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Transcriptional Regulatory Elements in the Noncoding Region of Human Papillomavirus Type 6

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We have identified three elements in the noncoding region of human papillomavirus type 6 (HPV-6) that regulate transcription when assayed in recombinant plasmids containing the bacterial gene for chloramphenicol acetyltransferase. One was a silencer that reduced expression in both a species- and tissue-dependent manner. The second was an enhancer element that was tissue specific. The third was a weak promoter that showed some tissue specificity. These elements have been localized within the noncoding region by analysis of 5′-to-3′ and 3′-to-5′ deletions with two HPV-6 subtypes, HPV-6e and HPV-6g. HPV-6g differs from HPV-6e by the presence of an additional copy in tandem of a 136-base-pair (bp) sequence and by an 8-bp sequence containing a 3-bp deletion. Silencer activity, assayed in plasmids with the simian virus 40 minimum promoter which were transfected into NIH 3T3 cells, could not be overcome by the enhancer activity of the simian virus 40 72-bp repeats. The 413-bp fragment A of HPV-6g showed silencer activity, while the corresponding HPV-6e fragment containing the 8-bp change did not. Enhancer activity of HPV-6g was localized to fragment C of 326 bp which contains the 136-bp repeat. Dot blot hybridizations reflected relative chloramphenicol acetyltransferase activities and demonstrated enhancer and silencer activities at the RNA level. Analysis of the interactions of these activities in naturally occurring variants should provide information on tissue specificity and regulation of gene expression of HPVs and may provide information on the mechanism of action of transcriptional regulatory elements in eucaryotic cells.

Human papillomaviruses (HPV) are naturally occurring tumor viruses that induce epithelial cell proliferation during the course of a productive infection. Replication of different HPV types is associated with particular anatomical sites. HPV type 6 (HPV-6) causes benign papilloma in the respiratory and genital tracts (12, 29), while different HPV types, for example HPV-16 (8), have been identified in malignant lesions of the respiratory (1) and genital tracts (9). These viruses also appear to have the ability to persist in a latent state. HPV DNA has been identified in apparently normal vocal cord epithelium in a patient with active recurrent respiratory papillomatosis (34), and disease has recurred in patients after as much as a 33-year remission (2). One approach to understanding the behavior of papillomaviruses is to investigate the virus-host cell relationship at the level of regulation of gene expression. Nucleotide sequence analysis of papillomavirus genomes from humans and other species has demonstrated a similar arrangement of potential open reading frames (ORFs) and a noncoding region (NCR), also called upstream regulatory region or long control region, of approximately 800 base pairs (bp), that has sequences associated with transcriptional regulatory elements such as TATA and CAAT (6). Several groups have demonstrated enhancer activity dependent on the interaction of E2 ORF product with the NCR in different types of HPV (20, 33, 36). For example, HPV-16, which was molecularly cloned from a cervical carcinoma (8), has been shown to contain a conditional enhancer that can be transactivated by an E2 ORF product (5, 30) and suppressed by an E2 ORF product that represses transcription (5). HPV-16 has also been shown (13) to contain a glucocorticoid-responsive enhancer activity that is active in HeLa cells, an HPV-18-containing cell line derived from a cervical carcinoma (3), but not in MCF-7 cells, a human breast-tumor-derived cell line. Two isolates of HPV-6, HPV-6b (7) and HPV-6vc (31), have been analyzed for enhancer activity (32). The enhancer activity in HPV-6vc was stronger than in HPV-6b, with two regions of approximately 300 and 400 bp in HPV-6vc having detectable enhancer activities in specific tissue culture cell lines (32).

We have been analyzing the behavior and transcriptional activities of naturally occurring variants of HPV-6 that are distinguishable by restriction endonuclease digestion patterns (28, 29). We report here the analysis of the NCRs of two HPV-6 subtypes. We have detected transcriptional silencer activity that reduces gene expression in both a species- and tissue-independent manner, tissue-specific enhancer activity, and weak promoter activity. The silencer activity could not be overcome by the enhancer activity of the simian virus 40 (SV40) 72-bp repeats and may be analogous to the silencers detected in the yeast *HMRE* locus (4), the rat insulin 1 locus (22), and the human beta interferon gene (14). Analysis of these transcriptional regulatory elements should provide information on the tissue specificity and regulation of gene expression of HPV and may provide a model system for regulation of gene expression in eucaryotic cells.

MATERIALS AND METHODS

Cells. The mouse fibroblast line NIH 3T3 and human osteosarcoma fibroblast line HOS (35) were obtained from Lou Ann Eader and Donald Blair (Frederick Cancer Research Facility). Cell lines derived from cervical carcinoma (HeLa, CaSki, C-33A, and HT-3) were obtained from the American Type Culture Collection, Rockville, Md. NIH 3T3, HOS, and HeLa cells were maintained in Dulbecco modified minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Hyclone; Sterile Systems Inc., Logan, Utah). CaSki,

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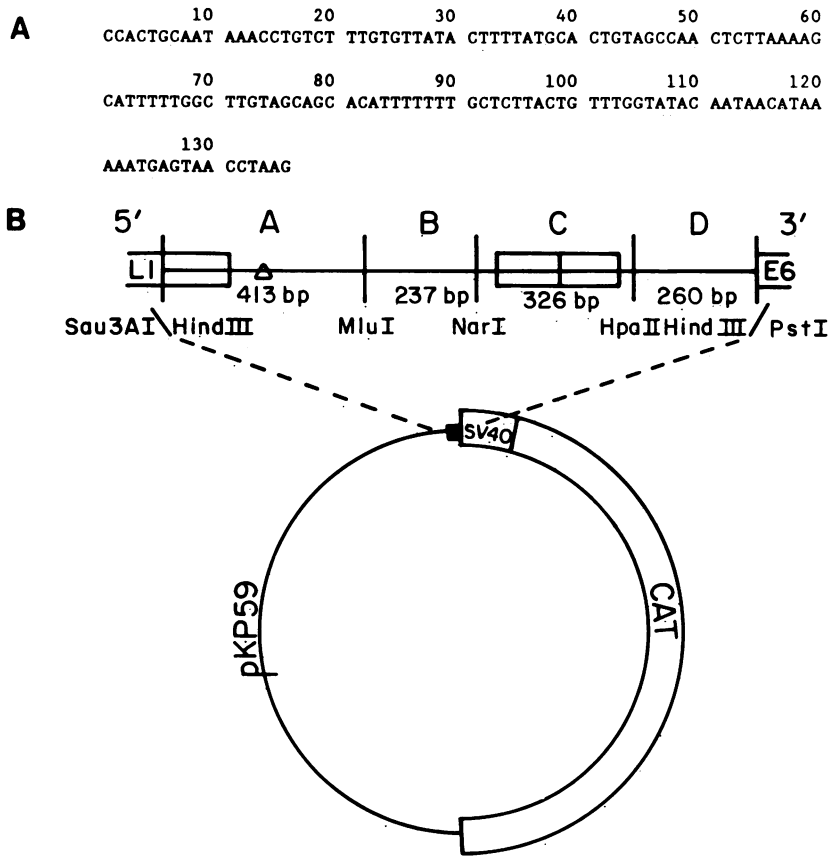

FIG. 1. Map of the NCR of HPV-6g contained in the *Sau*3AI-*Pst*I fragment that was cloned at *Hin*dIII sites as described in Materials and Methods, diagrammed with pertinent restriction enzyme sites that define the endpoints of four fragments, labeled A through D. The box in fragment C indicates the location of the 136-bp tandem repeat that is present in HPV-6g, and the sequence of one copy of this repeat (A) as it is present in HPV-6e is shown above the map (B). Symbol: △, the location of an 8-bp difference between HPV-6e and HPV-6g. Multiple cloning sites in the plasmid vector pKP59 were used to insert DNA to assay for enhancer or promoter activity. The plasmid shown here contains the *cat* gene and the SV40 minimum promoter constructed as described in Materials and Methods. L1 and E6, ORFs.

C-33A, and HT-3 cells were maintained in modified L15 (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% fetal bovine serum.

Plasmids. The plasmids pSV2cat (15), pA10*cat* (23), and pKP59, a 2-kilobase derivative of pBR322 containing a polylinker, were obtained from Keith Peden (Johns Hopkins University). The plasmid pTC consisted of the 1,634-bp *Bam*HI-*Hin*dIII fragment containing the *cat* coding sequences from pSV2cat cloned into the polylinker of pKP59. The plasmids pTC-6eABCD and pTC-6gABCD were generated by isolating the *Sau*3AI-*Pst*I fragment that contains the NCRs of the HPV-6e and HPV-6g genomes, adding *Hin*dIII linkers, and cloning the fragment into the *Hin*dIII site of pTC, which is 5′ to the *cat* gene. The plasmid pTCS was constructed by cloning into pKP59 the 1,784-bp *Bgl*II-*Bam*HI fragment of pA10*cat* that contains the SV40 minimum promoter and *cat* coding sequences. The series of 5′ and 3′ deletions of the HPV-6e and HPV-6g NCRs were generated by digestion of the NCRs with the appropriate restriction enzymes (Fig. 1B) and insertion into pTCS in the polylinker that was 5′ to the SV40 promoter. The same orientation was maintained for all of the deletions. For example, in the series of 5′ deletions, the plasmid pTCS-6gBCD was constructed by digesting the NCR of HPV-6g with *Hin*dIII and *Mlu*I to delete fragment A, pTCS-6gCD was constructed by digesting with *Hin*dIII and *Nar*I, and pTCS-6gD was constructed by digesting with *Hin*dIII and *Hpa*II. Fragment A refers to the DNA between the *Hin*dIII and *Mlu*I sites, fragment B refers to that between the *Mlu*I and *Nar*I sites, fragment C refers to that between the *Nar*I and *Hpa*II sites, and fragment D refers to that between the *Hpa*II and *Hin*dIII sites (Fig. 1B).

Transfections. DNA transfections were performed by the calcium phosphate precipitation method of Graham and van der Eb (16) with a 15% glycerol shock for 1 min (11). To subconfluent cells in a 60-mm dish were added 5 μg of test DNA and 3 μg of pCH110, a plasmid containing the *Escherichia coli lacZ* gene (17) that was used to monitor transfection efficiency (19). Cells were harvested 45 to 50 h post-transfection by being washed with phosphate-buffered saline and by being scraped with a rubber policeman in 1 ml of 40 mM Tris hydrochloride (pH 7.4)–1 mM EDTA–150 mM NaCl. Cells were pelleted, suspended in 0.25 M Tris hydrochloride (pH 7.8), and lysed by freeze-thawing. Debris was removed by centrifugation at 12,000 × *g* for 5 min, and the lysate was divided into two portions for assays to determine chloramphenicol acetyltransferase (CAT) and β-galactosidase (GAL) activities. CAT assays were performed as described by Gorman et al. (15), and GAL assays were performed as described by Miller (26), with units equal to $1{,}000 \times [OD_{420} - (1.75 \times OD_{550})/h]$, where OD_{420} is optical density at 420 nm. After autoradiographic exposure of the

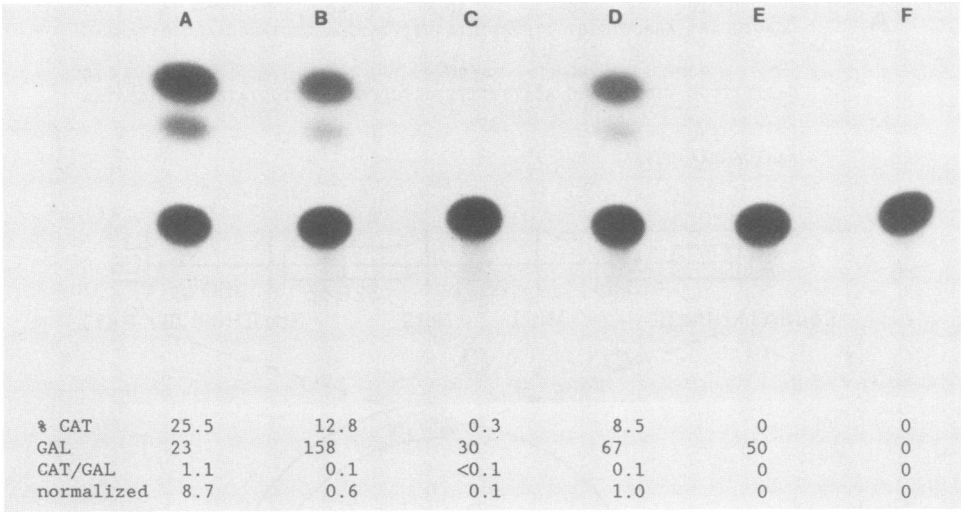

FIG. 2. An autoradiogram illustrating chromatographic separation of the acetylated forms of ^{14}C-chloramphenicol to quantify CAT activities obtained in transfections of NIH 3T3 cells as described in Materials and Methods. Each lane is a result of transfection with the recombinant plasmids pSV2cat (lane A), pTCS-6gABCD (lane B), pTCS-6eABCD (lane C), and pTCS (lane D), no test DNA (lane E), or a mock transfection (lane F). Beneath each lane are the data obtained for CAT activity determined by scintillation counting (% CAT), the GAL activity in units (GAL), the standardized value (CAT/GAL), and the standardized value normalized to the activity obtained with pTCS.

chromatographic separation of the acetylated forms of chloramphenicol, the spots were excised and CAT activity was computed as percent conversion based on liquid scintillation counts. The CAT activity was standardized by dividing by the number of units of GAL to correct for transfection efficiency. The data were normalized by dividing by the standardized value obtained for pTCS, which was included in each series of transfections.

Dot blot hybridizations. RNA was extracted approximately 48 h posttransfection and purified by guanidinium isothiocyanate-cesium chloride density gradient centrifugation as described previously (38). Total RNA (5 μg) was loaded onto nitrocellulose in duplicate and hybridized with either CAT- or GAL-coding sequences recovered from an agarose gel after restriction enzyme digestion and labeled in vitro by nick translation (38).

RESULTS

We are investigating the transcriptional regulatory elements of HPV-6 by conducting a structural and functional analysis of the NCR. Two of the HPV-6 genomes that we have molecularly cloned from benign condylomata acuminata of the genital tract have sequence differences in the NCR (T.-C. Wu and P. Mounts, submitted for publication). However, there is no in vitro system to support replication of HPV, and therefore cloned genomes cannot be assayed for infectivity. These isolates were molecularly cloned from the 8-kilobase viral DNA that was visible by ethidium bromide fluorescence in DNA extracted from benign condylomata from two women, and both of the lesions contained viral capsid antigen. The viral genome was molecularly cloned as a full-length linear molecule and was compared with the viral DNA in the biopsy specimen by restriction enzyme digestions and hybridizations to ensure that no structural rearrangements had occurred during the cloning. The NCR was isolated by digestion of the viral genome with *Sau*3AI and *Pst*I and recovery of the ~1-kilobase fragment from low-melting-temperature agarose. *Hin*dIII linkers were added, and the DNA was cloned into the plasmid vector pKP59. This DNA contained the whole NCR with the 3′ end of the L1 ORF and the 5′ end of the E6 ORF (Fig. 1B). Sequence analysis of this DNA in these two isolates demonstrated that relative to HPV-6e, HPV-6g contained a tandem duplication of a 136-bp sequence between the *Nar*I and *Hpa*II sites (Fig. 1A). The only other difference was an 8-bp sequence between the *Hin*dIII and *Mlu*I sites that included a 3-bp deletion in HPV-6g such that the sequence in HPV-6e was TAGTGTTA and in HPV-6g was ACTGT. We have not detected any changes in these sequences as a result of passage in bacteria; i.e., the tandem duplication is stable, and the 8-bp change in HPV-6g was also found in an isolate in another laboratory (HPV-6vc; 31).

To assay for functional activity, we cloned the NCR into recombinant plasmids containing the bacterial gene *cat* and transfected the plasmids into cells from different species and tissues. An example of the results obtained with transfections of NIH 3T3 cells is shown in Fig. 2. Controls included mock transfections with no DNA (lane F) and transfections with vector DNA as the test DNA (lane E). No CAT activity was obtained for these transfections. Because of variations in transfection efficiencies, it was necessary to standardize the results by including the plasmid pCH110 (17), which contains the bacterial gene for GAL activity. The GAL activity obtained, as described in Materials and Methods, for the transfection shown in lane E was 50 U. CAT activities for transfection with the plasmid pTCS, which contains the SV40 minimum promoter, and *cat* coding sequences in pKP59 were 8.5%, and as expected, the CAT activity for pSV2cat, which was constructed by Gorman et al. (15) and contains the SV40 enhancers and minimum promoter and *cat* gene, was higher, at 25.5% conversion of ^{14}C-chloramphenicol. However, the transfection efficiencies for pTCS and pSV2cat were different, as indicated by the GAL activities of 67 and 23 U, respectively. Therefore, to compare CAT activities for these two constructs it is necessary to standardize the data by dividing the CAT activity by the GAL activity. The data can be normalized to the standardized value for pTCS to facilitate a direct comparison of the

TABLE 1. Enhancer and promoter activities of HPV-6e and HPV-6g NCRs

Transfection recipient cell line	Activity[a] for plasmid:						
	pSV2cat	pTCS-6gABCD	pTCS-6eABCD	pTCS	pTC-6gABCD	pTC-6eABCD	pTC
NIH 3T3	6.7	0.6	0.1	1.0	<0.1	<0.1	0
HOS	2.5	<0.1	<0.1	1.0	<0.1	<0.1	0
HeLa	2.2	3.1	0.1	1.0	0.7	0.2	0
CaSki	29.4	2.3	0.6	1.0	0.6	0.5	0
C-33A	18.7	2.7	0.2	1.0	0.5	0.2	ND

[a] CAT activity standardized to GAL activity and normalized to the activity obtained with pTCS, as described in Materials and Methods. Results are an average of several transfections for each construct. ND, Not done.

activities for different constructs. Thus, the SV40 enhancers increased CAT activity by a factor of 8.7 relative to the SV40 minimum promoter. Results of an assay for CAT activity for the NCRs of HPV-6e (pTCS-6eABCD) and HPV-6g (pTCS-6gABCD) cloned in pTCS to examine enhancer activity are shown in Fig. 2 (lanes C and B, respectively). The CAT assay indicated 12.8% conversion for HPV-6g, and by examination of the autoradiogram, HPV-6g appeared to have about half of the enhancer activity of the SV40 enhancer. However, determining the GAL activity (158 U) revealed a much higher transfection efficiency and illustrated the need to include this assay to monitor transfection efficiency before any conclusions can be reached concerning relative levels of expression. Standardization (0.1) and normalization (0.6) of the data in fact revealed the presence of a silencer activity relative to the standardized value for pTCS (1.0) rather than an enhancer activity. Similarly, the results for HPV-6e in lane C when normalized (0.1) demonstrated a reduction in expression of the *cat* gene when the HPV-6 NCR is cloned 5′ to the SV40 minimum promoter. The averages of normalized results for several transfections of NIH 3T3 cells for pSV2cat, pTCS-6gABCD, pTCS-6eABCD, and pTCS are shown in Table 1 and confirm the silencer activity of the HPV-6 NCR illustrated by the example shown in Fig. 2.

Table 1 summarizes our data on the weak promoter activity that we observed in the NCR of both HPV-6e and HPV-6g. To assay for promoter activity, the NCR was cloned into the polylinker in pTC 5′ to the *cat* gene in pKP59. No CAT activity was seen for pTC (Table 1). The data were normalized to the pTCS value for comparison with the SV40 minimum promoter and are therefore defined as weak relative to the SV40 promoter. Little activity for either pTC-6gABCD or pTC-6eABCD was obtained in NIH 3T3 and HOS cells. However, higher levels were obtained in the cervical carcinoma cell lines. When the normalized CAT activities for pTC-6eABCD and pTC-6gABCD in HeLa, CaSki, and C-33A cells were compared, the activities for pTC-6gABCD were slightly higher, probably as a result of the stronger enhancer activity. Therefore, the values obtained for pTC-6eABCD can be considered to approximate promoter activity more closely. The higher levels of expression for pTC-6eABCD in the cervical carcinoma cell lines suggest that the HPV-6 promoter has some tissue specificity, since these values (e.g., 0.2 in HeLa cells) are comparable to those obtained in constructs with the SV40 promoter in pTCS-6eABCD (0.2) but lower than those obtained in HOS cells, i.e., <0.1 compared with 0.2 for pTCS-6eABCD. However, because of the low level of activity, constructs with the SV40 promoter were used to identify regulatory elements.

To localize the silencer activity within the HPV-6 NCR, 5′ and 3′ deletions were made by restriction enzyme digestion, with sites conveniently spaced (Fig. 1B). The CAT activities obtained for the 3′ deletions of HPV-6e are shown in Fig. 3. Again, CAT activity was standardized and normalized to facilitate comparison with pTCS (lane F) and pSV2cat (lane A). The CAT activity for pTCS-6eABCD, which contains the whole NCR, showed silencer activity with a normalized

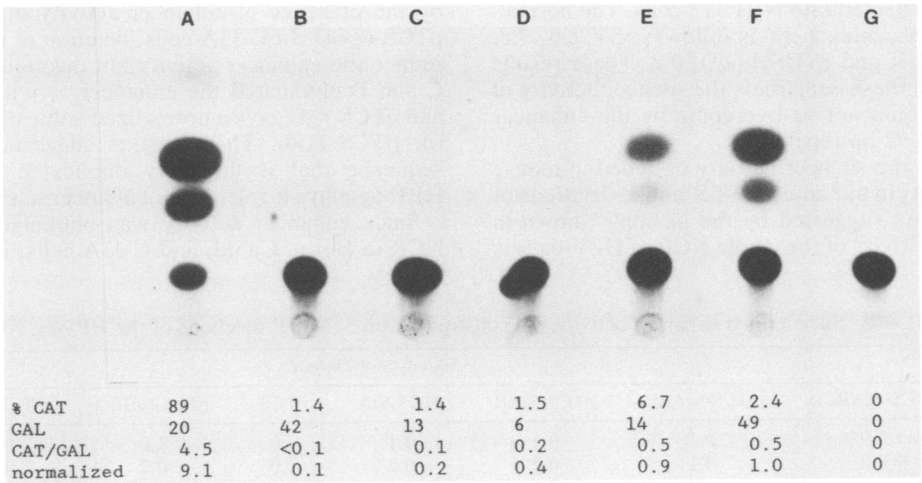

FIG. 3. Analysis of CAT activity in transfections of NIH 3T3 cells with a series of 5′ deletions of the NCR of HPV-6e. Plasmids pSV2cat (lane A), pTCS-6eABCD (lane B), pTCS-6eABC (lane C), pTCS-6eAB (lane D), pTCS-6eA (lane E), and pTCS (lane F) were tested, and a mock transfection (lane G) was done. Shown below each lane are the standardized and normalized CAT activities as described in the legend to Fig. 2.

TABLE 2. Silencer activity in constructs with 5′ and 3′ deletions of the HPV-6e NCR

Transfection recipient cell line	Activity[a] for plasmid:							
	pTCS-6eABCD	pTCS-6eABC	pTCS-6eAB	pTCS-6eA	pTCS	pTCS-6eBCD	pTCS-6eCD	pTCS-6eD
NIH 3T3	0.1	0.2	0.4	1.0	1.0	<0.1	0.1	<0.1
HOS	0.2	0.2	0.3	1.4	1.0	0.1	0.1	0.1
HeLa	0.2	0.1	0.1	0.9	1.0	0.5	0.3	<0.1
CaSki	0.8	0.9	0.7	1.8	1.0	0.6	0.6	0.9

[a] See Table 1, footnote *a*.

value of 0.1 (lane B). The plasmid pTCS-6eABC, which had fragment D deleted between the *Hin*dIII and *Hpa*II sites (Fig. 1B), showed silencer activity with a normalized value of 0.2 (Fig. 3, lane C). Deletion of fragments C and D in pTCS-6eAB retained silencer activity (lane D) with a normalized value of 0.4. However, fragment A alone, i.e., deletion of fragments B, C, and D, showed essentially the same level of activity as pTCS (lane E). These results demonstrated that fragment A of HPV-6e did not contain a silencer element, and in NIH 3T3 cells the effect of fragments B, C, and D on reducing gene expression was additive.

These results were confirmed by the analysis of 3′ deletions of the HPV-6e NCR, and normalized values of several transfections for both 5′ and 3′ deletions are presented in Table 2. Table 2 also presents data demonstrating that the silencer activity in HPV-6e was not cell type restricted. The results of transfections of human fibroblasts (HOS cells) and two human cervical carcinoma cell lines (HeLa and CaSki) are shown. Silencer activity was obtained in constructs containing the whole NCR (pTCS-6eABCD) in all four lines, and fragment A did not contain silencing activity, on the basis of the trend in the data as well as absolute numbers. However, the silencer activity in CaSki cells was not as strong, and in fact fragment A (pTCS-6eA) had some enhancing activity with a normalized value of 1.8.

As an indication of the strength of the silencer activity of HPV-6e, the SV40 enhancer element (nucleotides 37 to 270) was cloned into the 5′ end of the NCRs in pTCS-6eABCD to generate pTCSH-6eABCD and in pTCS-6eD, containing the 260-bp *Hpa*II-*Hin*dIII fragment, to generate pTCSH-6eD, by inserting the *Pvu*II-to-*Nco*I fragment from pSV2cat. These constructs were transfected into NIH 3T3 cells. The normalized values for the plasmids were as follows: pSV2cat, 7.8; pTCSH-6eABCD, 0.3; and pTCSH-6eD, 0.2. These results demonstrated that in these constructs the silencer activity of the HPV-6e NCR could not be overcome by the enhancer activity of the SV40 72-bp repeats.

Table 3 illustrates the silencer activity obtained in mouse and human fibroblasts in our analysis of 5′ and 3′ deletions of the HPV-6g NCR. As suggested by the example shown in Fig. 1, the silencer activity of the whole NCR of HPV-6g was not as strong as that for HPV-6e. The normalized data for all of the transfections with pTCS-6gABCD in NIH 3T3 cells gave a value of 0.9, i.e., essentially the same as for pTCS. However, in contrast to the results obtained with HPV-6e, fragment A of HPV-6g did have very strong silencer activity. In NIH 3T3 cells, the plasmid pTCS-6gA had a normalized value of 0.1 (Table 3), compared with a value of 1.0 for pTCS-6eA (Table 2). Plasmids containing fragments A and B (pTCS-6gAB) as well as C (pTCS-6gABC) also had strong silencer activities with values of normalized CAT activity of 0.1. Plasmids lacking fragment A but containing fragments B, C, and D (pTCS-6gBCD) or fragments C and D (pTCS-6gCD) did not demonstrate silencer activity in NIH 3T3 cells. Since fragment A of HPV-6e did not have silencer activity and the corresponding region in HPV-6g had very strong silencer activity, the 8-bp difference between these two subtypes in fragment A between the *Hin*dIII and *Mlu*I sites appears to be an important sequence for silencer activity.

Although the silencer activity was dominant when the HPV-6g constructs were transfected into fibroblasts, enhancer activity became apparent when cells derived from cervical carcinoma were transfected. The normalized CAT activity for pTCS-6gABCD with the whole NCR was 2.4 in HeLa cells, 3.9 in CaSki cells, and 5.3 in C-33A cells (Table 3). The enhancer was localized by analysis of the series of 5′ and 3′ deletions. In transfections of HeLa and CaSki cells, deletion of fragment D in pTCS-6gABC eliminated the enhancer activity (normalized value, 1.0), and when both fragments C and D were deleted in pTCS-6gAB and pTCS-6gA, the silencer activity became dominant. The location of the enhancer element in fragments C and D was confirmed by the presence of enhancer activity in pTCS-6gBCD and pTCS-6gCD. In C-33A cells, deletion of fragment D did not reduce the enhancer activity but deletion of both fragments C and D eliminated the enhancer activity, and fragment C and pTCS-6gC gave a normalized value of 5.0 relative to that for pTCS (1.0). These results suggested that the 136-bp sequence that is tandemly duplicated in fragment C of HPV-6g plays a role in the enhancer activity.

Since enhancer activity was obtained with the HPV-6g NCR in HeLa, CaSki, and C-33A cells, it was unlikely that

TABLE 3. Silencer and enhancer activities in constructs with 5′ and 3′ deletions of the HPV-6g NCR

Transfection recipient cell line	Activity[a] for plasmid:							
	pTCS-6gABCD	pTCS-6gABC	pTCS-6gAB	pTCS-6gA	pTCS	pTCS-6gBCD	pTCS-6gCD	pTCS-6gD
NIH 3T3	0.9	0.1	0.1	0.1	1.0	1.0	1.0	<0.1
HOS	0.2	0.1	0.2	0.2	1.0	0.2	0.2	<0.1
HeLa	2.4	1.0	0.1	0.1	1.0	1.5	2.4	<0.1
CaSki	3.9	0.8	0.6	0.5	1.0	4.4	2.5	0.3
C-33A	5.3	5.7	0.4	1.4	1.0	1.9	0.9	0.6

[a] See Table 1, footnote *a*.

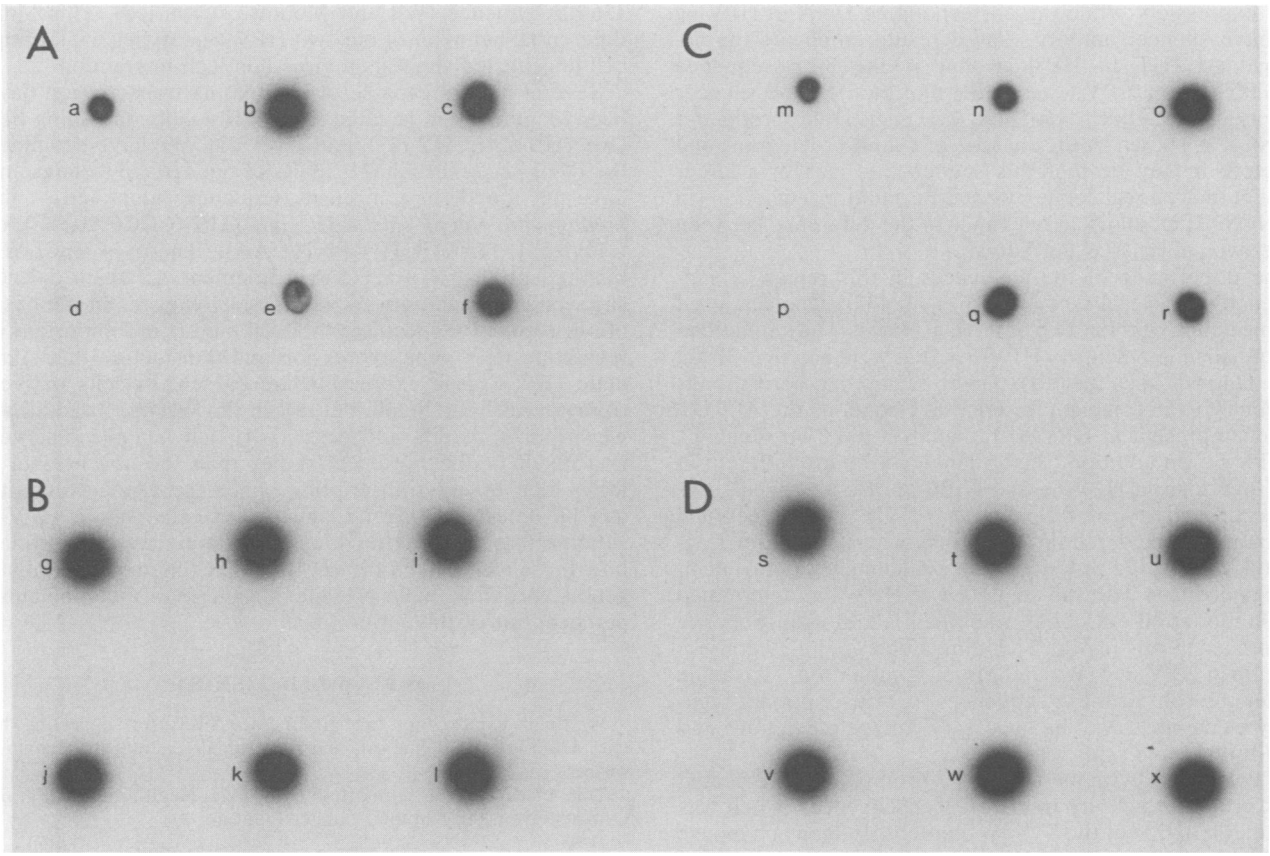

FIG. 4. Dot blot hybridizations of CAT and GAL RNA. Resulting autoradiogram of duplicate dots of RNA from transfected C-33A cells (A and B) and HeLa cells (C and D). The filters in panels A and C were hybridized with a probe specific for CAT sequences, and the filters in panels B and D were hybridized with a probe specific for GAL sequences. The plasmids cotransfected with pCH110 were pTCS (blots a, g, m, and s), pTCS-6gC (blots b and h), pTCS-6gABCD (blots c, i, o, and u), pKP59 (blots d, j, p, and v), pTCS-6gD (blots e and k), pTCS-6gCD (blots f and l), pTCS-6gA (blots n and t), pTCS-6eA (blots q and w), and pTCS-6eABCD (blots r and x).

an HPV gene product was involved in the enhancer activity, because HeLa cells contain HPV-18 DNA (3), CaSki cells contain HPV-16 DNA (39), and C-33A cells have not been demonstrated to contain HPV DNA. To confirm this, transfections were performed with an additional cell line derived from cervical carcinoma, HT-3, which also has not been shown to contain HPV DNA. Enhancer activity was detected for HPV-6g NCR, but no enhancer activity was detected for HPV-6e NCR.

To demonstrate that activity of the enhancer element was relatively independent of position and orientation, the NCR of HPV-6g was placed 3′ to the *cat* gene in two orientations. Relative to that for pTCS (1.0), normalized CAT activity for pTCS-6gABCD was 5.2, and placing the NCR 3′ gave activities of 2.8 and 3.0 in the two orientations.

To determine whether CAT activities were reflecting differences at the transcriptional level, dot blot hybridizations were used to examine RNA levels. Figure 4 shows the analysis of RNA from C-33A and HeLa cells cotransfected with pCH110 and test plasmids as indicated. The uniform intensity of hybridization with the probe specific for GAL (Fig. 4B and D) demonstrated a uniform transfection efficiency. Control transfections with the plasmid vector pKP59 (blots d and p) showed no hybridization as expected. Relative to that of pTCS (blots a and m), the NCR of HPV-6g (blots c and o) showed enhancer activity that was greater than that of HPV-6e (blot r). The enhancer activity was localized to fragment C of HPV-6g (blot b), which was reduced by the presence of sequences in fragment D (blot f). Fragment D (blot e) showed silencer activity relative to pTCS (blot a). Fragment A of HPV-6e (blot q) showed more enhancer activity than did fragment A of HPV-6g (blot n), which contains the 8-bp change.

DISCUSSION

We have detected a transcriptional silencer activity in the NCR of HPV-6. The detection of this activity was facilitated by the ability to correct for the variations in transfection efficiencies with different plasmids in different cell lines. The CAT activity was standardized by including the plasmid pCH110, which was constructed by Hall et al. (17) and contains the gene for GAL, and was used by Herbomel et al. (19) to monitor transfection efficiencies of eucaryotic cells. The demonstration of silencer activity was also facilitated by the differential behavior of two HPV-6 subtypes. We have determined the nucleotide sequence of the NCR for several HPV-6 subtypes and have identified insertions, deletions, and base substitutions (Wu and Mounts, submitted). The functional analysis is reported here for two of these subtypes that differ only in the presence of a tandem copy of a 136-bp sequence and an 8-bp change that includes a 3-bp deletion. Although the whole NCR of both subtypes has silencer activity, the 416-bp fragment A of HPV-6e did not reduce

gene expression, while the corresponding DNA in HPV-6g did have silencer activity. These results implicate the sequence ACTGT in HPV-6g that is the 8-bp sequence TAGTGTTA in HPV-6e as being important in the silencer activity. The silencer element(s) was distributed throughout the NCR as shown by the analysis of 5′ and 3′ deletions, and therefore it may be that this sequence is part of a larger element that we will define by creating additional deletions in the NCR. The biological relevance of the difference between these two subtypes is not known.

The importance of the sequences in this region is supported by the results of Rando et al. (32), who analyzed enhancer activity for two HPV-6 isolates. They identified two enhancer elements in HPV-6vc that were active in HeLa cells. One was localized to a 336-bp region that corresponds to sequences in fragment A. HPV-6vc contains the ACTGT sequence present in HPV-6g rather than the 8-bp sequence of HPV-6e. In addition, the 5′-flanking 4 bp are different in HPV-6vc than in HPV-6e and HPV-6g. We observed some enhancer activity in fragment A of HPV-6e in cervical carcinoma-derived cell lines and enhancer activity for fragment A of HPV-6g in C-33A cells. Alignment of our nucleotide sequences with the sequence of HPV-6vc determined by Rando et al. (31) has identified additional sequence differences in this region, including a deletion of 19 consecutive bp in HPV-6vc (Wu and Mounts, submitted). To define further the role of these sequences as transcriptional regulatory elements, it will be necessary to create deletions and point mutations in this region.

In constructs without the SV40 promoter, we detected weak promoter activity in the HPV-6 sequences which was less than 1/10 that of the SV40 promoter in human and mouse fibroblasts but was about 1/2 that of human cervical carcinoma cell lines. Thierry et al. (37) have reported cell type differences in an HPV-18 promoter which had activity between 1% of that of Rous sarcoma virus promoter in HeLa and monkey COS-1 cells containing SV40 T antigen and 10% of that of the Rous sarcoma virus promoter in human adrenocortical carcinoma cells. To define the location of the promoter, we are mapping the 5′ ends of the RNA and constructing additional plasmids to explore the cell specificity of the promoter.

The 136-bp difference between HPV-6e and HPV-6g was shown to be involved in the strongest enhancer activity that was detected in HPV-6g and only in cell lines derived from cervical carcinoma. Since the only sequence difference in this region between HPV-6g and HPV-6e is the tandem duplication, this sequence must be an important component of the enhancer element. The second enhancer element in HPV-6vc was localized to a region of 427 bp (32) that is contained within fragments B and C of HPV-6g and HPV-6e. HPV-6vc, like HPV-6e, has only one copy of the 136-bp sequence, but there is a deletion of 1 bp adjacent to this sequence in HPV-6vc.

Investigations of the transcriptional regulatory regions of papillomaviruses have demonstrated a transcriptional transactivation by the E2 ORF that interacts with a sequence ACCGN$_4$CGGT that is identified as the E2 binding site (18, 25). Our evidence to date indicates that HPV gene products are not involved in the enhancer activity that we have detected. First of all, the transcriptional activation was detected in four cervical carcinoma cell lines, one of which contains HPV-18, one of which contains HPV-16, and two in which no HPV genome has been identified. Second, there are no differences in the number or location of E2 binding sites in HPV-6e and HPV-6g, and none is located in the 136-bp sequence (Wu and Mounts, submitted). Third, the differential behavior of our two HPV-6 subtypes in all of the cell lines tested suggests a virus-host cell interaction.

Several factors capable of stimulating transcription have been identified and isolated from HeLa cells, including SP1 (10), AP1 (24), AP2 (27), and CTF (21). We have examined the DNA sequences in the NCRs of our HPV-6 isolates and have not found the consensus sequences identified as the binding sites for SP1 (GGGCCGG), AP2 (CCCCAGGC), or CTF/NF-1 (TTGGCT[N$_3$]AGCCAA). There is one AP1 binding site (T[T/G]AGTCA) in fragment A, which did not show enhancer activity for HPV-6g. However, on the basis of our results we speculate that cell factors are important in regulating HPV gene expression and that factors that stimulate HPV-6 gene expression are present in cells derived from cervical carcinoma but not in fibroblasts. In addition, we speculate that the silencer activity that we have observed is a result of the presence, rather than the absence, of a factor that blocks transcription, since the silencer activity can be detected in HeLa and CaSki cells for an HPV-6 subtype that does not have enhancer activity. Determining how these elements in the HPV-6 NCR function to regulate gene expression may provide insight into the molecular pathogenesis of this virus.

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