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Nuclear Factor I and Epithelial Cell-Specific Transcription of Human Papillomavirus Type 16

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The transcription of human papillomavirus type 16 (HPV-16) is mediated by the viral enhancer. Epithelial cell-specific activation is achieved by the cooperative interaction of apparently ubiquitous transcription factors. One of them, nuclear factor I (NFI), binds seven sites within the HPV-16 enhancer. Point mutations on enhancer fragments, which retain epithelial cell specificity, verify the functional contribution of NFI. In band shift experiments, the epithelial cell-derived NFI proteins CTF-1, CTF-2, and CTF-3 form a characteristic pattern of heterodimeric complexes which are observed in all epithelial cells tested. Divergence from this pattern in fibroblasts, liver cells, and lymphoid cells correlates with the lack of HPV-16 enhancer activation. The HPV-16 enhancer can be activated by CTF-1 in SL-2 cells, which lack NFI-like proteins. However, exogenous CTF-1 fails to overcome the inactivity of the viral enhancer in fibroblasts. Western immunoblot and supershift analysis shows that exogenously introduced CTF-1 proteins form different heterodimer complexes with the given subset of endogenous NFI proteins in epithelial or fibroblast cells. Polymerase chain reaction analysis and cDNA library screens identified the endogenous fibroblast type NFI as NFI-X, an NFI family member originally cloned from hamster liver cells. The strict correlation between the activation or lack of activation of the HPV-16 enhancer and cell-specific subsets of NFI proteins argues for the pivotal role of NFI binding sites in the epithelial cell-specific function of the viral enhancer.

Human papillomavirus type 16 (HPV-16), which plays a central role in the etiology of cervical carcinomas, contains a circular DNA genome with a length of 7,904 bp. Its epithelial cell-specific enhancer is located between positions 7454 and 7854 and directs viral transcription through the promoter for the early genes (10, 18). This epithelial cell-specific enhancer may form the principal component of HPV-16 tropism for mucosal epithelia.

The 400-bp enhancer segment has been mapped by DNase I protection studies (9, 19, 36, 47) to reveal the binding sites for transcription factors. This resulted in identification of the transcriptionally active factors of the HPV-16 enhancer as the progesterone and glucocorticoid receptors (5), nuclear factor I (NFI) (20), AP1 (4), TEF-2, Oct-1 (6), TEF-1 (25), and possibly yet unidentified factors (such as X and Y; Fig. 1A) (9, 36). The multiple, closely spaced, and in some cases redundant *cis* elements that bind these transcription factors cooperate (7), resembling the modular nature of other transcriptional enhancers (14). Surprisingly, our work to date does not support the possibility that activity of the HPV-16 enhancer depends critically upon factors that bind DNA in epithelial cells but are absent elsewhere. Rather, functional specificity appears to be brought about by transcription factors that are ubiquitous. This apparent contradiction may possibly be explained by a combination of transcription factor synergism, concentration differences of qualitatively identical factors, and functional variation of factors through means other than their DNA recognition specificity (15). Experimental data support several of these concepts: synergism is suggested by the observation that HPV enhancer activity is eliminated by mutations in alternative transcription factor binding sites, although the remaining sites within the enhancer are unaltered (6, 9). Functionally relevant variations of factors have also been observed: AP1 has a different subunit composition in epithelial and nonepithelial cells (50), TEF-1 requires a cofactor with cell type (although not epithelial cell) specificity (25), and NFI shows differences in band shift experiments in a cell-type-specific manner (6). In the experiments reported here, we have concentrated on this latter factor, since NFI binding sites are a particularly conspicuous feature of the enhancer of all HPVs (7, 13, 20).

The DNA-binding protein NFI was identified because of its importance in directing the initiation of adenovirus (Ad) DNA synthesis by binding to the consensus recognition sequence 5′-TGG(A/C)N_5GCCAA-3′ (11, 22, 35). NFI is also a transcription factor essential for the optimal expression of a number of cellular and viral genes (1–3, 39, 41). NFI is a family of proteins cloned from HeLa cells (44) and several animals species (17, 37, 42). Sequence homology enabled identification of four factors in the NFI family: NFI-A, -B, -C, and -X. Among these four factors, the N-terminal DNA binding and dimerization domain is highly conserved. Conversely, the C-terminal proline-rich transactivating domains are heterogeneous even between different NFI forms in HeLa cells, which are generated by differential RNA splicing of one gene product (33, 44). HeLa NFI-C was isolated by binding to the CCAAT box of the human α-globin gene promoter; hence, the term NFI/CTF was used (28). However, the identity of the exact binding sequence in this promoter fragment has been recently disputed (51).

The long control regions of all papillomaviruses that have been examined contain epithelial cell-specific enhancers that correlate with NFI clusters. Seven half palindromic NFI binding sites of the sequence 5′-TTGGC-3′ are found within the HPV-16 enhancer, making it the most abundant transcription factor binding site of the enhancer. Epithelial cell specificity was retained by nonoverlapping subclones of the HPV-16 enhancer, which had only NFI sites in common (6).

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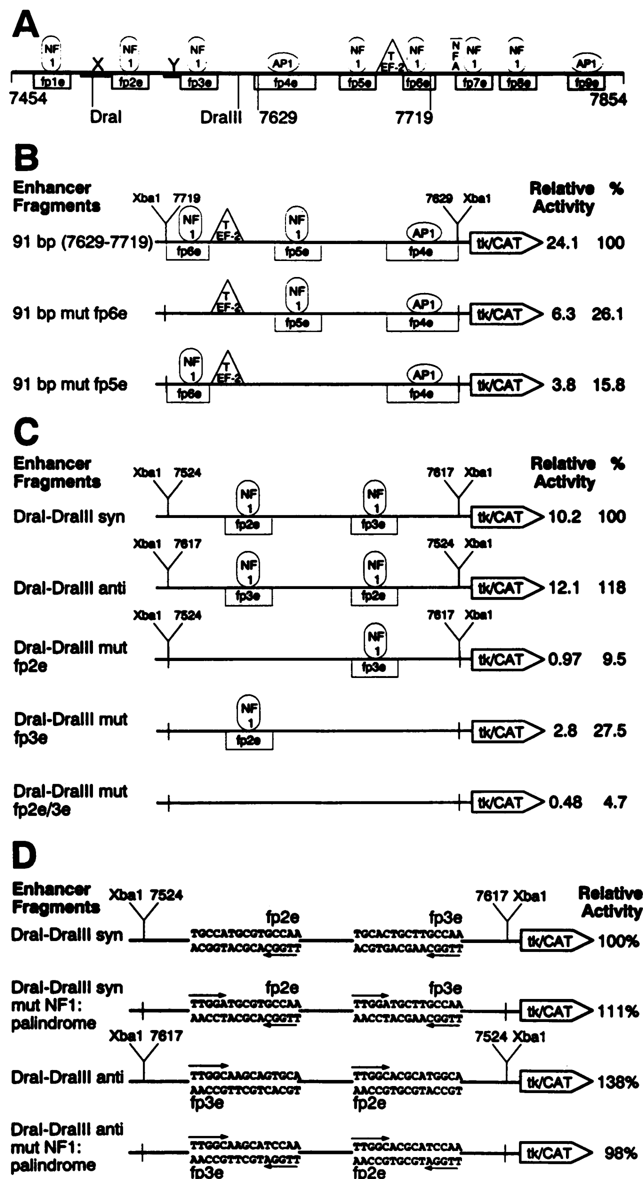

FIG. 1. The complete HPV-16 enhancer (A) and functional analysis of two subclones with and without mutations of individual NFI binding sites (B to D). (A) Diagram showing the enhancer fragments *Dra*I-*Dra*III (genomic positions 7524 to 7617) and the 91-bp enhancer (positions 7629 to 7719) and the relative positions of transcription factor binding sites (labeled fp1e, etc., consistent with previous publications [6, 19]). The 91-bp fragment has also been recently shown to contain two binding sites for TEF-1 (25). The symbols X and Y represent footprints resulting from unidentified factors (9, 36). (B and C) Results of NFI binding site mutation studies on the 91-bp and *Dra*I-*Dra*III enhancer fragments, respectively. The activity of each CAT construct is expressed relative to that of the parent plasmid ptkCATdH/N, which was assigned an activity of 1.0 (1.54 pmol/min per mg of protein). (D) The NFI binding sequences of the HPV-16 *Dra*I-*Dra*III enhancer were changed to the consensus palindromic NFI sequence. All CAT activities were derived from four separate transfections into HeLa cells for each construct.

Significantly, complexes that bind the NFI consensus sequence show differences in band shift profile between extracts from epithelial cells, which support enhancer activity, and fibroblasts, which do not (6). Here we verify the contribution of NFI to HPV-16 enhancer function by point mutation and gain-of-function experiments. The complexity of NFI-binding activities in different cell types is examined and discussed as a possible mechanism of differential transcriptional activation.

MATERIALS AND METHODS

Plasmid constructs. The HPV-16 enhancer constructs p*Dra*I-*Dra*III and p91bp have been described elsewhere (7). The NFI binding site mutations of the HPV-16 enhancer fragments (Fig. 1) were achieved by using two overlapping pairs of oligonucleotides with point mutations cloned into the *Xba*I site of ptkCATdH/N (46). The NFI binding sites were mutated 5′-TTGGC-3′ to 5′-TGTTC-3′, previously shown to abolish binding of HeLa nuclear proteins to the site (6). The construct pαCAT3×Ad contains the wild-type α-globin promoter and three Ad NFI binding sites in pBLCAT3 (33). Plasmid p113-CTF-1 contains the Rous sarcoma virus long terminal repeat promoter directing the transcription of CTF-1, an NFI cDNA clone from HeLa cells (31). Plasmids pPADH-CTF-2 and pPADH-CTF-3 direct the expression of cDNA clones CTF-2 and CTF-3 in *Drosophila* cells (44). Plasmids pαCAT3×Ad, p113-CTF-1, pPADH-CTF-2, and pPADH-CTF-3 were generous gifts from N. Mermod; pHPV16-fp4el-15-5× and the full-length 400-bp HPV-16 enhancer construct used in this study have been described elsewhere (4, 19). Plasmid pCGN (48), a gift from A. Stenlund, was used to construct the expression vector pCGND-CTF-1, in which NFI is expressed as a fusion protein with the hemagglutinin epitope. To subclone CTF-1 in frame with the hemagglutinin epitope, the pCGN polylinker was reversed in orientation, giving rise to the vector pCGND. CTF-1 was subcloned as a partially digested 1.8-kb *Bam*HI-*Kpn*I fragment of p113-CTF-1.

Cell culture, transfection, and CAT assays. The epithelial cell lines HeLa, Siha, Caski, HaCat, and MCF-7, the fibroblast lines MRHF, SF-4, and H68, and the lymphoid cell line Daudi were cultured by standard procedures (6). The cervical carcinoma cell line C-33A and the liver carcinoma cell line HepG2 were grown in minimal essential medium supplemented with 10% fetal calf serum, nonessential amino acids, and sodium pyruvate in a 1:100 dilution. HeLa and MRHF cells were transfected by electroporation with a Bio-Rad Gene Pulser. Functional studies of the HPV-16 enhancer fragments and mutants in HeLa cells were made with 10 μg of plasmids for each transfection. In cotransfection experiments, 10 μg of reporter plasmid was cotransfected with various amounts (1 to 10 μg) of effector plasmid. Chloramphenicol acetyltransferase (CAT) assays were done as previously described (6). *Drosophila* Schneider SL-2 cells were grown in Schneider's insect medium (Sigma) supplemented with 10% fetal calf serum at 25°C. Twenty-four hours before transfection, the cells were seeded at a density of 10^6 cells per ml and then transfected by calcium phosphate coprecipitation as described previously (5). Two micrograms each of expression vector p113-CTF-1, pPADH-CTF-2, or pPADH-3 and/or reporter plasmids was cotransfected in the presence of pBSSK+ carrier (Stratagene) to a total of 15 μg of DNA. Cells were harvested 36 h after transfection, and 20 μg of protein was used for CAT assays.

Nuclear extracts and DNA binding assay. Nuclear extracts

were prepared according to Schreiber et al. (45). About 10^6 cells were used for each preparation, and nuclear proteins were dissolved in a final volume of 50 μl. Band shift assays were performed as described previously (4) except that 3 μg of poly(dI-dC) was used as competitor. One microliter of nuclear extract was used for each binding reaction. For supershifts, the nuclear extracts were first incubated with the competitor and the labeled oligonucleotide for 15 min on ice and then further incubated with 0.1 μl of the monoclonal antibody for 1 h at room temperature. The band shift mixes without antibody were left on ice for the same period before separation on a polyacrylamide gel.

Immunoprecipitations and Western immunoblotting. HeLa or MRHF cells were transfected with 5 μg of the expression plasmid pCGND-CTF-1 and 15 μg of carrier DNA (pBSSK+) by electroporation. Forty-two hours after transfection, the cells were washed with phosphate-buffered saline and lysed on the plate with 0.5 ml of radioimmunoprecipitation assay buffer (0.5% Nonidet P-40, 0.5% Tween 20, 0.5% deoxycholic acid, 150 mM NaCl, 50 mM Tris [pH 7.5], 0.1% sodium dodecyl sulfate [SDS]) for 30 min at 4°C. The lysates were centrifuged for 10 min at 10,000 × *g*, and the supernatant was precleared with protein A-Sepharose. Precipitations were carried out with 0.1 μl of monoclonal antibody C12A5 (made available by The Scripps Research Institute) for 1 h at 4°C. For Western blotting, 30 μl of the nuclear extracts and the immunopurified proteins dissolved in 30 μl of Laemmli buffer were separated in an SDS-10% gel and electroblotted on nitrocellulose filters. The blots were blocked with 3% bovine serum albumin, and monoclonal antibody C12A5 was added at a 1:5,000 dilution. After incubation with the second antibody (alkaline phosphatase-conjugated goat anti-mouse) at a 1:5,000 dilution, the blot was processed as described previously (24).

Library screening and DNA sequencing. Two commercially available human skin fibroblast cDNA libraries (Clontech H1052a and H1052b) were screened with a 1.5-kb *Bam*HI fragment of pCTF-1 (44). The hybridization solution contained 30% (vol/vol) formamide, 5× Denhardt's solution, 5× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7]), 0.1% SDS, 100 μg of salmon sperm DNA per ml, and 10^6 cpm of the random-primed ^{32}P-labeled (Boehringer Mannheim) DNA probe per ml. After hybridization for 16 h at 42°C, the filters were washed three times in 1× SSC-0.1% SDS for 20 min at 42°C. From a total of 5×10^5 individual plaques, eight cDNAs were isolated by polymerase chain reaction (PCR), using cDNA insert screening amplifiers (Clontech) according to the manufacturer's recommendations. The PCR products were subcloned in pUC19, and about 250 bp of both strands were sequenced from both ends as described by Sanger et al. (43), with two primers which correspond to pUC19 sequences on both ends of the polylinker.

Reverse transcriptase-coupled PCR and Southern blotting. Oligonucleotides were synthesized on a Pharmacia gene synthesizer. Primer oligonucleotide Do4 (ATGGATGAGT TCCACCCGTT) matches the 5′ start of the open reading frames of CTF-1 (44) and NFI-X (17). Oligonucleotides Do5 (ATCCCACAGCAGTCTCAGTC) and RT-2 (CAAACCGT TCCTTTGTGGGA) are located at the 3′ termini of the open reading frames of NFI-X and CTF-1, respectively.

First-strand cDNA was prepared from HeLa, MRHF, and Daudi RNA by using oligo(dT) and oligonucleotides Do5 and RT-2 as primers. Synthesis was carried out in a 30-μl reaction mixture containing 4 μg of total RNA, 70 pmol of each primer, nucleoside triphosphates (dNTPs; 1 mM each at final concentrations), 1 μl of reverse transcriptase (Superscript; Bethesda Research Laboratories), 6 μl of 5× buffer (Bethesda Research Laboratories), and 10 mM dithiothreitol for 1 h at 42°C. At the end of the reaction, 70 μl of 1× PCR buffer (Boehringer Mannheim) was added. One microliter of the cDNA was used for amplification with *Taq* DNA polymerase (Boehringer Mannheim) in a 50-μl reaction mixture containing 200 μM each dNTP and 10 pmol of primer. Forty cycles were applied under the following conditions: 2 min at 95°C, 2 min at 55°C, and 2 min at 72°C. The PCR products were separated in a 1% agarose gel and blotted on nitrocellulose filters (Hybond-C; Amersham) in 0.2 N NaOH. The filter was probed with a 631-bp *Bst*XI fragment of pCTF-1 in a hybridization solution containing 0.5 M sodium phosphate (pH 7.9), 7% SDS, 15% formamide, and 10^6 cpm of the random-primed labeled (Boehringer Mannheim) DNA per ml. After 16 h of hybridization at 65°C, the filters were washed three times for 20 min each time in 50 mM sodium phosphate (pH 7.9)-0.1% SDS at 65°C. After being stripped at 95°C in 10 mM sodium phosphate-0.1% SDS solution, the filter was reprobed with a 753-bp *Bam*HI-*Eco*RI fragment of cDNA clone FN6.

RESULTS

The functional contribution of NFI to the HPV-16 enhancer is shown by mutations of individual NFI binding sites. A fragment of 91 bp retained significant enhancer activity with two binding sites for NFI, an AP1 site, and a TEF-2 site (6). The same genomic region has been shown recently to contain two functionally relevant binding sites for TEF-1 (25). Point mutations of the NFI sites reduced transcriptional activity significantly. Both sites, fp5e and fp6e, contribute independently to function, as the point mutation of the respective sites reduced activity to 16 and 26% of the wild-type activity (Fig. 1B). This finding complements those of our earlier study in which both sites were mutated together; in that instance, the activity dropped to 8% of the wild-type activity (7). Another segment of the enhancer of approximately the same size, defined by *Dra*I-*Dra*III restriction enzyme sites, retains enhancer activity albeit at a lower level than that of the 91-bp fragment, 10.2-fold versus 24.1-fold (Fig. 1). Mutation of its two NFI binding sites, fp2e and fp3e, alternatively and then simultaneously (Fig. 1C) shows that NFI is also a key factor in the function of this enhancer fragment. The full-length HPV-16 enhancer was included in parallel transfections as a positive control. A consistent average 110-fold enhancement over the activity of the enhancerless CAT vector ptkCATdH/N was obtained from seven separate transfections over the course of this study.

Qualitatively, the half palindromic HPV-16 NFI sites bind NFI similarly to palindromic sites (6). The only difference observed in such band shift experiments was that the HPV-16 NFI elements bind NFI with a lower affinity. Beyond these binding studies, a functional comparison of nonpalindromic and palindromic NFI binding sites seemed warranted because HPV enhancers contain exclusively nonpalindromic recognition sequences (21). Also, a transcriptional function of nonpalindromic NFI sites had been disputed (51). Figure 1D shows that point mutations that re-create palindromic NFI binding sites in the context of the *Dra*I-*Dra*III HPV-16 enhancer fragment do not significantly change transcriptional activity in comparison with the wild-type fragment. The HPV-16 half palindromic NFI binding

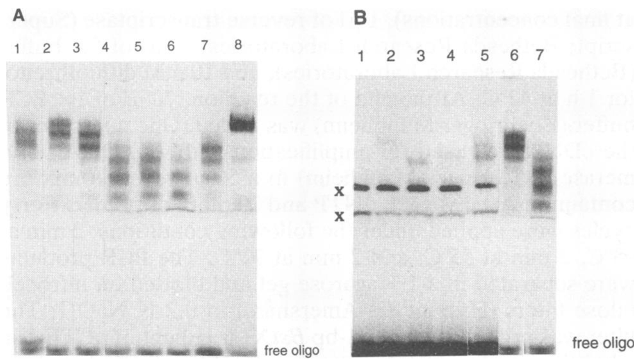

FIG. 2. (A) DNA-binding activities of NFI proteins from cell lines of different differentiation origins. Band shift experiments using an α-^{32}P-labeled Ad NFI binding site oligonucleotide with nuclear extracts from three epithelial lines (HeLa, C33-A, and MCF-7; lanes 1 to 3), one liver cell line (HepG2; lane 4), three fibroblast cell lines (MRHF, SF-4, and H68; lanes 5 to 7), and one lymphoid cell line, Daudi (lane 8), were performed. Similar DNA-binding activities were seen with oligonucleotides containing NFI half sites of the HPV-16 enhancer (6). (B) DNA-binding activity of CTF-1, CTF-2, and CTF-3. Lanes: 1, untransfected SL-2 cells; 2 to 4, SL-2 cells transfected with CTF-1, CTF-2, and CTF-3, respectively; 5, coexpression of CTF-1, CTF-2, and CTF-3 in SL-2 cells compared with nuclear extracts from HeLa cells (lane 6) and MRHF cells (lane 7). Bands X result from nonspecific binding.

site can be functionally replaced by the consensus palindromic NFI binding site.

The NFI band shift profile differs in a consistent manner between cells that support HPV-16 enhancer activity and cells that do not. Our earlier work has indicated that there are significant qualitative differences between NFI proteins binding to the DNA recognition sequence in nuclear extracts from HeLa cells compared with MRHF fibroblast cells. Here we have expanded our previous study to include the epithelial cell lines HeLa, C-33A, MCF-7, HaCat, SiHa, and Caski, the liver cell line HepG2, the fibroblast cell lines MRHF, SF-4, and H68, and a lymphoid cell line, Daudi. The epithelial cell lines were chosen because they support HPV-16 enhancer activity whereas human liver cells, fibroblasts, and lymphocytes do not (6). Slowly migrating NFI complexes are consistently present in epithelial cells (Fig. 2A, lanes 1 to 3, shows data for HeLa, C-33A, and MCF-7 cells; data for HaCat, SiHa, and Caski cells are not shown), while these complexes are not present in liver cells (lane 4) and fibroblasts (lanes 5 to 7). Homologous competition with the Ad NFI oligonucleotide and NFI half sites and competition with an unrelated AP1 binding site showed that all complexes seen are specific for NFI sites (data not shown). Similar experiments with the same nuclear extracts and binding sites for AP1, TEF-2, and Oct-1 gave rise to bands in HeLa and MRHF cells that did not differ in a cell-specific manner (6). This finding suggests that the higher mobility of NFI complexes in fibroblasts is not due to degradation of the nuclear extracts.

The lymphoid cell line Daudi seems to express only one species of protein that binds specifically to the NFI site (Fig. 2A, lane 8). We confirmed by Northern (RNA) blot analysis (data not shown) evidence from another laboratory that lymphoid cell lines do not transcribe the NFI-C gene. The protein binding to NFI sites in lymphocytes differs in proteolytic clipping assays from those isolated from HeLa cells (32).

To determine whether expression of the NFI proteins CTF-1, CTF-2, and CTF-3, originally cloned from HeLa cells (44), is sufficient to reconstitute the basic pattern of NFI complexes found in all epithelial cells, we transfected these cDNAs in SL-2 cells, which lack endogenous NFI proteins. Band shift analysis with nuclear extracts from untransfected SL-2 cells (Fig. 2B, lane 1) and SL-2 cells transfected with expression plasmids for CTF-1, CTF-2, and CTF-3 (lanes 2, 3, and 4, respectively) shows the correct expression and DNA-binding ability of the three NFI proteins. Cotransfection of all three plasmids in SL-2 cells leads to a band shift profile similar to the one seen with nuclear extracts from HeLa cells when the three proteins are present (compare lanes 5 and 6). The complex with the slowest mobility, which is formed only in the presence of CTF-1, is clearly missing in fibroblast cells (lane 7).

Modulation of the HPV-16 enhancer in different cellular environments by exogenous NFI CTF-I protein. The functional dependence of the HPV-16 enhancer on the NFI CTF-1 protein from HeLa cells would be supported if complementation of an NFI-deficient transcriptional environment with NFI resulted in enhancer activation. Undifferentiated *Drosophila* Schneider SL-2 cells lack Sp1, NFI, and AP-2 but contain AP1 and the necessary machinery for transcription (8, 38). The CTF-1-expressing clone p113-CTF-1 activates the reporter construct pαCAT3×Ad, which contains four NFI binding sites, eightfold in SL-2 cells (Fig. 3A) (33). The same NFI-expressing clone can activate the HPV-16 *Dra*I-*Dra*III fragment and the 91-bp fragment, which both contain two NFI sites, fivefold in SL-2 cells (Fig. 3A) but not the construct *Dra*I-*Dra*III mut (Fig. 1C) and the construct pHPV16-fp4el-15-5×, which carries five active AP1 binding sites (data not shown). This result shows that cotransfection of CTF-1 into a transcriptional background void of NFI is necessary for transcriptional activity of HPV-16 enhancer fragments. Interestingly, the background activity of the *Dra*I-*Dra*III enhancer in SL-2 cells is the same as that of its parent vector, ptkCATdH/N, but slightly lower than that of the 91-bp enhancer. This difference corresponds with the enhancer strengths in HeLa cells and could be accounted for by the binding of factors other than NFI, e.g., to the AP1 sites.

To investigate whether the HPV-16 enhancer can be activated in fibroblasts by exogenous CTF-1 in a similar manner, the target HPV-16 enhancer CAT constructs with the *Dra*I-*Dra*III and 91-bp segments as well as the reference plasmid pαCAT3×Ad were transfected into MRHF cells together with the expression clone for CTF-1. As a control for transfection efficiency, we included pORFEXCAT. This vector led to high CAT activities similar to those observed in HeLa or other epithelial cells (7). Figure 3B shows that none of the HPV-16 enhancer clones could be activated by exogenous CTF-1 in fibroblast.

One explanation for the failure of exogenous CTF-1 to activate the HPV-16 enhancer fragments in fibroblasts in a manner similar to that seen in SL-2 cells could be that NFI requires a cofactor for activation which is missing in fibroblasts. The involvement of a limiting soluble coactivator can be tested by intracellular competition or squelching, i.e., titrating it through its interaction with the activation domain of the overexpressed activator. To this end, we transfected increasing amounts of CTF-1 in HeLa cells. Figure 3C shows that coexpression of exogenous CTF-1 had no effect on the reporter pαCAT3×Ad or on the 91-bp fragment. Activation by the *Dra*I-*Dra*III fragment, which is mainly dependent on NFI (Fig. 1A and C), was slightly stimulated with increasing

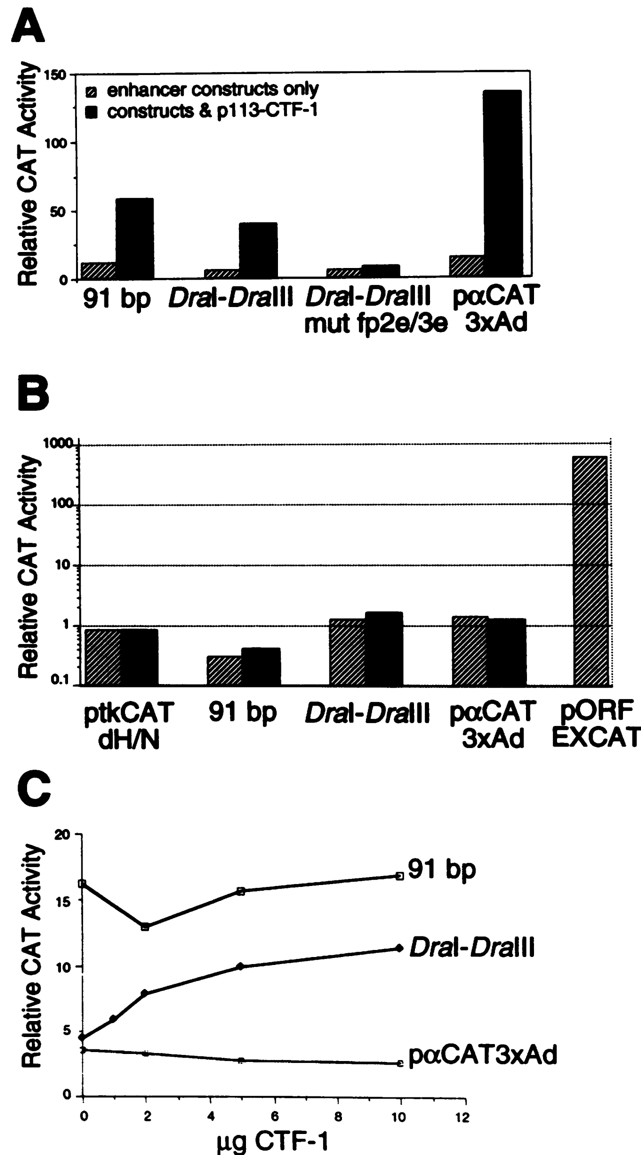

FIG. 3. Activities of HPV-16 enhancer fragments in different cell lines cotransfected with CTF-1. (A) Activation of two nonoverlapping HPV-16 enhancer fragments (*Dra*I-*Dra*III and 91 bp) by cotransfection with CTF-1 in SL-2 cells. Plasmids *Dra*I-*Dra*III mut fp2e/3e and pαCAT3×Ad, a known target for NFI (33), are negative and positive controls for the assay conditions used. Striped bars indicate activities of the individually transfected constructs; black bars represent CAT activities after cotransfection with the CTF-1-expressing construct p113-CTF-1. CAT activities (vertical axis) are given as picomoles per minute per milligram of protein. (B) Effects of CTF-1 on enhancer fragments cotransfected into MRHF cells. As negative and positive controls, the parent vector for the HPV-16 enhancer constructs ptkCATdH/N and pαCAT3×Ad were included. Plasmid pORFEXCAT served as a control for transfection efficiency. (C) Effect of CTF-1 overexpression on the HPV-16 enhancer in HeLa cells. Five micrograms of the reporter plasmids was cotransfected with 1, 2, 5, and 10 μg of expression plasmid. The relative stimulation values were calculated from at least three different CAT assays.

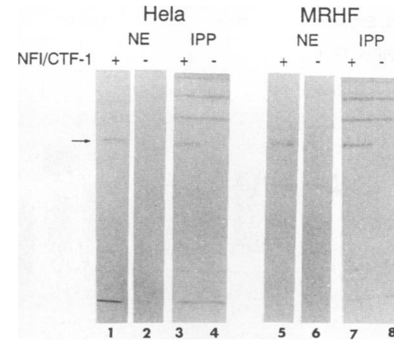

FIG. 4. Western blot of nuclear extracts and immunoprecipitated CTF-1 expressed in HeLa and MRHF cells. Expression from plasmid pCGND-CTF-1 results in a fusion protein with a hemagglutinin epitope which allows its detection by monoclonal antibody C12A5. The position of CTF-1 fusion is marked by an arrow. Nuclear extracts (NE) from mock-transfected cells (lanes 2, 4, 6, and 8) do not show this band. Immunoprecipitation from whole cell extracts was carried out with the same hemagglutinin-specific antibodies. The three larger bands seen in the immunoprecipitation (IPP) lanes resulted from the reaction of the secondary antibody with excess C12A5.

amounts of CTF-1. This finding could be attributed to the increase of the concentration of the CTF-1 factor, which is known to contain a transcriptional activation domain with higher activity than that of CTF-2 or CTF-3 (31, 44). The lack of a squelching effect indicates that NFI does not need a limiting coactivator for HPV-16 enhancer activation, but our data do not completely rule out the possibility that NFI needs a cofactor.

Posttranscriptional modification is another potential level for regulation of transcription factor activity. Since fibroblast extracts contain only NFI complexes which run with higher mobility in gel retardation assays than does epithelial cell NFI (Fig. 2), one possibility is that NFI proteins are processed in fibroblasts to lower-molecular-weight forms that may not support HPV-16 transcription. To investigate this possibility, we decided to monitor the fates of CTF-1 proteins in HeLa (epithelial) and MRHF (fibroblast) cells.

Properties of exogenous CTF-1 in epithelial and fibroblast cells. To monitor the fate of transfected CTF-1, we designed a system by which we could detect the exogenous NFI protein with an antibody which does not recognize endogenous NFI forms. CTF-1 was expressed as a fusion protein with a small epitope from influenza virus hemagglutinin at the N-terminal end (see Materials and Methods). A specific monoclonal antibody, C12A5, exists for this epitope (16). Western blots of immunopurified CTF-1 of transfected HeLa from whole cells show a band of 68 kDa, the expected size of CTF-1 including the hemagglutinin epitope (Fig. 4, lane 3; marked with an arrow). We observed in MRHF the same full-size protein as that obtained from transfection of HeLa cells (lane 7). This finding negates the hypothesis that there are specific proteases for NFI in fibroblasts or that this particular mRNA for CTF-1 is further processed at the RNA level in MRHF cells. The integrity of the protein was furthermore confirmed by blotting nuclear extracts of both cell lines (lanes 1 and 5). Under these experimental conditions, we repeated the CAT assay cotransfection experiments by using the pCGND-CTF-1 construct with the target HPV-16 fragments and confirmed the result obtained when the p113-CTF-1 construct was used (data not shown), i.e.,

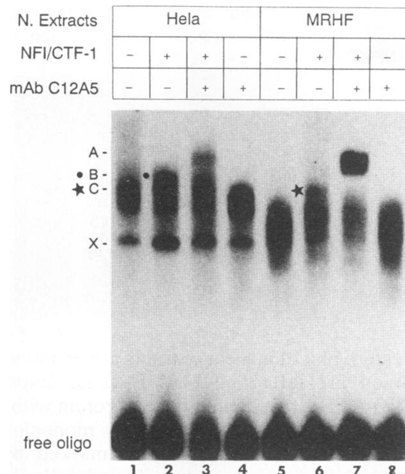

FIG. 5. Band shift experiments with nuclear extracts from untransfected and CTF-1-transfected HeLa and MRHF cells. Supershifts with monoclonal antibody C12A5 identify the complexes formed between transfected CTF-1 and endogenous NFI in HeLa (band B) and MRHF (band C) cells. Both bands can be retarded by the antibody to form band A. Band X results from nonspecific binding. The constituents of individual binding reactions before resolution on a polyacrylamide gel as indicated (N. Extracts, nuclear extracts; NFI/CTF-1, transfection with hemagglutinin-tagged CTF-1; mAb C12A5, monoclonal antibody against the hemagglutinin epitope).

exogenous CTF-1 cannot activate the HPV-16 enhancer in fibroblasts. We therefore investigated the behavior in band shift experiments of transfected CTF-1 in the fibroblast background to examine why, even when correctly expressed, CTF-1 cannot activate the HPV enhancer.

Transfected CTF-1 forms heterodimers with endogenous fibroblast NFI. Nuclear extracts from HeLa or MRHF cells that had been transfected with pCGND-CTF-1 led, in band shift experiments, to complexes with apparently unchanged, as well as lower, mobility (Fig. 5; compare lanes 1 and 2 and lanes 5 and 6). Complexes with lower mobility can be attributed to the transfected form of NFI carrying the hemagglutinin epitope (bands B and C; Fig. 5). Interestingly, the novel DNA-protein complex in HeLa extracts (band B) differs from the complex obtained with MRHF extracts (band C). Since the exogenous NFI protein is of identical size in HeLa and MRHF cells (Fig. 4), the most likely explanation for this finding is the dimerization of exogenous NFI with an endogenous NFI monomer, resulting in a deficiency of homodimers of exogenous NFI in these cells. The lower-mobility complex B formed by NFI proteins present in HeLa extracts may also result from heterodimerization between the exogenous and endogenous CTF-1. Supershifts with monoclonal antibody C12A5 directed against the hemagglutinin epitope of the transfected CTF-1 show that the new high-molecular-weight complexes have indeed resulted from the dimerization of exogenous proteins, as both bands B and C were retarded by the addition of specific antibodies to produce band A. The complexes that ran below band B and C were also reduced by the addition of the antibody. This finding further suggests the presence of transfected NFI proteins in these complexes. The addition of antibodies to untransfected extracts did not lead to any alteration of the individual complexes.

We therefore conclude from these experiments that NFI can be expressed from pCGND-CTF-1 in MRHF fibroblasts in a form that highly resembles HeLa CTF-1 (Fig. 4). However, these exogenous NFI monomers form complexes different from those found in HeLa cells, most likely by dimerizing with endogenous, fibroblast-specific proteins. These different complexes may have different functional properties. Synergistic activation of individual enhancers involving NFI proteins may thus be dependent not only on the presence of a certain type of NFI but also on a specific heterodimeric interaction.

NFI expression differs between human fibroblast and epithelial cells. The divergence of the NFI protein pattern seen in band shift analysis led to the conclusion that human fibroblasts may express different members of the NFI family than do epithelial cells. To address this question, we screened a commercially available human skin fibroblast library under low-stringency conditions with cDNA clone CTF-1. Eight cDNA clones were isolated and partially sequenced. Three cDNA clones did not align to any known sequence in GenBank. Sequence alignment of the other five cDNAs to known forms of NFI, NFI-A, -B, -C, and -X (42), showed that fibroblast cells express members of the NFI-C and NFI-X families. The NFI-C family includes the NFI proteins CTF-1, CTF-2, and CTF-3 from HeLa cells (44), while NFI-X was isolated from a hamster liver cDNA library (17). The cDNA clone FN10-3, a 1.2-kb fragment, matched perfectly to CTF-1 from HeLa cells (EMBL accession number X12492). FN6, a 1.2-kb cDNA clone, aligned best to the hamster *ha*NFI-X (GenBank accession number J04123). The other three cDNAs represented shorter forms (0.4 to 0.8 kb) of the above-mentioned clones. Sequence alignment of 120 bp of the 5′ and 3′ ends of cDNA clone FN6 to *ha*NFI-X and *h*NFI-C is shown in Fig. 6A.

We confirmed the expression of NFI-X and NFI-C in the fibroblast cell line MRHF, which we used for our functional studies, by reverse transcriptase-coupled PCR. In this study, we included RNA from HeLa cells to confirm that epithelial cells do not express NFI-X type proteins. Using a 5′ consensus primer for both forms and a specific primer for NFI-C and NFI-X on the 3′ end (see Materials and Methods), two bands with the predicted sizes of 1.42 kb for CTF-1 and 1.27 kb for CTF-2 could be detected in an agarose gel for both HeLa and MRHF cells. The predicted reaction product of 1.37 kDa, obtained with use of primers for NFI-X, was detectable only when cDNA from MRHF cells was used as the template for the PCR. The PCR for both sets of primers did not result in any product when cDNA synthesized from Daudi lymphocyte RNA was used. The integrity of the Daudi cDNA was confirmed by using primers specific for the transcription factor Oct-1 (data not shown). The specificity of the PCRs was confirmed by probing a Southern blot of the reaction products with 3′ DNA fragments of CTF-1 and FN6 cDNA. These fragments code for the heterogeneous transactivation domains of NFI-C and NFI-X and do not cross-react under stringent hybridization conditions. Figure 6B shows a Southern blot probed with the CTF-1 DNA fragment. The same filter was stripped and reprobed with the NFI-X-specific DNA probe (Fig. 6C).

DISCUSSION

The transcription of HPV-16 is epithelial cell specific. However, no unique cell-specific enhancer element, i.e., an element that binds a factor present in epithelial cells but absent in cell lines in which the enhancer is inactive, has yet been identified. Studies on the HPV-18 enhancer led to the

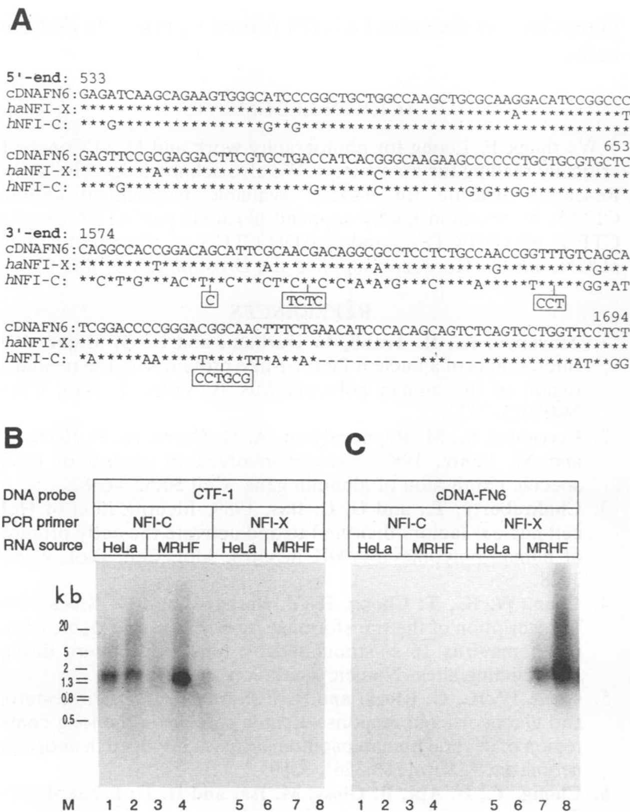

FIG. 6. (A) Sequence alignment of the 5′ and 3′ ends of clone FN6, from a human fibroblast cDNA library, to the hamster NFI-X sequence (*ha*NFI-X; GenBank accession number J04123) and the homologous sequence of the human NFI-C gene (*h*NFI-C; EMBL accession number X12492). Dots represent identical nucleotides. Nucleotides which differ between the different NFI clones are indicated. The genome position number refers to that of *ha*NFI-X. Boxed sequences are insertions in NFI-C which are not present in NFI-X. (B and C) Southern blots of PCR products from HeLa and MRHF cells probed with NFI-C (B) and NFI-X (C). Primers used for cDNA synthesis: RT-2 for NFI-C (lane 1 and 3); Do5 for NFI-X (lanes 5 and 7); oligo(dT) (lanes 2, 4, 6, and 8). M, DNA marker (*Hin*dIII-*Eco*RI-digested λ).

observation of an epithelial cell-specific band shift which, however, does not have a sequence equivalent in other papillomaviruses, such as HPV-16 (30). Furthermore, we do not believe that this HPV-18-specific factor, called KRF-1, constitutes the decisive element of HPV enhancers, since it does not accommodate multiple levels of cooperativity as observed with HPV enhancers (see above and references 6 and 7). Indeed, recent studies have presented evidence that differential expression of the JunB subunit of AP1 (50) and the availability of a cofactor for TEF-1 (25), both ubiquitous factors, can alter HPV enhancer activity in a cell-type-specific manner. The data presented in this report describe the contribution made by the NFI family of transcription factors to the transcriptional activation of the HPV-16 enhancer and furthermore assign an important role for NFI in determination of the viral enhancer's epithelial cell specificity.

Cell type specificity of the HPV-16 enhancer resides in two nonoverlapping enhancer domains, the *Dra*I-*Dra*III fragment and a 91-bp fragment (6), that have only NFI sites in common (Fig. 1A). Point mutations of the individual NFI *cis* elements of both fragments reduce enhancer activity in HeLa cells dramatically (Fig. 1B and C). Furthermore, converting the HPV-16 half palindromic NFI binding sites to consensus palindromic NFI binding sites does not markedly alter the enhancing potential of the wild-type fragment. We have also observed that the activation of the enhancer by NFI is dependent on cooperation with additional factors. In the case of the 91-bp enhancer domain, these factors include AP1 (7) and TEF-1 (25), which do not bind to the *Dra*I-*Dra*III enhancer fragment. For the *Dra*I-*Dra*III fragment, other, unidentified factors are implicated, both by footprints X and Y seen in Fig. 1 (6, 9, 36) and by the observation that multimers of HPV-16 NFI sites alone cannot bring about transcriptional activation (unpublished results). The *Dra*I-*Dra*III and 91-bp clones are weak enhancers compared with the full-length 400-bp enhancer (6), but this is not surprising as these fragments are nonoverlapping subsets of the full-length enhancer. Full enhancer activity is a result of synergism of the various enhancer domains.

In the fibroblast cell line MRHF, we initially observed NFI site-protein complexes of a higher mobility than those found in HeLa cells. We extended this study by examining various cell lines of epithelial and fibroblast origin to show the consistency of this difference. To further substantiate the correlation between the presence of unique epithelial NFI complexes and an ability to support HPV-16 enhancer activity, a liver and a lymphoid cell line, which do not support HPV-16 activity, were included. The liver cell line, HepG2, showed the same protein complex pattern as observed for fibroblast cells. The lymphoid cell line, Daudi, revealed an NFI probe binding profile that again lacked the pattern seen in epithelial cells. In other lymphoid cell lines, this binding activity has been shown to be a protein other than CTF, even though it binds specifically to NFI sites (32).

We infer from these data that the ability of a cell line to support HPV-16 activation correlates with the presence of a specific subset of NFI complexes. The slowly migrating gel shift complexes, which are present in epithelial cells but absent in liver and fibroblast cells, are formed only when CTF-1, the largest of three differentially spliced mRNAs with the full-length proline-rich activation domain (31), is present. This could be shown by reconstituting the band shift pattern in SL-2 cells, which lack endogenous NFI. While gain-of-function experiments have shown that expression of CTF-1 can activate the HPV-16 enhancer (e.g., the complementation of an NFI-deficient transcriptional environment in *Drosophila* SL-2 cells with transfected CTF-1), similar transfection experiments in MRHF fibroblasts did not lead to enhancer activation above the basal level. One possible explanation for these results could be ascribed to the failure of transfection or expression of CTF-1 in MRHF cells. However, we have shown that transfected CTF-1 is correctly expressed and can be detected in Western blot experiments in both HeLa and MRHF cells.

Another possibility for the differences in the activation potential of CTF-1 in different cellular environments is that NFI requires the presence of a cofactor (40, 49) and that such a cofactor is missing in MRHF cells. However, increasing the amount of transfected CTF-1 did not lead to any squelching effect (Fig. 3C), indicating that NFI may not use a limiting cofactor for HPV-16 enhancer activation. However, our results do not completely rule out the involvement of a cofactor for NFI in HeLa cells, since we may not have established in our transfection experiments a sufficiently high level of exogenous CTF to titrate out a very abundant cofactor.

An alternative explanation is that the function of NFI

proteins is regulated by posttranscriptional mechanisms. CTF-1 is subject to O-linked glycosylation (26) as well as phosphorylation (27), and while we cannot exclude that CTF-1 is not modified to the same extent in fibroblasts and epithelial cells, major modification differences seem to be unlikely, as we see no shifting in SDS-polyacrylamide gel electrophoresis when this protein is expressed in HeLa or MRHF cells.

A likely explanation for the dysfunction of CTF-1 in MRHF cells became apparent when nuclear extracts of HeLa and MRHF cells, transfected with CTF-1, were examined for the integrity of the proteins and their interaction with NFI binding sites. Western blot analysis showed that exogenous CTF-1 proteins had the same molecular weight in the two cell lines (Fig. 4) but formed different complexes in band shift analysis (Fig. 5, band B versus band C). The NFI complexes which contained transfected CTF-1 were identified by using antibodies in a series of supershift experiments. These data implied that exogenous CTF-1 dimerizes with endogenous monomers of the NFI population. Cloning of fibroblast type NFI showed that fibroblast cells express, in addition to NFI-C proteins, the NFI type cloned from HeLa cells, NFI-X, a member of the NFI family originally cloned from hamster liver cells (17). This is the first documentation of expression of NFI-X in human cells. Epithelial cells, which have a different DNA-protein pattern, formed by the three different members of the NFI-C family, do not express NFI-X proteins. Overexpression of CTF-1 in fibroblast cells did not lead to the band shift pattern seen in epithelial cells, which shows that CTF-1 preferentially forms heterodimeric complexes with endogenous fibroblast-type NFI proteins. As the dimerization domains of CTF and NFI-X proteins are conserved (33, 42), sequestering of CTF-1 proteins by heterodimerization with endogenous NFI-X is possible. This could explain why there is no activation of the HPV-16 enhancer fragments in CTF-1-transfected fibroblast cells in contrast to SL-2 cells, which do not express endogenous NFI. Our cDNA screening does not exclude that other, not yet identified members of the NFI family participate in the NFI complexes seen in band shift analysis. Additionally, NFI-X proteins may also exist in different forms generated by differential splicing (unpublished observations).

Several models could explain how heterodimerization can alter the function of NFI in a cell-type-specific way. Heterodimers of NFI-C and NFI-X proteins may assume a different conformation in complexes with DNA than do NFI-C–NFI-C complexes and may in turn not be able to cooperate with other transcription factors during the cooperative activation of the HPV-16 enhancer (6). Different members of the NFI family differ extensively in their transcriptional activation domains, which specify the interaction potential of each particular NFI protein with other factors of the transcription machinery. A well-documented example of how transcription can be regulated in a cell-type-specific manner by the presence of different homo- and heterodimers of a transcription factors family is represented by the AP1/ATF family of transcription factors and its interaction with various nuclear receptors (23), e.g., the glucocorticoid receptor (12).

The dependence of NFI binding sites in the cell-type-specific activation of other cellular and viral enhancers was reported recently (21, 29, 34). Since the presence of a specific subset of NFI proteins, composed of three different splice forms of the NFI-C family, correlates strictly with HPV-16 enhancer activity, we infer that the transcription of HPV-16, and most likely of all genital papillomaviruses, is dependent on the subset of NFI proteins present in epithelial cells.

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