 100 amino acids of the proline rich C-terminal end of the protein (6). Since NFI/CTF and NFI-X proteins have a similar organization, the chimeric NFI-X constructs where designed according to NFI/CTF. For comparison with NFI/CTF, we used the construct p[GAL4]−CTF-1, containing 101 amino acids (residues 399−499) of the activation domain of NFI/CTF-1 (21). The activation domain of NFI-X1, the most abundant form of NFI-X in fibroblast cells, was represented by 160 amino acids between residues 282 and 441, and was fused to the GAL4 DNA binding domain, resulting in the vector p[GAL4]−NFI-X1*c*. To test the activation potential of the chimeric proteins in proximal 'promoter' and distal 'enhancer' position, the OVEC reporter gene system (26) was used. Reporter plasmid p1GAL/SV40 contains one GAL4 binding site upstream of a TATA-box (promoter position) and a SV40 enhancer downstream of a β-globin gene, while plasmid pβ/5GAL contains five GAL4 binding sites downstream of the β-globin gene (enhancer position). These reporter constructs, and a reference plasmid (OVEC Ref.), were cotransfected into HeLa and MRHF cells with the GAL4-transactivator fusion constructs. To assay for transcriptional activation, the amount of correctly initiated β-globin gene transcript was determined by quantitative RNAse protection. Figure 3 shows the results obtained from transfection into HeLa cells. The data from MRHF cell transfections are not presented in a figure, because they show similar functional properties as those obtained from HeLa cell transfections. Figure 4A shows that the NFI-X1 construct (lanes 2 and 4) can stimulate transcription only weakly, compared with the NFI/CTF-1 activation domain (lanes 3 and 5). Compared with the DNA binding domain alone (lanes 1 and 6), the activation domain of NFI-X1 stimulates transcription threefold from promoter proximal position, while an 11-fold stimulation is observed for the activation domain of NFI/CTF-1. From a distal enhancer position, activation results in a twofold and a fourfold stimulation for NFI-X1 and NFI/CTF-1, respectively.

To compare the activation domain of NFI-X1 to the shorter spliced isoform, NFI-X2, 160 amino acids of NFI-X2 from positions 241 to 441 where used to construct the fusion protein p[GAL4]−NFI-X2. This allowed us to compare fragments of the activation domains of similar size. In contrast to the activation domain representing NFI-X1, which has only a weak activation potential (Fig. 4A, lanes 2 and 4), the activation domain of NFI-X2 could stimulate transcription strongly, about 14-fold, both from proximal and remote positions (Fig. 4B, lanes 8 and 10). To confirm, that the observed difference between NFI-X1 and NFI-X2 was due to the internal 41 amino acids spliced out in NFI-X2, and not to the 41 amino acids from positions 241 to 282 (which are missing in the construct p[GAL4]−NFI-X1*c*) we included a second construct, p[GAL4]−NFI-X1, containing, like p[GAL4]−NFI-X2, amino acids from positions 241 to 441 of NFI-X1. Figure 4B shows that p[GAL4]−NFI-X1 can stimulate transcription from promoter positions in a similar manner as p[GAL4]−NFI-X1*c* (compare Fig. 4B, lane 7 with Fig. 4A, lane 2), but fails to stimulate transcription from enhancer position at all (Fig. 4B, lane 9).

As a positive control of our assay conditions we included a chimeric construct containing the full length acidic activation domain of VP16, which has a strong activation potential and stimulates transcription from one GAL4 binding site in the

promoter position about 180-fold and from five GAL4 binding sites in the enhancer position more than 1000-fold (Fig. 4C, lanes 11 and 12).

In summary, the activation domain of NFI-X1 can stimulate transcription only weakly from proximal promoter positions and fails to activate altogether from distal enhancer positions, while the activation domain of NFI-X2, the shorter spliced product, activates transcription to a similar extent as NFI/CTF-1 from both positions.

Exogenous NFI-X1 can downregulate the HPV-16-p91mer enhancer construct in HeLa cells

We have demonstrated the functional dependence of the HPV-16 enhancer construct pHPV-16/91mer on the subset of NFI proteins expressed in epithelial cells (15). To support our hypothesis, that the subset of NFI proteins expressed in human fibroblast cells fails to activate the viral enhancer, we cotransfected the NFI-X expression plasmids pXJ-NFI-X1 and pXJ-NFI-X2 together with the viral core-enhancer construct pHPV-16/91mer into HeLa cells. Figure 5 shows, that the expression of NFI-X2 has no effect on enhancer activity, as it is the case for the overexpression of NFI/CTF-1 in HeLa cells (15). In contrast, the viral enhancer construct is repressed 2.3-fold in the presence of NFI-X1. No effect of NFI-X1 could be observed on the activity of the construct pαCAT3XAd (Fig. 5, last two lanes), which contains NFI binding sites in the promoter position.

We conclude that downregulation of the HPV-16 enhancer segment by NFI-X1 in HeLa cells correlates with the lack of activation in SL-2 cells and an inability of the isolated activation domain of NFI-X1 to stimulate transcription from enhancer positions.

Expression of NFI-X in HeLa cells changes the DNA binding profile of NFI proteins to a fibroblast-like phenotype

Our earlier studies showed that the HPV enhancer activity correlated strongly with a characteristic DNA binding profile of NFI proteins in epithelial versus fibroblast cells (15). Additionally, NFI/CTF-1 which could activate the viral enhancer in a cellular background void of endogenous NFI proteins failed to activate transcription in fibroblast cells. A combination of Western blot and bandshift analysis suggested heterodimeric complex formation with endogenous NFI proteins in fibroblast cells. The functional interference of fibroblast NFI-X1 with NFI/CTF proteins in HeLa which we observed in this study may be also explained by heterodimerization of the exogenous and endogenous proteins. To investigate this hypothesis, we transfected NFI-X1 and NFI-X2 into HeLa cells. Bandshift analysis shows that the endogenous NFI protein complex pattern in Hela cells (Fig. 6, lane 1) disappears, and is replaced by complexes with lower mobility after transfection of NFI-X1 (lane 2) and the shorter spliced isoform NFI-X2 (lane 3), resembling the complexes formed with endogenous NFI proteins in fibroblast cells (15). As a control we overexpressed NFI/CTF-1, the longest form of NFI proteins in HeLa cells, which resulted in the expected shift to lower mobility complexes (lane 4).

DISCUSSION

NFI constitutes a family of polypeptides which are regarded as ubiquitous transcription factors due to their expression in a variety of different tissues. However, several reports demonstrate their importance, not only in the regulation of epithelial cell specific transcription (15,27), but also for the expression of liver specific genes (1,14), for the activity of an adipocyte specific enhancer (28) and for brain specific gene expression (13,29,30). In the context of such cell-type specific promoters and enhancers, NFI proteins often cooperate with other transcription factors whose binding sites are located in close proximity to the NFI sites. Because some of these transcription factors showed cell-type specific DNA binding profiles (14,27), NFI was never really regarded as a major determinant in mediating the cell-type specificity. In our studies on the HPV-16 enhancer we developed the concept that seemingly ubiquitous factors might mediate epithelial specificity, as no transcription factor specific for epithelia could be identified (23,31).

The enhancers of human papillomaviruses exert a strict epithelial specific response in transfection experiments. This property is probably a reflection of the natural tropism of these viruses (32,33). HPV enhancers are synergistically activated by heterologous transcription factors, which include AP-1 (34,35), TEF-1 (36), and SP1 (22), and the most conspicuous property being a cluster of four to seven half-palindromic binding sites for NFI. Strict sequence conservation of these sites among all sequenced human papillomaviruses, i.e. more than 40 different types, (16, and our unpublished observations) and extensive mutational studies in a variety of cell lines (23) provided evidence for the functional importance of these sites. Furthermore, we observed that the activity of the HPV-16 enhancer correlated with a characteristic pattern of NFI protein/DNA complexes in gel retardation assays, which were formed by NFI/CTF-1, CTF-2 and CTF-3 from epithelial cells (15).

In this report, we have followed up on the observation of the expression of the NFI-X gene in fibroblasts (15). Northern blot and PCR analysis demonstrate that human fibroblasts express NFI-X mRNA to a much higher level than epithelial cells. This differential expression may be relevant *in situ*, because different human tissues express dramatically different levels of NFI-X mRNA. We also show that in human fibroblasts NFI-X is expressed in two forms which differ through differential splicing in a 41 amino acid segment of the transcription activation domain. It seems to be a frequent scenario that transcription factors with different, or even missing transactivation domains, can be derived from the same gene by either alternative splicing (37–39), usage of different polyadenylation signals (40) or different translation start sites (41). Such transcription factor isoforms may then play a different or even opposing function on a given promoter or enhancer (for review see ref. 42). It could be demonstrated that the three different spliced forms of NFI/CTF all activated transcription, albeit to a different extent, when tested for their ability to activate a construct containing the α-globin promoter (7). Functional data for NFI-X proteins cloned from hamster (8) and chicken cells (11), have so far not been presented. We therefore decided to compare the function of the two forms of NFI-X activators with one another, and with NFI/CTF.

The activation potential of the newly isolated NFI-X proteins were tested in *Drosophila* SL-2 cells, which contain the necessary machinery for transcription, but lack some transcription factors including NFI (44). While NFI-X2 can activate transcription of the reporter plasmid pαCAT3XAd, which contains four NFI sites in the promoter position, to the same extent as NFI/CTF-1, the activation potential of NFI-X1 is weak. Additionally, NFI-X1 fails to activate the HPV-16 enhancer construct in SL-2 cell.

Fusion proteins based on the DNA binding domain of GAL4 with the transcription activation domains of NFI-X have enabled

us to study the different activation domains of NFI-X in HeLa cells, independent of the endogenous background of NFI proteins. Seipel *et al.* (21) demonstrated a considerable activity for the proline-rich activation domain of NFI/CTF-1 from promoter positions, and a weaker, but still significant, activation potential from enhancer positions. By using the same OVEC reporter system we show that a similar fragment of the proline-rich activation domain of NFI-X1 stimulates transcription only very weakly from the promoter position and completely fails to activate transcription from the enhancer position. However, the activation domain of the shorter spliced isoform, NFI-X2, can stimulate transcription efficiently from the promoter, as well as enhancer, position. This different effect may reflect the behavior of the full length proteins in SL-2 cells. Similar experiments, performed in MRHF fibroblast cells, show functional properties indistinguishable to those observed in HeLa cells (data not shown), indicating that the activation domains of NFI-X and NFI/CTF-1 proteins do not have any cell-type specific functional properties, which may, for example, have arisen if different cell-type specific coactivators were used. This is of interest as it could be demonstrated that NFI/CTF requires cofactors for transcriptional activation (19), which have subsequently been purified from HeLa cells (43).

At present, we do not understand the molecular basis for the differential activation properties of spliced NFI proteins. For example, NFI/CTF-1, which contains the longest activation domain of the NFI/CTF variants, forms the strongest activator. However, NFI/CTF-3, with the shortest activation domain, has a higher activation potential than the intermediate form NFI/CTF-2 (6, and our own unpublished observations), resembling the observed functional properties of NFI-X1 and NFI-X2. Similar differences, where isoforms with shorter activation domains represent the more potent transcriptional activators, have been observed for other transcription factors, such as the liver enriched transcription factor HNF1 (40).

To address the significance of the observed differences for epithelial cell specific HPV gene expression, we tested the effect of NFI-X1 on the HPV-16 enhancer by transient transfection assays in Hela cells. We have published previously, that NFI/CTF-1 from HeLa cells transfected into fibroblast cells becomes quantitatively entrapped in heterologous complexes, most likely due to the abundance of NFI-X in these cells, and becomes unavailable for activation (15). Here we show that exogenous expression of NFI-X1 in HeLa cells leads to a significant down-regulation of the viral enhancer construct. Bandshift analysis supports the hypothesis, that this functional interference of NFI-X with NFI/CTF is brought about by heterodimer formation between the exogenous and the endogenous proteins. We believe that this negative effect is the principal function exerted by NFI-X proteins in our test system *in vivo*, because both our library screens and PCR analysis suggest that NFI-X1 is the predominant form of NFI-X in human fibroblasts. Thus competition between NFI-X1 and the activator NFI/CTF is apparently responsible for functional differences observed for the HPV-16 enhancer in epithelial and fibroblast cells (23).

Several publications from other laboratories report on the role of NFI in cell-type specific gene expression, but do not explicitly address the possibility of differential roles for different NFI factors. For example, the activation of the liver specific albumin enhancer by an interaction of NFI and HNF3α differed in two cell types, liver cells and HeLa cells (14). The authors propose a decisive role for NFI/CTF. In the light of our data a reappraisal may be necessary with the possible involvement of different NFI proteins in these two cell lines, particularly when one considers that NFI-X was originally isolated from liver cells (8). Furthermore, cell type specific gene expression in the brain may be also influenced by yet other members of the NFI family, as different factors derived from the NFI-A and NFI-B gene were found to be involved in the activity of two brain specific genes, the myelin basic promoter (45), and the proenkephalin enhancer (30), respectively.

In summary, in spite of the ubiquitous expression of NFI, there is increasing evidence that NFI proteins do not constitute a family of transcription factors with mostly constitutive functions but rather play an essential role in the cell-type specific gene expression of many cellular and viral genes. Modulation of cell-type specificity cannot be viewed in the context of a single type of NFI, but rather has to be looked upon as a complex composition of specific subsets of NFI proteins, which can vary in a cell-type specific manner by differential expression, by differential splicing and by heterodimerization.

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