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Transcriptional Silencer of the Human Papillomavirus Type 8 Late Promoter Interacts Alternatively with the Viral *trans* Activator E2 or with a Cellular Factor

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The noncoding region of the highly oncogenic, epidermodysplasia verruciformis-associated human papillomavirus type 8 contains a negative regulatory element (NRE). Quantitative RNase protection analysis confirmed that the NRE sequence acts as a silencer of transcription. A 38-bp sequence upstream of late promoter P_{7535} down-regulated expression from the homologous P_{7535} promoter, as well as the heterologous *tk* gene promoter, independently of its orientation relative to the test promoters. It also reduced gene expression when cloned downstream of the transcription units. Transient expression assays with keratinocytes and fibroblasts of epidermodysplasia verruciformis patients and controls demonstrated that the NRE activity is not cell specific. Gel retardation tests suggested that NRE specifically interacts with only one nuclear factor. Mutational analysis identified three NRE mutants which no longer formed a detectable DNA-protein complex but still repressed transcription, indicating that protein-DNA interaction is not relevant for the silencer function. The NRE contains a binding site of viral *trans* activator protein E2. It was shown that expression of E2 overrides the inhibitory effect of the NRE sequences. Binding of E2 and that of the cellular factor were mutually exclusive. The bifunctional nature of NRE acting as a silencer and a target site for viral *trans* activator E2 offers an interesting opportunity to regulate the switch from early to late transcription in the human papillomavirus life cycle.

Analyses of numerous promoters have almost consistently revealed activator and enhancer sequences which bind nuclear proteins that stimulate the rate of transcription initiation by RNA polymerase II (23, 25, 30, 41). Evidence is accumulating that negatively acting elements play an equally important role in the regulation of cellular and viral promoters (16, 28, 43). A negative control can be expected to be particularly important in viruses that are able to establish latent infections characterized by strongly down-regulated transcription. Silencers of transcription were indeed detected in herpesviruses, adenoviruses, papovaviruses, hepadnaviruses, and retroviruses and in particular in human tumor viruses such as Epstein-Barr virus (34, 35) and hepatitis B virus (14).

Human papillomaviruses (HPV) induce a variety of benign proliferations of the skin and mucosa, some of which may progress to cancer (15, 40). A large proportion of HPV infections do not lead to histological abnormalities. The state of the virus in these cases without clinical consequences is not clear but is assumed to represent latent persistence (45). Viral transcription in benign lesions is highly dependent on the differentiation state of the epithelium (39). The so-called early genes, like oncogenes E6 and E7, are weakly expressed in proliferation-competent cells of the basal layer, whereas the late genes encoding structural proteins are transcribed only in terminally differentiated keratinocytes (9, 18, 21, 47). Enhanced expression of viral oncogenes has generally been observed in premalignant lesions and cancers.

Most of the transcription control sequences of papillomaviruses reside within the noncoding region (NCR) of the genome. Initial dissection of the NCRs revealed, besides enhancer elements, several interspersed negative modules, indicating that the regulation of transcription involves a network of counteractive signals (22, 42, 52). More specific analyses with genital HPV type 16 (HPV16) and HPV18 identified negative transcriptional control elements that were responsive to the epidermal growth factor receptor (53) or cellular DNA-binding proteins YY1 (4, 31), retinoic acid receptor (3), and NF-IL6 (27). All of these elements were shown to down-regulate the activity of the early E6-E7 promoters.

HPV8 belongs to the virus types that frequently occur in skin cancers of epidermodysplasia verruciformis (EV) patients (12, 36). EV-associated HPVs are distinguished by inducing disease only in the small group of specifically predisposed EV patients. We recently demonstrated that the HPV8 NCR contains two promoters active in epithelial cells: P_{7535}, a late promoter localized in the 5′ part of the NCR, and P_{175}, residing in front of open reading frame E6 (48). The constitutive activity of P_{7535} is increased by the HPV8 E2 protein (48). In this report, we characterize a negative regulatory element (NRE) that down-regulates the activity of P_{7535}. This element was found to be a close neighbor of the M33-AP1 motif, the main activator sequence of the HPV8 P_{7535} promoter (20). Evidence is presented that the inhibitory effect of the silencer is modulated by viral *trans* activator protein E2 (33), which binds to its palindromic cognate sequence within the NRE.

MATERIALS AND METHODS

Cell culture, transfections, and CAT assays. All cell types were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% fetal calf serum, 120 mg each of

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penicillin and streptomycin per liter, and 100 mg of Ciprobay (Bayer, Leverkusen, Germany) per liter.

Cervical carcinoma cell line HT3 (10), primary human foreskin fibroblasts, EVK (a cell line derived from an eccrine skin carcinoma of an EV patient [19]), primary skin fibroblasts of an EV patient (EVF), and human embryonal fibroblasts (diplP8) were grown in 100-mm-diameter tissue culture dishes and transfected by the standard calcium phosphate method (1) with 20 μg of a chloramphenicol acetyltransferase (CAT) reporter plasmid and 1 μg of a luciferase expression vector (pRSV-LUC) as an internal transfection control (7). To test for *trans* activation by E2, 5 μg of plasmid pCE2 or an equivalent amount of vector pCB6 was coprecipitated. When cotransfecting the expression vector for E2, the luciferase plasmid was omitted because of its susceptibility to *trans* activation by HPV8 E2.

CAT assays were performed as previously described (20), with the enzyme-linked immunosorbent assay system (Boehringer GmbH, Mannheim, Germany). The expression rate was standardized against the luciferase activity of the corresponding extract (7). Each transfection was repeated at least three times.

Oligonucleotides and plasmid constructions. The nucleotide numbering of the HPV8 sequences is that of Fuchs et al. (11). The following double-stranded oligonucleotides were used for plasmid constructions and for band shift analysis: NRE (nucleotides [nt] 7384 to 7421), NRE-MT1 to NRE-MT7 (nt 7384 to 7421 with the point mutations indicated in Fig. 4), NRE-S (nt 7397 to 7421), and HOR (nt 199 to 218). Sequence analysis of pNCR8-CATΔNRE was performed with the pre-NRE primer (nt 7361 to 7383) and the M13(−20) primer (Stratagene, La Jolla, Calif.). All in vitro-synthesized oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany).

Plasmids pNCR8CAT-7378, pNCR8-CAT-7413, and pNCR8-CAT-7422 are 5′ deletions of pNCR8-CAT and were previously described (20, 48). pNCR8-CAT-7378SP and pNCR8-CAT-7422SP were constructed by cloning a 330-bp *Bst*UI fragment from pBR322 (nt 3103 to 3433; 50) into the blunt-ended *Hin*dIII sites of pNCR8-CAT-7378 and pNCR8-CAT-7422, respectively. To generate pNRE-U and pNRE-D, the NRE oligonucleotide was inserted into the unique *Hin*dIII site of pNCR8-CAT-7422 located 12 bp upstream from viral sequences or into the unique *Sma*I site downstream from the *cat* gene, respectively. For pNRE-U, the oligonucleotide was cloned in the sense (pNRE-U-s) and antisense (pNRE-U-a) orientations relative to the promoter. pNRE-3U contains a trimer of the NRE oligonucleotide. The NRE oligonucleotide was also cloned into the *Xba*I (monomer, pNRE-U-*tk*; trimer, pNRE-3U-*tk*) and *Sma*I (pNRE-D-*tk*) sites of pBLCAT2 (29), upstream and downstream of the herpes simplex virus thymidine kinase gene (*tk*) promoter, respectively. Oligonucleotides with NRE point mutations and PCR amplification products, NRE* (nt 7361 to 7421) and NRE** (nt 7361 to 7432), were cloned upstream of the *cat* gene. Shortened oligonucleotide NRE-S was inserted in the antisense orientation relative to the *tk* promoter (pNRE-S-*tk*).

To generate internal deletion mutant pNCR8-CATΔNRE, we PCR amplified the pNCR8-CAT sequence between the binding site of the M13(−20) primer and HPV8 nt 7383, directly upstream of the NRE motif. The PCR product was cloned into *Hin*dIII-*Sph*I-linearized deletion mutant pNCR8-CAT-7422 to reconstitute the upstream sequences of pNCR8-CAT except for the NRE.

pBS-NCR82, used for RNase protection analysis, contains the *Nla*IV-*Alu*I fragment (nt 7359 to 243) of HPV8 cloned into the Bluescribe M13+ vector (48).

To construct the expression vector for the full-length HPV8 E2 open reading frame (pCE2), a DNA fragment comprising the HPV8 sequences from nt 2682 to 4222 was inserted into vector pCB6 (a gift from M. Stinski, University of Iowa) downstream of the cytomegalovirus early gene promoter. For in vitro transcription and translation, the HPV8 E2 gene was cloned into the *Bam*HI site of the Bluescribe M13+ vector (Stratagene).

All new plasmid constructs were verified by direct sequence analysis as described by Sanger et al. (44).

RNA analysis. Total RNA from HT3 cells was isolated by the guanidinium lysis method of Chirgwin et al. (6). RNase protection experiments were performed essentially as previously described (48), with riboprobes synthesized with T7 RNA polymerase after cutting of template plasmid pBS-NCR82 with *Bam*HI. As an internal control for transfection efficiency, 2 μg of plasmid pOVEC was cotransfected (51). Its β-globin transcript was detected parallel to the CAT mRNA by using a riboprobe transcribed from vector SP6βTS (51). Protected RNA fragments were separated by 5% urea-polyacrylamide gel electrophoresis. To quantify the results, RNA bands were cut out from the gel and radioactivity was determined by scintillation counting.

Gel retardation analysis. Band shift assays were performed as previously described (32), with crude nuclear extracts prepared as described by Sealey and Chalkley (46) or Dignam et al. (8). Briefly, an aliquot of a ^{32}P-labelled oligonucleotide equivalent to 2×10^4 Cerenkov cpm was incubated with 15 μg of a nuclear extract and 0.5 or 1.0 μg of poly(dI-dC) · poly(dI-dC) in a total volume of 20 μl of binding buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9], 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.25 mM dithiothreitol, 0.1 mM spermidine, 100 μg of bovine serum albumin per ml, 5% glycerol). For competition experiments, the reaction was supplemented with different amounts of homologous and heterologous unlabelled oligonucleotides. After 20 min at room temperature, the samples were electrophoresed in 5% native polyacrylamide gels. Competitive band shift analyses with in vitro-synthesized E2 protein were performed with 2 to 6 μl of rabbit reticulocyte lysate and a mixture of 0.7 μg of poly(dI) · poly(dC) and 0.3 μg of poly(dA-dT) · poly(dA-dT).

In vitro synthesis of HPV8 E2 protein. In vitro transcription and translation of HPV8 E2 DNA were performed with kits in accordance with the instructions of the manufacturers (Stratagene and Promega [Madison, Wis.]). An *Eco*RI-linearized Bluescribe vector containing E2 DNA served as the template for in vitro transcription. E2 protein was translated in a reaction volume of 50 μl with 2 μl of RNA, a 1 mM amino acid mixture, and 35 μl of rabbit reticulocyte lysate. In control experiments, the translation procedure was performed without addition of RNA (unprogrammed lysate).

RESULTS

The upstream region of the HPV8 P_{7535} promoter contains a transcriptional silencer. Analysis of 5′ deletion mutants of the HPV8 NCR suggested the occurrence of a negative *cis*-active element in the upstream region of late promoter P_{7535} (48). Removal of 44 bp between HPV8 nt 7378 and 7422 resulted in a 17-fold increase in promoter activity of HPV8 NCR-CAT test constructs. Analysis of additional deletion mutants showed that pNCR8-CAT-7400 was nearly as re-

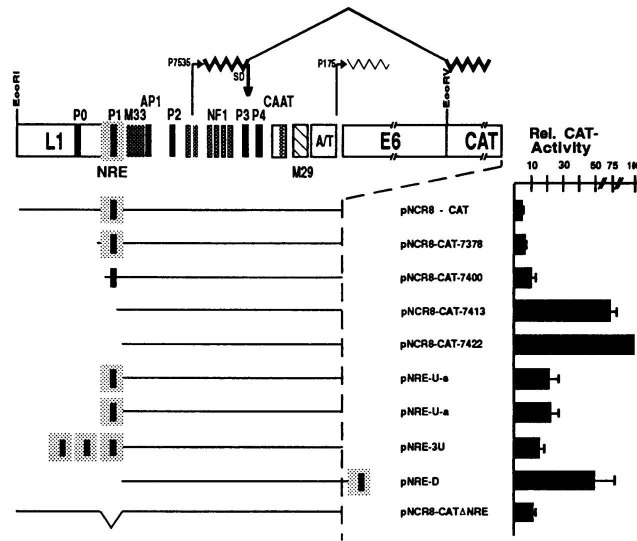

FIG. 1. Negative regulation of *cat* gene expression by the NRE of HPV8. The diagram at the top shows the organization of the HPV8 NCR between open reading frames L1 and E6. P0 to P4, E2-binding palindromes (filled boxes); M33, M29, and A/T, conserved sequence motifs in the NCR of EV-associated virus types; CAAT, CAAT box equivalent; AP1, AP1-binding site; NF1, NF1-binding sites; P7535 and P175, HPV8 NCR promoters; SD, splice donor; NRE, silencer element. The structures of the P_{7535}- and P_{175}-specific transcripts are shown above. The lower diagrams represent silencer test plasmids. Promoter test construct pNCR8-CAT was generated by inserting the HPV8 *Eco*RI-*Eco*RV fragment into pBLCAT3 upstream of the *cat* gene. Each deletion mutants thereof is designated with the position of the first nucleotide of the shortened HPV8 fragment. Silencer test constructs pNRE-U contains HPV8 NRE oligonucleotides in the sense (pNRE-U-s) and antisense (pNRE-U-a) orientations upstream of position 7422 of promoter plasmid pNCR8-CAT-7422. pNRE-3U contains a trimer of the NRE oligonucleotide. For pNRE-D, the oligonucleotide was cloned downstream to the *cat* gene. For pNCR8-CATΔNRE, the NRE was deleted. CAT activities relative to that of pNCR8-CAT-7422 (100%) are shown to the right. The error bars are based on at least five independent experiments.

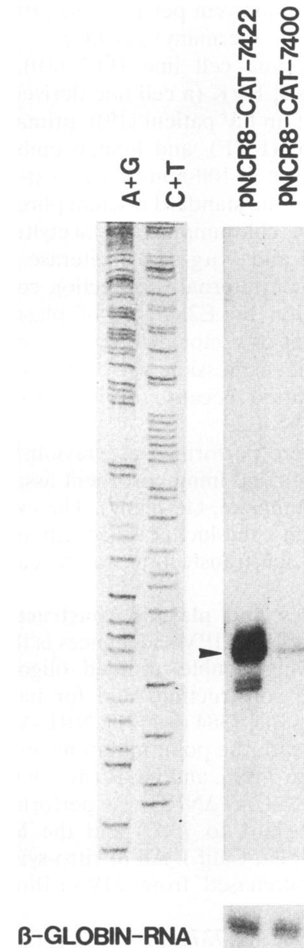

FIG. 2. RNase protection analysis of steady-state levels of P_{7535}-specific mRNA. HT3 cells were transfected with 20 μg of deletion mutants pNCR8-CAT-7422 and pNCR8-CAT-7400. Fragments corresponding to the correctly initiated transcripts with 124 nt are indicated by the arrowhead. As an internal control for transfection efficiency, 2 μg of plasmid pOVEC was cotransfected with the reporter plasmids. The β-globin mRNA was detected in RNase protection reactions by using a riboprobe transcribed from plasmid SP6βTS. A sequencing ladder of pNCR8-CAT was used as molecular length markers (A+G and C+T).

pressed as pNCR8-CAT-7378, whereas pNCR8-CAT-7413 (20) already reached 75% of pNCR8-CAT-7422 activity. This locates the 5′ end of the NRE core 13 bp downstream of position 7400 (Fig. 1).

Using a quantitative RNase protection assay, we compared the steady-state levels of *cat* mRNA in HT3 cells transiently transfected with pNCR8-CAT-7422 and pNCR8-CAT-7400 and observed drastically reduced amounts of *cat* mRNA with the latter (Fig. 2). This indicates that the NRE exerts its function at the level of transcription.

To characterize the identified HPV8 NRE further, we synthesized an oligonucleotide corresponding to the HPV8 sequence between nt 7384 and 7421 and cloned it upstream and downstream of the HPV8 NCR-*cat* cassette of the most active promoter plasmid, pNCR8-CAT-7422. All of these constructs showed substantially reduced CAT enzyme expression compared with the parental plasmid. An NRE oligonucleotide cloned in the upstream position as a monomer (pNRE-U) and as a trimer (pNRE-3U) resulted in fourfold and sevenfold decreases of CAT expression, respectively. There was essentially no difference in the activities of plasmids containing the NRE sequence in opposite orientations (pNRE-U-s and pNRE-U-a). A twofold reduction was observed with the NRE element cloned 3′ to the *cat* gene (pNRE-D). According to these data the NRE displays properties of a transcriptional silencer.

To test the role of the NRE in the context of the entire HPV8 NCR, we removed the corresponding sequence from pNCR8-CAT, generating plasmid pNCR8-CATΔNRE. As revealed by transient CAT assays, the promoter activity of the NCR was stimulated 2.5-fold after deletion of the NRE motif (Fig. 1). The difference in the stimulation of HPV8 transcription due to deletion of the NRE element from pNCR8-CAT-7378 (17-fold) and from the context of the NCR (2.5-fold) could be a consequence of changed spacing between the promoter and sequences lying upstream of the HPV8 NCR. To test this possibility, we inserted a 330-bp spacer consisting of an extraneous sequence into the *Hin*dIII sites of constructs pNCR8-CAT-7378 and pNCR8-CAT-7422, thus restoring the distance between vector parts and the HPV8 promoter in

TABLE 1. Silencer activity of NRE on the *tk* gene promoter[a]

Plasmid	Mean relative CAT concn (%) ± SD	
	Without E2	With E2
pBLCAT2	100	80 ± 20
pNRE-U-*tk*	24 ± 6.0	180 ± 20
pNRE-3U-*tk*	17 ± 5.1	1,530 ± 200
pNRE-D-*tk*	55 ± 0.6	ND[b]
pNRE*-U-s-*tk*	26 ± 5.4	ND
pNRE**-U-s-*tk*	26 ± 2.4	ND

[a] CAT concentrations were determined in cell extracts from at least three independent transfection experiments and normalized against luciferase activity in assays without E2. The concentrations are expressed relative to that obtained with pBLCAT2.
[b] ND, not done.

pNCR8-CAT. Transient CAT expression experiments revealed no significant change in the relative activities of pNCR8-CAT-7378 and pNCR8-CAT-7422 and their spacer-containing derivatives, suggesting that vector sequences have no influence on the silencing activity of the NRE.

Down-regulation of transcription by the NRE could be a property intrinsic to the control sequences of the homologous HPV8 late promoter. To investigate whether the NRE also influences the activity of a heterologous promoter, we cloned the NRE oligonucleotide into vector pBLCAT2, which contains the *cat* gene controlled by the herpes simplex virus *tk* promoter. The constructs were designed analogous to the HPV8 promoter plasmids and were tested for transient CAT expression in HT3 cells. Plasmids carrying the NRE monomer and trimer 5′ to the promoter-reporter gene unit presented four- and sixfold-reduced CAT expression in comparison with pBLCAT2 (Table 1). A twofold reduction of expression was observed with the NRE in the downstream position. These results demonstrate that the NRE can repress both homologous and heterologous promoters comparably. However, this inhibitory effect was considerably lower than expected from the analysis of the deletion mutants.

To determine if surrounding sequences may enhance the activity of the NRE, we synthesized two PCR amplification products, extending the NRE oligonucleotide in both cases to position 7361 at the 5′ end and in one case additionally to position 7432 at the 3′ end. The choice of the new 3′ boundary was directed by the results of Horn et al. (20), who identified an activator element starting between positions 7422 and 7432. The PCR products were cloned into pBLCAT2 analogous to pNRE-U-*tk* to generate the constructs pNRE*-U-s-*tk* and pNRE**-U-s-*tk*, respectively, which were tested in transient CAT assays. The flanking sequences did not improve NRE activity (Table 1).

To test for possible cell type specificity of NRE sequences, we analyzed the activity of pNRE-U-a relative to that of pNCR8-CAT-7422 in a skin keratinocyte cell line of an EV patient (EVK), in primary human foreskin fibroblasts, in primary skin fibroblasts of an EV patient (EVF), and in human embryonal fibroblasts (diplP8). In all of these cells, pNRE-U-a achieved only 16 to 26% of the CAT expression of the silencerless control construct.

Interaction of the HPV8 NRE with nuclear proteins. To gain insight into the mechanisms that mediate the negative function of the NRE, we subjected the corresponding oligonucleotide to gel retardation analyses with the nuclear extract of HT3 cells (Fig. 3). Two DNA-protein complexes were demonstrated. Only the upper complex was successfully competed against by a nonlabeled homologous nucleotide but not by heterologous

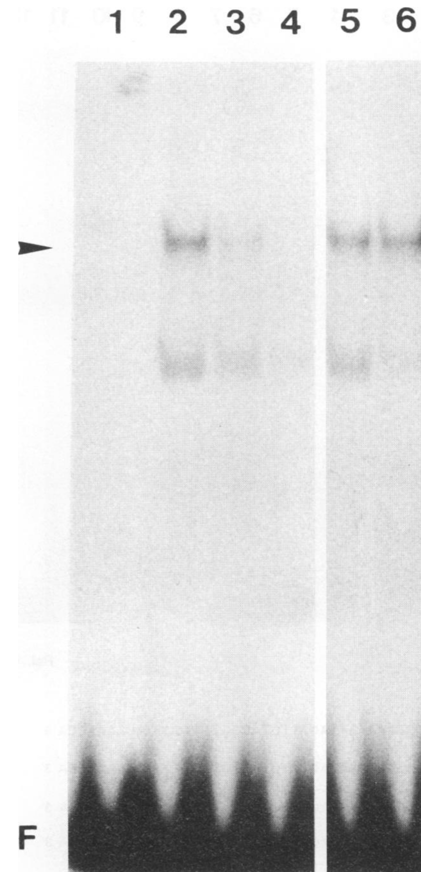

FIG. 3. The NRE forms one specifically retarded DNA-protein complex. Gel mobility shift assays were performed with the ^{32}P-labelled NRE oligonucleotide and 15 μg of nuclear extract of HT3 cells. No protein was added in lane 1. Binding reactions were performed in the absence of competitor DNA (lane 2), with 10 (lane 3) or 50 (lane 4) ng of an unlabelled NRE oligonucleotide, or in the presence of 10 (lane 5) or 100 (lane 6) ng of the unlabelled HOR oligonucleotide. The arrowhead points to the position of the specific DNA-protein complex. F, free oligonucleotides.

oligonucleotides and is therefore specific. To test for the importance of the protein-DNA interaction, we synthesized seven mutated double-stranded NRE oligonucleotides which contained two or four base substitutions in various positions (Fig. 4B). Additionally, we synthesized a shortened version of the NRE oligonucleotide lacking about one-third of the sequences at its 5′ end (NRE-S). The NRE-MT4 and NRE-S oligonucleotides failed to form the specific complex, and oligonucleotides NRE-MT6 and NRE-MT7 were severely disabled (Fig. 4A).

Functional analysis of NRE mutants. To determine the functional consequences of the NRE mutations, we introduced all of the mutated NRE oligonucleotides into pBLCAT2 upstream of the *cat* gene and tested the constructs in transient assays. All of the mutants, except NRE-MT3 and NRE-MT4, showed wild-type silencer activity. Mutation NRE-MT3 led to a slight increase, whereas mutation NRE-MT4 resulted in 2.4-fold stimulation of CAT expression relative to that of pNRE-U-*tk* (Fig. 4B).

Only one mutation (MT4) affected both protein binding and silencer activity. Three mutants (NRE-MT6, NRE-MT7, and NRE-S) no longer bound the NRE-specific protein but still

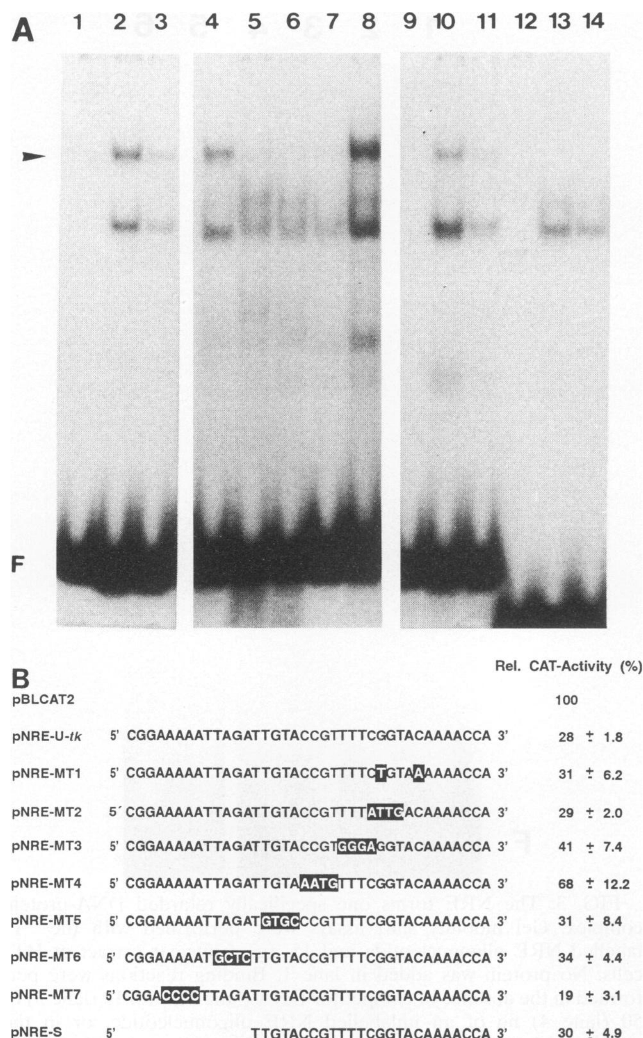

FIG. 4. Protein-binding capacity and functional analysis of the wild-type and mutated NRE oligonucleotides. (A) Band shift analysis for protein binding to wild-type and mutated NRE oligonucleotides (see panel B). Lanes: 1, 2, and 3 NRE; 4, NRE-MT3; 5, NRE-MT6; 6, NRE-MT7; 7, NRE-MT4; 8, NRE-MT5; 9, 10, and 11, NRE-MT1; 12, 13, and 14, NRE-S. No nuclear protein was present in lanes 1, 9, and 12. The other lanes showed band shifts after incubation with nuclear extract. In lanes 3, 11, and 14, the amount of unspecific competitor poly(dI-dC) · poly(dI-dC) was raised to 1.5 μg. The arrowhead marks the NRE-specific complex. F, unbound oligonucleotides. (B) NRE mutants and their activities in transient expression assays. Mutations introduced into the NRE sequence are boxed. All double-stranded NRE oligonucleotides were inserted into the *Xba*I site of vector pBLCAT2. Mean CAT activities relative to that of pBLCAT2 and standard deviations are shown to the right.

repressed transcription to the same extent as the wild-type NRE, whereas one mutation (NRE-MT3) reduced silencer activity without impeding protein binding. This result suggests that the protein-binding domain and the functional silencer core of the NRE overlap without negative regulatory function dependence on this DNA-protein interaction.

Silencer activity of the NRE motif can be modulated by viral *trans* activator E2. An intriguing aspect of the NRE sequence is the presence of a perfect binding site for viral *trans* activator protein E2. All of the experiments described so far were

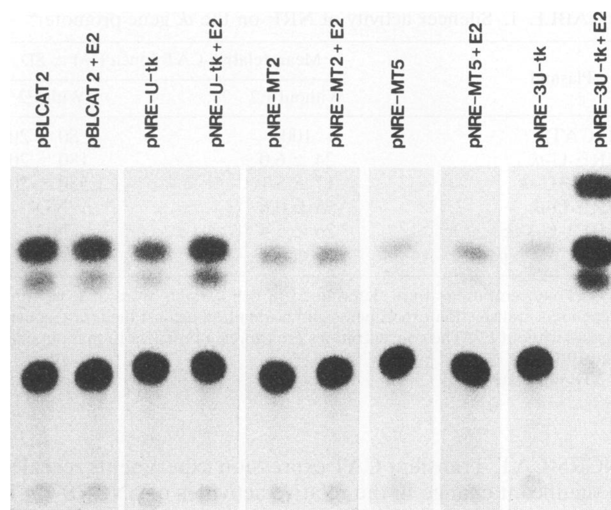

FIG. 5. Effect of HPV8 E2 on the activity of wild-type and mutated NREs. Monolayers of HT3 cells were transfected with wild-type (pNRE-U-*tk* or pNRE-3U-*tk*) or mutated (pNRE-MT2 or pNRE-MT5) NREs cloned into test vector pBLCAT2 with and without the HPV8 E2 expression vector. The CAT activity expressed was determined 48 h after transfection by enzyme assays with [^{14}C]chloramphenicol as the substrate as described previously (48).

performed without viral E2. Cotransfection of HT3 cells with pNRE-U-*tk* and expression vector pCE2 for HPV8 E2 resulted in a sevenfold increased CAT concentration compared with pNRE-U-*tk* alone (Fig. 5 and Table 1). Viral E2 stimulated the CAT expression of pNRE-3U-*tk* with three copies of the NRE 90-fold, which can be explained by the synergism of the three E2 binding sites generating an E2-dependent enhancer (17). This indicates that the negative regulatory activity of the NRE can be overridden by viral *trans* activator E2. No effect of E2 could be demonstrated for silencer constructs pNRE-MT2 and pNRE-MT5 containing mutated NREs (Fig. 5). This demonstrates that E2 *trans* activation is due to DNA binding within the NRE and not to the general transactivational activity of E2.

The E2 site and the previously defined binding site of the NRE-specific protein (Fig. 4B) overlap. We therefore investigated if binding of both proteins is mutually exclusive. We performed a band shift of the NRE oligonucleotide with HT3 nuclear extract and increasing concentrations of in vitro-translated E2 protein. Figure 6 shows that band A, corresponding to the NRE-specific complex, disappeared after addition of increasing amounts of E2 protein, leading to complexes B1 to B4 (lanes 8 to 11). In analogy, disappearance of the E2-specific DNA complexes was observed with increasing concentrations of HT3 protein (lanes 2 to 6). No E2-induced dissociation of the NRE factor was found for the complexes formed with oligonucleotides NRE-MT2 (lanes 12 to 17) and NRE-MT5 (lanes 18 to 23), which contain mutations in the E2-binding palindrome resulting in complete loss (NRE-MT2) or severe reduction (NRE-MT5) of E2-binding capacity. We conclude from this experiment that E2 protein competitively displaces the NRE-specific factor from the adjacent binding site and vice versa.

DISCUSSION

A NRE in the upstream region of late HPV8 promoter P_{7535} was active independently of its orientation, in both upstream

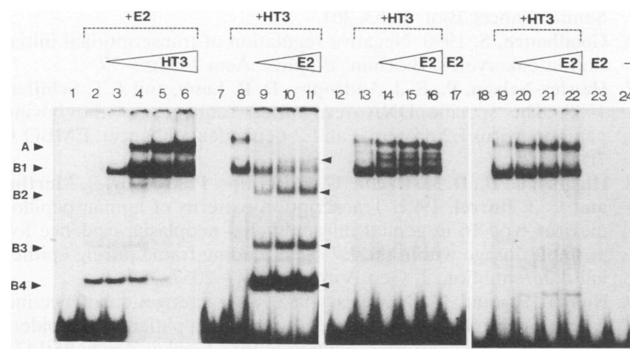

FIG. 6. Competitive interaction of the cellular factor and viral E2 with the NRE sequence. To investigate whether viral E2 and the NRE factor could bind simultaneously to their target sequences, band shift analyses were performed with wild-type NRE (lanes 1 to 11), NRE-MT2 (lanes 12 to 17), and NRE-MT5 (lanes 18 to 23) oligonucleotides by using in vitro-translated E2 protein and HT3 nuclear extract. In lanes 2 to 6, the NRE oligonucleotide was incubated with 2 μl of rabbit reticulocyte lysate and increasing amounts (0, 1, 5, 10, and 15 μg) of HT3 protein. In lanes 8 to 11, 13 to 16, and 19 to 22, binding reactions were performed by addition of 5 μg of HT3 extract and increasing concentrations of E2 protein (0, 2, 4, and 6 μl of lysate). Lanes: 2, 17, and 23, oligonucleotides incubated with 2 μl of lysate alone; 1, 12, and 18, NRE, NRE-MT2, and NRE-MT5 oligonucleotides without addition of protein; 24, unprogrammed lysate with the NRE oligonucleotide. A, complex with NRE-binding cellular protein; B1 to B4, complexes formed by the E2 protein. The binding affinity of E2 and the cellular protein varied with experimental conditions. Band shifts 1 to 6 were run with 1.0 μg of poly(dI) · poly(dC), and assays 7 to 24 were done with 0.7 μg of poly(dI) · poly(dC) and 0.3 μg of poly(dA-dT) · poly(dA-dT). The two extra bands observed with NRE-MT2 (lanes 13 to 16) are specific for the mutated oligonucleotide. They were successfully competed against by homologous NRE-MT2 but not by the wild-type NRE.

and downstream positions relative to the homologous promoter. Essentially the same results were obtained when the influence of the NRE on the activity of the heterologous *tk* gene promoter was tested, indicating that the inhibitory properties of this element are not specific for the HPV8 late promoter. NRE-mediated reduced expression of the *cat* reporter gene was shown to result from decreased levels of *cat*-specific mRNA. The NRE thus fulfills all of the requirements of a transcriptional silencer (5). The decrease of CAT expression was less pronounced with the NRE oligonucleotide cloned 3′ to the NCR-*cat* cassette. Such a position effect was also reported for other silencer elements (2, 24, 37). The HPV8 silencer was active in both keratinocytes and fibroblasts of EV patients and non-EV donors.

Analysis of 5′ deletion mutants of the HPV8 NCR mapped the 5′ boundary of the HPV8 silencer between nt 7400 and 7413. This was confirmed by the comparable negative effects of oligonucleotides NRE-S, NRE, and NRE* (5′ ends at positions 7397, 7384, and 7361) in front of the *tk* promoter and by mutations NRE-MT6 and NRE-MT7 (nt positions 7388 to 7396) showing no effect on silencer activity. Among the 5′ deletion mutants, pNCR8-CAT-7422 showed maximal expression (20, 48), indicating that there is no functional NRE module downstream of position 7422. Extension of the standard NRE oligonucleotide beyond position 7421 did not even reveal auxiliary sequences between nt 7422 and 7432. Exhaustive mutagenesis of NRE (Fig. 4B) finally localized the silencer core between positions 7402 and 7409 (mutants NRE-MT3 and NRE-MT4).

Negative regulation of HPV8 late transcription by the NRE in the natural context is supported by a 2.5-fold increase in *cat* expression after deletion of the NRE from pNCR8-CAT (Fig. 1). A 1.7-fold enhancement was observed when mutation NRE-MT4 (Fig. 4B) was introduced into pNCR8-CAT (49). This effect is quantitatively comparable to the down-regulation achieved by cloning the NRE in front of the homologous P_{7535} promoter and the heterologous *tk* promoter. In view of these data, the 10- to 17-fold-increased promoter activity of pNCR8-CAT-7422 relative to pNCR8-CAT-7400 and pNCR8-CAT-7378 is rather surprising. As pNRE-U-s with the reinserted NRE and pNCR8-CAT-7400 differ in only a few nucleotides because of the cloning procedure, the extraordinary silencer activity of the NRE in the context of the 5′ deletion mutants seems to be highly dependent on correct spacing downstream of the NRE. As shown by cloning of extraneous spacer sequences in front of the NRE, this effect is not mediated by changing the distance to the vector backbone.

Band shift analyses with wild-type and mutated NRE oligonucleotides revealed one DNA-protein complex (Fig. 3) dependent on sequences between positions 7388 and 7405 (mutants NRE-MT4, NRE-MT6, and NRE-MT7). Since NRE-MT6, NRE-MT7, and NRE-S no longer bind the only detectable NRE-specific protein but are still active as transcriptional silencers, it is tempting to speculate that protein binding is not involved in the mediation of silencer activity. For technical reasons, it is difficult to exclude definitely the possibility that a protein(s) plays a role in NRE activity. One can think in this regard, e.g., about a labile protein(s) that undergoes rapid degradation upon attempted isolation or about factors against which the extraction protocol selects. However, it should be noted that identical band shift results have been observed with nuclear extracts prepared by two different methods (8, 46). A possible functional independence of any cooperating protein would be a very unusual feature of the HPV8 NRE. Most known eukaryotic silencers depend on binding of specific repressor factors, but an NRE of the mammalian dihydrofolate reductase gene promoter was recently shown to down-regulate transcription in the absence of protein binding because of its rigid DNA structure, which probably limits interactions between proteins that bind adjacent to this region (38).

NREs that reduce the levels of polyadenylated cytoplasmic RNA have been identified in bovine papillomavirus type 1 and in genital mucosa-specific HPV16 (13, 26). Their genomic locations are similar to that of the HPV8 NRE, but they have to be cotranscribed as parts of the polyadenylated mRNA molecules and the bovine papillomavirus type 1 element works only in the sense orientation. The HPV8 silencer does not appear in the CAT-specific mRNA and also lacks the characteristic AT-rich stretches. Except for localization, there are consequently no analogies between the NREs of bovine papillomavirus type 1 and HPV16 and the NRE of HPV8 concerning their modes of action.

The most intriguing aspect of the HPV8 silencer is the finding that its negative activity on late promoter P_{7535} may be counterbalanced by binding of viral *trans* activator E2 to its cognate sequence within the NRE. It seems possible that this effect accounts, at least in part, for the stimulation of the late promoter by protein E2. In view of these data, the NRE motif appears as a functionally bivalent element whose activity might vary depending on the presence and/or the level of the viral *trans* activator E2. This scenario can be expected to be further complicated by the cellular protein whose binding site overlaps with the cognate sequence of E2. Binding of both proteins to the NRE was shown to be mutually exclusive, indicating that

the cellular factor prevents E2 activation at appropriate relative concentrations.

The presence of the NRE in the proximity of the late promoter suggests a role in the early-to-late-gene switch in HPV8. It can be assumed that the NRE acts as a silencer early in the life cycle in the absence of the viral E2 protein. Even at low levels of E2, the negative role of the NRE may be supported by the cellular E2 competitor. Following increased expression of E2 in the more differentiated cell layers of the epithelium and—maybe—decreased expression of the cellular antagonist, HPV8 E2 will finally override the negative effect of the NRE and stimulate the late promoter.

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