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Enhancer-promoter Activity of Human Papillomavirus Type 16 Long Control Regions Isolated from Cell Lines SiHa and CaSki and Cervical Cancer Biopsies

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Expression of human papillomavirus 16 (HPV-16) oncogenes is markedly higher in cervical cancer cells than in precancerous cells, and the elevated expression is believed to be required for the malignant phenotypes. We compared cancer cell lines CaSki (with 200 to 400 copies of HPV-16 DNA per cell) and SiHa (with one to two copies of HPV-16 DNA per cell) for the E7 expression in cells and the enhancer-promoter activity of the isolated viral long control region (LCR). Although these parameters per cell were 10-fold higher in CaSki than in SiHa, the levels of the E7 mRNA and protein per HPV DNA copy were 10- to 20-fold higher in SiHa than in CaSki. Characterization of the isolated LCRs showed that, whereas the LCR from CaSki resembled the prototype in structure and activity, the LCR from SiHa, with a deletion of 38 base pairs, enhanced transcription from P97 as assayed by using a plasmid capable of expressing luciferase. The upregulation appeared to be due to removal of one of the silencer YY1-binding sites. Furthermore, we isolated and characterized LCRs from 51 cervical cancer patients' biopsies. Among them, one with a deletion including YY1-binding sites and the other with a substitution in a YY1-motif were found to enhance the transcription. These findings suggest that mutation affecting YY1-motifs in the LCR is one of the mechanisms enhancing the viral oncogene expression in the course of progression of cancer cells.

Key words: HPV-16 — Long control region

The high-risk human papillomaviruses (HPVs), such as HPV-16 and -18, are known to be a major causative agents for cervical cancer and its precursor lesions, cervical intraepithelial neoplasia.[1)] The high-risk HPVs encode two oncoproteins E6 and E7.[1)] E6 associates with p53 and mediates its degradation through a ubiquitin pathway.[2, 3)] E7 binds to pRb and induces release of E2F from the pRb/E2F complex.[4)] Continuous high-level expression of E6 and E7 is required for primary human keratinocytes to be immortalized,[5)] and for cells derived from cervical cancers to grow *in vitro*[6–8)] and to maintain tumorigenicity in nude mice.[9)] Although the transcripts for E6 and E7 are hardly detectable in the basal epithelial layers of low-grade squamous intraepithelial lesions induced by HPV-16, they are abundant in cervical cancer biopsies.[10–12)] It is likely, therefore, that high-level expression of E6 and E7 is required for progression and maintenance of cancer cells.[1)]

Increased expression of E6 and E7 in a cell persistently infected with HPV could be brought about by the presence of more copies of HPV DNA and/or by augmentation of the transcription. In fact, cancer cells which contain multiple copies of HPV DNA per cell have been frequently found. For example, cell line CaSki has been reported to contain 60 to 600 copies of HPV-16 DNA per cell[13, 14)] and the prototype HPV-16 DNA has been cloned from cancer biopsy in which multimeric HPV-16 DNA was present in an episomal state.[15)] On the other hand, augmentation of the transcription is achieved by destruction of the viral *E2* gene that encodes a protein repressive to the transcription of *E6* and *E7* genes and by mutations in the long control region (LCR) that regulates the transcription from promoter P97 for the *E6* and *E7* genes.[16)] The LCR contains multiple binding sites for transcription factors (TFIID, SP-1, AP-1, NF1, Oct-1, TEF-1, 2)[16)] and for silencer YY1 (ten sites, #1 to #10, were identified by gel shift assays)[17)] and the papillomavirus silencing motif (PSM).[18)] Up-regulation of the transcription from the PSM-less LCR by the experimental disruption of one of the five YY1-binding sites (#4, #5, #7, #8, and #9) strongly suggests that transcriptional activity of the LCR is suppressed by YY1.[17)] Among the LCRs isolated from episomal HPV-16 DNA in cervical carcinomas, some of the LCRs with mutations in the YY1-binding sites have shown augmented transcriptional activities.[19, 20)] Further analyses of the LCRs in cervical carcinomas are required to confirm the involvment of naturally occurring mutations within YY1-binding sites in the up-regulation of the transcription from the LCR.

In this study we compared two cervical cancer cell lines, CaSki and SiHa, for HPV-16 expression in the cells

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and for the enhancer-promoter activity of the isolated LCRs, using a luciferase-expressing reporter plasmid that was designed to monitor expression of the *E7* gene. These two cell lines, widely used as cancer cells harboring HPV-16 DNA, have very different viral copy numbers (integrated in cell DNA) per cell. CaSki and SiHa cells have been reported to contain approximately 60–600 and 1–2 copies per cell, respectively.[13, 14] If expression of HPV oncoproteins needs to be enhanced during progression from precancerous states, cells like SiHa may have some mechanism to augment the oncogene expression from a low copy number of viral DNA. In an attempt to clarify a possible enhancing mechanism working in SiHa cells, we characterized isolated LCRs. The LCR from SiHa was found to have a short deletion affecting a YY1-motif and to show enhanced promoter activity for the viral oncogenes. To see whether such changes can be found in LCRs from clinical materials, we isolated and characterized LCRs from HPV-16-positive cancer biopsies from 51 Japanese patients. Two LCRs were found to have mutations in regions containing YY1-binding sites and to show augmented enhancer-promoter activity.

MATERIALS AND METHODS

Real-time PCR HPV-16 DNA and cDNA encoding E7 were quantitated with an ABI-PRISM 7700 sequence detector (PE Applied Biosystems, Foster City, CA). Total DNA and mRNA were isolated from approximately 2×10^6 cells of the human cervical carcinoma cell lines, SiHa and CaSki, and the spontaneously transformed human epithelial cell line HaCaT with QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) and with QuickPrep mRNA Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden), respectively. For HPV-16 DNA quantitation, 50 ng of total DNA was included in 50 μl of PCR mixture containing 25 μl of TaqMan universal PCR master mix (PE Applied Biosystems), oligonucleotides (final concentration in parenthesis): forward primer: 5′-GCGTGCCAAATCCCTGTT-3′ (900 n*M*), reverse primer: 5′-TAAGGCGTTGGCGCATAGT-3′ (900 n*M*), and TaqMan probe: 5′-FAM (6-carboxyfluorescein)-GCACTGCTTGCCAACCATTCCATTGTT-TAMRA (6-carboxytetramethylrhodamine)-3′ (200 n*M*). PCR reaction conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. For mRNA quantitation, 12.5 ng of mRNA was included in 25 μl of One-step RT-PCR mixture containing 1× TaqMan Buffer A, 5.5 m*M* $MgCl_2$, 300 μM dATP, dCTP, dGTP, 600 μM dUTP, 0.25 U/ml MultiScribe reverse transcriptase, 0.4 U/ml RNase inhibitor, 0.025 U/ml AmpliTaq Gold (PE Applied Biosystems) and oligonucleotides (final concentration in parenthesis): forward primer: 5′-TGCGACGTGAGATCATCAAGA-3′ (900 n*M*), reverse primer: 5′-ATATATTCATGCAATGTAGGTGTATCTCC-3′ (900 n*M*), and TaqMan probe 5′-FAM-CACGTAGAGAAACCCAGCTGTAATCATGCA-TAMRA-3′ (225 n*M*). PCR reaction conditions were 48°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Standard curves for HPV-16 DNA and mRNA were generated using plasmid containing HPV-16 DNA and sequentially diluted CaSki mRNA, respectively, and analysis of data was done according to the manufacturer's instructions. Relative levels of HPV-16 DNA and mRNA in CaSki to SiHa were calculated. DNA and mRNA isolated from HaCaT were used for negative controls.

Immunoblotting SiHa, CaSki, and HaCaT were grown in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal calf serum. Cellular proteins were extracted by incubation at 4°C for 20 min with a buffer consisting of 10 m*M* Tris (pH 7.4), 150 m*M* NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF), and 0.005% aprotinin. The samples were electrophoresed on a 15 % SDS-polyacrylamide gel and the resulting protein bands were transferred electrophoretically onto a Hybond-P membrane (Amersham Pharmacia Biotech). The membranes were incubated with anti-HPV-16 E7 mouse monoclonal antibodies, #730[21] (1:1000) in PBST [8.1 m*M* Na_2HPO_4, 1.47 m*M* KH_2PO_4, 2.68 m*M* KCl, 137 m*M* NaCl, and 0.1% Tween 20] containing 5% (wt/vol) skim milk for 1 h at room temperature, washed six times with PBST, incubated with horseradish peroxidase-conjugated anti-mouse IgG in PBST containing 5% skim milk for 1 h at room temperature, and then washed. Protein bands were visualized with the ECL Plus western blotting detection reagent (Amersham Pharmacia Biotech).

Amplification of LCR by PCR and sequence analysis DNA of SiHa and CaSki cells, a human cell line derived from cervical cancer, was extracted from monolayer cell cultures with a QIAamp DNA Blood Mini Kit (QIAGEN GmbH). Tissue DNA was extracted by a standard SDS-proteinase K procedure from specimens diagnosed histologically as cervical carcinoma that had been stored at −80°C at the Department of Obstetrics and Gynecology, Tokyo University Hospital and Department of Gynecology, Saitama Cancer Center. Types of HPV DNA in the samples were examined using the L1-PCR method[22] and DNA samples having HPV-16 were used in this study.

The HPV-16 LCR in the DNA sample was amplified by PCR using primers having the nucleotide sequence from nucleotide (nt) 7215 to 7249 (primer-1: 5′-ATGTGCTTGTAAATATTAAGTTGTATGTGTGTTTG), from nt 301 to 272 (primer-2: 5′-ACATTTATCACATACAGCATATGGATTCCC), from nt 7055 to 7087 of Asian-American variant (primer-3: 5′-GGATTTAAGGCCAAACCAAAATTTACATTAGGA), and from nt 301 to 272 of Asian-American variant (primer-4: 5′-CATTTATCACACACTG-

CATATGGATTCCCA) and the Advantage-HF PCR Kit (CLONTECH Laboratories, Inc., Palo Alto, CA). The LCR regions of European and Asian variants were amplified using primer-1 and primer-2 and those of Asian-American variants were amplified using primer-3 and primer-4. The nucleotide sequence of the DNA fragment amplified by PCR was examined after purification of the PCR products with a QIAquick PCR Purification Kit (QIAGEN GmbH). Sequence analysis was done using at least two independently amplified PCR products for each sample to avoid artifacts. Numbering of HPV-16 nucleotides is according to the revised sequence of HPV-16 (HPV-16R) registered in the HPV Sequence Database of Los Alamos National Laboratory.

Construction of expression plasmids An expression plasmid for monitoring the transcription from P97 in terms of firefly-luciferase activity was constructed by replacement of the *E7* gene with the luciferase gene at the first ATGs of the two genes (Fig. 2A). A 1776-bp *Pst*I-B fragment (nt 7010 to 7906, 1 to 879) of HPV-16 containing LCR, E6 and E7 open reading frame was inserted into the *Pst*I site of modified pUC19 that was constructed by insertion of a *Pst*I-linker between two *Pvu*II sites of pUC19. Firefly-luciferase gene with the SV40 polyA signal sequence was amplified by PCR using synthetic primers (sense: 5′-CGCAGCTGTAATCATGGAAGACGCCAAAAACATAA, antisense: 5′-CGCAGCTGTCGGTCGACGGATCCTTATCG) and pGL3Basic (Promega Corp., Madison, WI) for a template, cleaved with *Pvu*II, and inserted into the *Pvu*II site (nt 554) in the *Pst*I-fragment of HPV-16 to generate pLCR-luc. To measure the transcriptional activity of the LCR with mutations, the DNA fragment between *Eco*RI (nt 7457) and *Eco*T22I (nt 258) of the LCR amplified by PCR using primer-1 and primer-2 was substituted for the corresponding region of pLCR-luc.

The LCR with a short deletion was generated by means of a combination of two PCRs. The 5′-part was amplified using primer-1 and a reverse primer whose nucleotide sequence lacked the portion to be deleted. The 3′-part was amplified using a forward primer that was complimentary to the reverse primer for the PCR to generate the 5′-part and primer-2. The upper-strand of the 5′-part was annealed with the lower-strand of the 3′-part by incubation at 95°C for 3 min, followed by immediate cooling to 4°C, and then incubated at 60°C for 4 min in a PCR-reaction mixture to make the annealed DNA completely double-stranded. The resultant double-stranded DNA was used as the template for the second PCR using primer-1 and primer-2. The product was digested with *Eco*RI and *Eco*T22I, and the resultant fragment was used to construct the expression plasmid for luciferase.

Assay for luciferase activity SiHa and HaCaT cells, which were grown in D-MEM supplemented with 10% fetal calf serum, were used for assay. Four hundred nanograms of pLCR-luc plasmid was cotransfected with 500 pg of control vector containing CMV promoter-driven *Renilla*-luciferase into cells (80% confluency) per one well of a 24-well plate using transfection reagent, Effectene Transfection Reagent (QIAGEN GmbH). Forty-eight hours after transfection, luciferase activity was measured with a PicaGene Dual SeaPansy Luminescence Kit (Toyo Ink Co., Ltd., Tokyo) according to the manufacturer's protocol. Efficiency of transfection was normalized using *Renilla*-luciferase activity. Transcription from P97 controlled by LCR with mutation was presented as relative activity to that of pLCR-luc.

RESULTS

Expression of HPV-16 *E7* gene in CaSki and SiHa cells The HPV-16 genome number per cell was estimated as 200−400 and 1−2 for CaSki and SiHa cells used in this study, respectively. Total DNA per cell was measured by FACS Calibur (Becton Dickinson Immunocytometry Systems Inc., Franklin Lakes, NJ). CaSki and SiHa were found to have 1.76 and 1.51 times, respectively, as much DNA per cell as normal human diploid cells, indicating that they have abnormal karyotypes. The amount of HPV-16 DNA relative to total cell DNA was estimated from reaction curves made by real-time PCR with an ABI-PRISM 7700 (PE Applied Biosystems).[23] The estimated HPV-16 DNA copy numbers for these cancer cells are consistent with previous estimates.[13, 14]

Expression of the HPV-16 *E7* gene in CaSki and SiHa cells was examined by measuring mRNA and protein. The amount of E7 mRNA relative to total cell mRNA was estimated by a similar real-time PCR method to that used for estimation of HPV-16 copy numbers. The steady-state level of E7 mRNA per cell, which is generated by splicing within the *E6* gene, was found to be 10-fold higher in CaSki cells than in SiHa cells. Levels of E7 protein in CaSki and SiHa were compared by immunoblotting using anti-HPV-16 E7 mouse monoclonal antibody #730.[21] The extracts from cells were electrophoresed on a 15% SDS-polyacrylamide gel and transferred to a nylon membrane. E7 was detected as a 20 kDa protein in the lysate from CaSki and SiHa (Fig. 1). Like the steady-state E7 mRNA, the steady-state E7 protein level per cell was 10-fold higher in CaSki than in SiHa.

Despite the higher E7 expression in CaSki cells, the E7 transcription from a single viral genome appears to be more efficient in SiHa than in CaSki. If most of the 200–400 HPV genomes in a CaSki cell,[14] which are tandemly arranged in a head-to-tail fashion, were transcriptionally active, the 1−2 HPV genomes in a SiHa cell should be transcribed nearly 20 times more efficiently than those in a CaSki cell. Since changes in the LCR could affect the efficiency of transcription, the HPV-16 LCRs isolated from

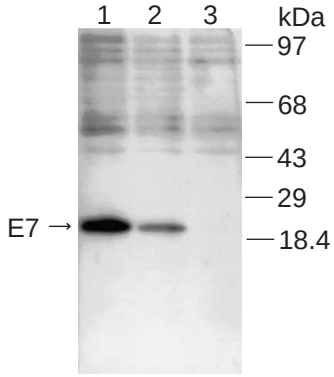


Fig. 1. HPV-16 E7 protein detected by immunoblotting. The extracts from 2×10^6 cells of CaSki (lane 1), SiHa (lane 2), and HaCaT (lane 3) were separated by SDS-polyacrylamide gel electrophoresis and E7 protein was detected using anti-HPV-16 E7 mouse monoclonal antibody.

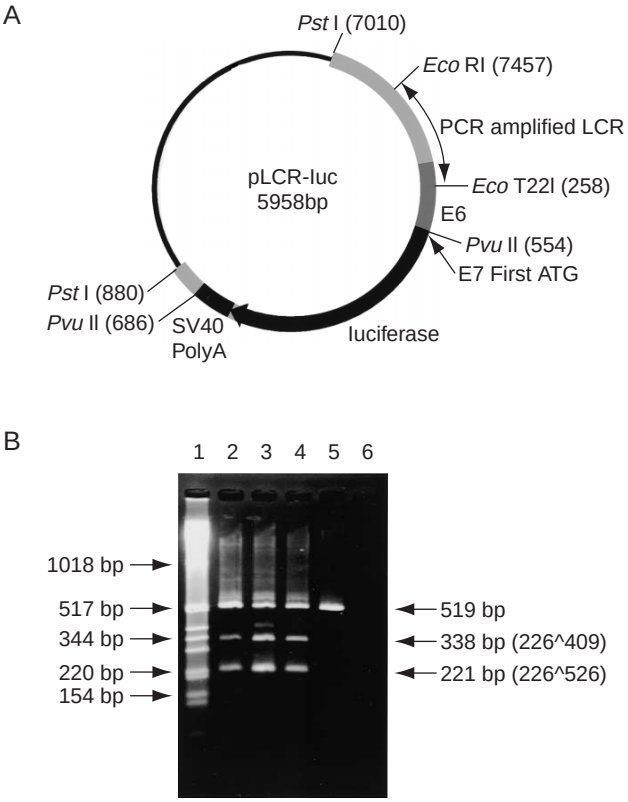


Fig. 2. (A) Structure of the plasmid for LCR-activated luciferase expression. Numbers in parentheses indicate nucleotide number of HPV-16R resistered in the HPV Sequence Database of Los Alamos National Laboratory. (B) cDNAs synthesized by RT-PCR using mRNAs obtained from SiHa cells transfected with the expression plasmids for luciferase. DNA size marker (lane 1); PCR-amplified cDNA generated from mRNA isolated from SiHa cells transfected with pLCR-luc (lane 2), with pSiHa-LCR (lane 3), with pCaSki-luc (lane 4); DNA amplified by PCR using pLCR-luc DNA as the template (lane 5); cDNA generated from mRNA isolated from mock-transfected SiHa cells (lane 6). Splicings were confirmed by nucleotide sequencing.

CaSki and SiHa cells were characterized, and their structure and enhancer-promoter activity were compared as described in the following section.

Transcription activated by LCRs from CaSki and SiHa

The enhancer-promoter activity of cloned HPV-16 LCRs from CaSki and SiHa was assayed by using a luciferase gene as a reporter. To monitor LCR-activated transcription from P97 by measuring luciferase activity, we replaced the *E7* gene in the *Pst*I-B fragment of prototype HPV-16 DNA[24] with luciferase gene at the position of the first ATGs of the two genes to generate pLCR-luc (Fig. 2A). The region of the LCR from *Eco*RI (nt 7457) to *Eco*T22I (nt 258) in pLCR-luc was replaced with the corresponding regions of DNA fragments isolated from SiHa and CaSki cells to produce pSiHa-luc and pCaSki-luc, respectively. SiHa-LCR had a deletion of 38 bp (from nt 7757 to 7794) and CaSki-LCR had a nucleotide substitution of T at nt 12 for C, as described previously[25] (Fig. 3). A at nt 7521 of SiHa and CaSki substituted for G of the prototype DNA, as described.[25] Transcripts produced in SiHa cells transfected with pLCR-luc, pSiHa-luc, or pCaSki-luc were examined by RT-PCR (Fig. 2B) followed by sequence analysis. Two micrograms of each plasmid was transfected into SiHa cells (50% confluency) in a 10-cm dish. Forty-two hours after transfection, mRNA was isolated with a QuickPrep mRNA Purification Kit (Amersham Pharmacia Biotech), transcribed to cDNA using random hexamer, and amplified by PCR using primers having sequences of the E6 region (sense primer) and the luciferase gene (antisense primer). The presence of two species of mRNAs that were generated by alternative splicing in the E6 region (nt 226 to 409 or to 526) and mRNA without splicing was confirmed by nucleotide sequencing. These splicings are consistent with those described for authentic mRNAs transcribed from P97 in cervical cancer cells positive for HPV-16 DNA.[26] Therefore, we concluded that the level of LCR-activated *E7* gene-expression from P97 could be monitored in terms of luciferase activity.

Transcription activated by SiHa-LCR and CaSki-LCR was measured and compared with that by the prototype LCR using SiHa and HaCaT cells. Luciferase activities of the cells transfected with pSiHa-luc and pCaSki-luc are presented as values relative to those of the cells transfected with pLCR-luc in Table I. Luciferase activity under the control of SiHa-LCR was higher by 4-fold than that under prototype LCR or CaSki-LCR in both cell lines used

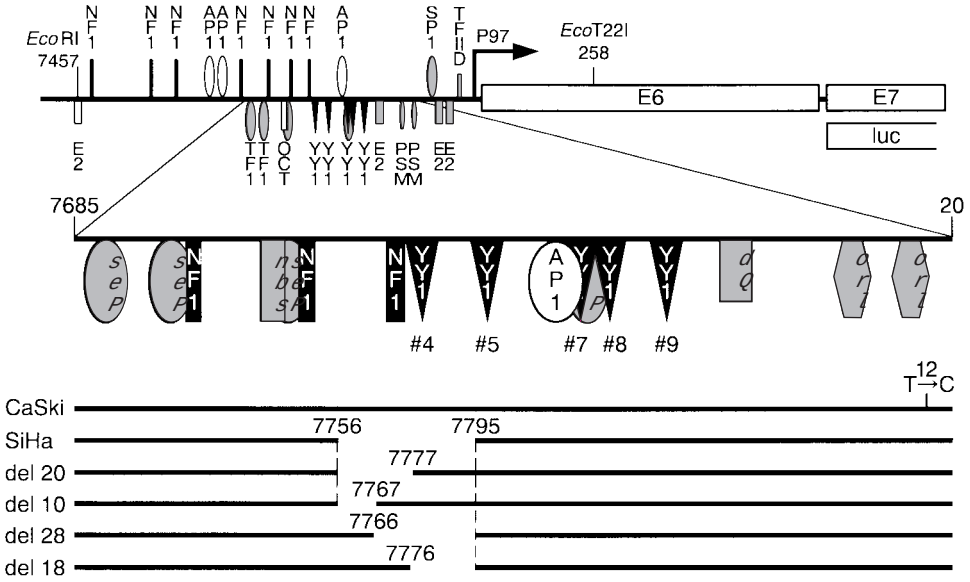


Fig. 3. Schematic representation of the HPV-16 LCR upstream of the promoter P97 and ORFs E6 and E7. The LCR spans from nucleotide 7154 to 7096, 1 to 103 in a total of 7906 base pairs of cellular DNA genome. The upper part shows binding sites for TEF-1, NF-1, Oct-1, YY1, AP-1, TFIID, and viral E2 protein and papillomavirus silencing motif (PSM). *E7* gene was replaced with luciferase gene to monitor transcription from P97. The lower part illustrates the positions of the deletions and point mutations (sense strand for the downstream gene) in the LCRs isolated from CaSki and SiHa cells, and in the experimentally generated LCRs. Numbering of the nucleotides follows that of HPV-16R (the HPV Sequence Database of Los Alamos National Laboratory).

Table I. Luciferase Activity under the Control of HPV-16 LCR

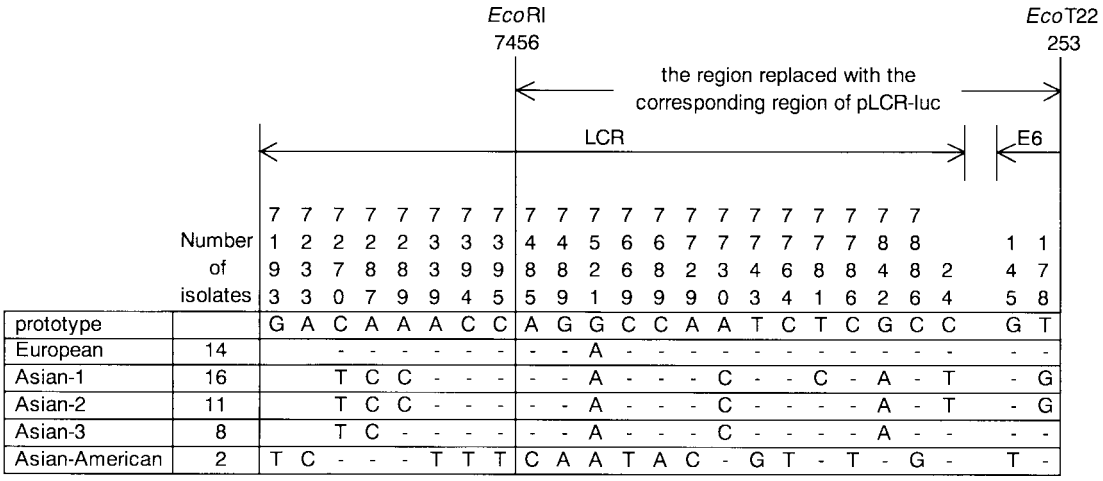
Plasmid	Luciferase activity[a)]	
	SiHa	HaCaT
pLCR-luc	1.0±0.4	1.0±0.5
pCaSki-luc	1.5±0.2	0.8±0.8
pSiHa-luc	4.6±0.8	3.6±0.5
pdel-20-luc	0.7±0.2	ND
pdel-10-luc	1.4±0.2	ND
pdel-28-luc	2.5±0.5	ND
pdel-18-luc	2.9±0.3	ND

a) Luciferase activities relative to that of the prototype are shown.

for the assay. Similar data were obtained from CaSki cells transfected with the plasmids (data not shown because of the low transfection efficiency with CaSki cells). SiHa, which showed the highest transfection efficiency among the cell lines tested, was used in the following experiments. The data indicate that the deletion of 38 bp in the SiHa-LCR augmented the transcription. Substitutions of T at nt 12 for C (CaSki) and A at nt 7521 for G (prototype) did not significantly affect the transcription.

In an attempt to identify the region responsible for the enhanced transcription under the SiHa-LCR, luciferase activities under LCRs having deletions shorter than 38 bp were measured (Fig. 3 and Table I). LCRs with deletions from nt 7757 to 7776 (del-20), from 7757 to 7766 (del-10), from 7767 to 7794 (del-28), and from 7777 to 7794 (del-18) were constructed and introduced into the expression plasmid for luciferase. Although luciferase activities under del-20-LCR and del-10-LCR were decreased to a comparable level to that under prototype LCR, luciferase activities under del-18-LCR and del-28-LCR were significantly higher. The results indicate that the transcription under LCR having deletion of YY1-binding sites #4 and part of #5 is up-regulated. The deletion of 38 bp is more effective to augment the transcription than the deletion of the 18 bp, suggesting that deletion in LCR causes complex enhancing effects on the enhancer-promoter activity of LCR.

Characterization of the LCRs has shown that the SiHa-LCR, containing the deletion described above, is a more efficient enhancer-promoter for E7 expression from P97 than CaSki or the prototype LCR. This probably accounts, at least partly, for the elevated E7 transcription per HPV-16 genome copy in SiHa cells. If that is the case, changes like that in SiHa LCR may be related to carcinogenesis by HPV and may be found in clinical samples from cervical cancer. HPV-16 LCRs were isolated from cancer biopsies and characterized as shown in the subsequent section.



	Number of isolates	7 1 9 3	7 2 3 3	7 2 7 0	7 2 8 7	7 3 8 9	7 3 9 9	7 3 4 4	7 3 4 5	7 4 8 8	7 4 5 9	7 5 6 1	7 6 8 9	7 6 2 9	7 7 3 9	7 7 4 0	7 7 6 3	7 7 8 4	7 8 4 1	7 8 8 6	7 8 2 4	1 4 5	1 4 7 8
prototype		G	A	C	A	A	A	C	C	A	G	G	C	C	A	A	T	C	T	C	G	C	C	G	T
European	14			-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-
Asian-1	16			T	C	C	-	-	-	-	-	A	-	-	-	C	-	-	C	-	A	-	T	-	G
Asian-2	11			T	C	C	-	-	-	-	-	A	-	-	-	C	-	-	-	-	A	-	T	-	G
Asian-3	8			T	C	-	-	-	-	-	-	A	-	-	-	C	-	-	-	-	A	-	-	-	-
Asian-American	2	T	C	-	-	-	T	T	T	C	A	A	T	A	C	-	G	T	-	T	-	G	-	T	-

Fig. 4. Nucleotide sequences of representative HPV-16 variants. Nucleotide positions at which variations were observed are written vertically across the top. Numbering of the nucleotides follows that of HPV-16R (the HPV Sequence Database of Los Alamos National Laboratory).

LCRs derived from cervical cancer biopsies The LCR regions of HPV-16 DNAs in cancer biopsies from 51 Japanese patients with cervical cancer were amplified by PCR and their nucleotide sequences were analyzed. The nucleotides that differed from those of prototype HPV-16[24] are shown in Fig. 4. According to the classifications described by Yamada *et al.*,[27] the HPV-16 DNAs isolated from Japanese patients consisted of European (14 cases, 27.5%), Asian (35 cases, 68.6%) and Asian-American (2 cases, 3.9%) variants (Fig. 4). It should be noted that the prevalence of Asian variants in Japanese patients was much higher than that in Southeast Asian countries (Indonesia, Philippines, and Thailand) (25.7%).[27] The Asian branch was found to be divided into 3 subgroups, based on the nucleotides at 7289, 7781, and 24; subgroup-1 (representative SA85, C at 7289, C at 7781, and T at 24), subgroup-2 (SA28, C, T, and T, respectively), and subgroup-3 (SA15, A, T, and C).

Mutations found in the samples in this study and the three variants described previously [19, 20] are shown in Fig. 5. Nucleotide substitutions found in SA108 and #3342 are within and adjacent to YY1-binding site #5, respectively, and those in #3341 and #1411 are within YY1-binding site #8. LCR from patient #216 has a deletion from nt 7790 to 7856 and LCR from #2166 has a deletion from nt 7791 to 7837 and a nucleotide substitution of G for A at nt 7838. Since LCR DNA amplified by PCR from samples #216 or #2166 migrated and formed a single band in agarose gel-electrophoresis (data not presented), these LCRs having a deletion were likely to be major species. LCRs with deletions similar to that of SiHa-LCR were not found.

Transcription activated by the selected LCRs from cancer biopsies was examined by using expression plasmids for luciferase constructed similarly to pSiHa-luc using pLCR-luc as a backbone (Table II and Fig. 5). Luciferase activities under the control of LCRs of Asian-American variants (#2723) and Asian variants (#SA85, #SA28, and #SA15) were higher by approximately 2-fold than those of prototype LCR and SA71-LCR (European variant), being consistent with the data in a recent report.[28]

Among the LCRs with nucleotide substitutions, SA108-LCR (Asian variant subgroup-1) showed significantly higher luciferase activity than did SA85-LCR (standard type of the subgroup-1). The nucleotide substitution, C for G at nt 7799, in the LCR of SA108 is presumably responsible for the enhanced transcription. This is consistent with a previous report that transcription under the LCR with a substitution of A at nt 7799 for G (referred to as LCR390)[19] was enhanced. The #3342-LCR (Asian variant subgroup-2) which had nucleotide substitution outside of the YY1-motif #5 showed slightly higher luciferase activity than did SA28-LCR (standard type of the subgroup-2).

In the two LCRs having a deletion, #2166-LCR (Asian variant subgroup-2) induced 2-fold higher luciferase activity than SA28-LCR, and #216-LCR (European variant) did not induce significantly higher luciferase activity than SA71-LCR (standard type of European variant). Since #2166-LCR contains the YY1-motif #9 that was removed in the #216-LCR, the data suggest that high-level transcription under #2166-LCR was not ascribable simply to removal of the YY1-binding sites.

Although the transcription under the control of #216-LCR was not augmented, the previously described LCR having the deletion from nt 7792 to 7856 (designated as 1326B by Dong *et al.*, 1994),[19] which is very similar to the deletion in #216-LCR (from nt 7790 to 7856), has

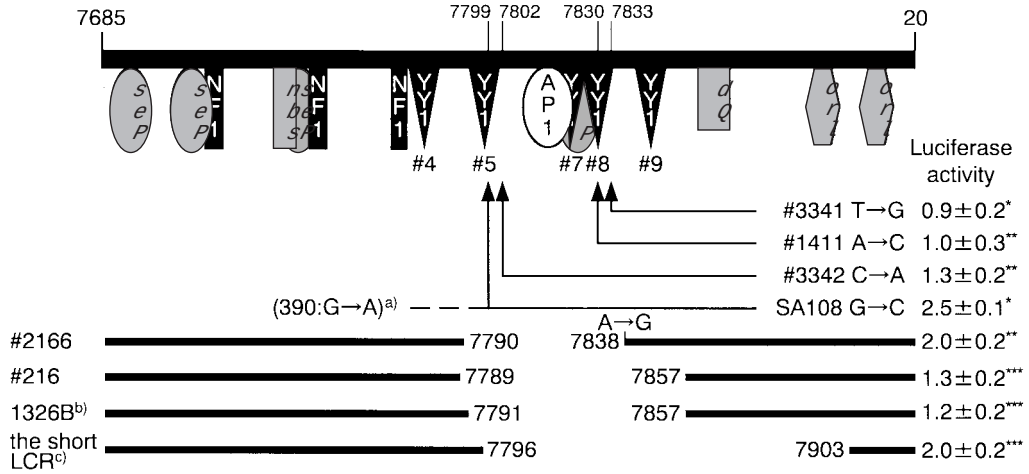


Fig. 5. Schematic representation of the HPV-16 LCR isolated from cancer biopsies. Binding sites for TEF-1, NF-1, Oct-1, YY1, AP-1, and viral E2 protein in the region from nucleotide 7685 to 7906, 1 to 20 are shown together with PSM. For comparison, two samples (390[a] and 1326B[b]) described by Dong *et al.*[19] and one (the short LCR[c]) described by May *et al.*[20] are included. Numbering of the nucleotides follows that of HPV-16R (the HPV Sequence Database of Los Alamos National Laboratory). Luciferase activities relative to pSA85-luc (standard type of Asian-1 variant) (*), to pSA28-luc (Asian-2) (**), and to the prototype (European) (***) are shown.

Table II. Luciferase Activity under the Control of HPV-16 LCRs Isolated from Cancer Biopsies

Plasmid	Subtype	Luciferase activity[a)]
pLCR-luc	European	1.0±0.1
pSA71-luc	European	1.3±0.2
pSA85-luc	Asian-1	2.4±0.3
pSA28-luc	Asian-2	2.3±0.2
pSA15-luc	Asian-3	1.9±0.4
p#2723-luc	Asian-American	2.5±0.2

a) Luciferase activities relative to that of the prototype are shown.

been reported to enhance transcription from P97. To measure the effects of previously reported deletions on the function of the LCR using the construct in this study, two expression plasmids having LCRs with the same deletions as those of 1326B and with the short LCR (deletion from nt 7797 to 7902) that has also been reported to enhance transcription[20] were constructed by the use of the same backbone as in pLCR-luc in this study. Luciferase activity under the 1326B-LCR (European variant) was comparable to that under #216-LCR and not higher than that under SA71-LCR (Fig. 5) in SiHa, CaSki, HaCaT, or HeLa cells. Luciferase activity under the short-LCR (European variant) was higher by 2-fold than that under SA71-LCR (Fig. 5) in the cells used for the assay. In the previous studies, the plasmids used for the assay of transcription were constructed by ligation of a DNA fragment containing HPV-16 LCR (from nt 7010 to 7096, 1 to 123) with the luciferase gene through a *Bam*HI linker[19, 20] and, consequently, expression of luciferase fused with the N-terminal 9 amino acids derived from *E6* gene was analyzed. Disagreement of the result for 1326B-LCR in the previous study with that in this study may be ascribable, at least partly, to the difference in the structure of the reporter plasmids.

DISCUSSION

In this study we have found that SiHa cells, containing 1–2 copies of HPV-16 DNA per cell, express more viral oncoprotein E7 per HPV-16 genome harbored in the cell than CaSki cells, containing 200–400 tandemly repeated HPV-16 DNA per cell, and that the cloned LCR from SiHa can serve as a more efficient promoter-enhancer for E7 expression than the LCR from CaSki or the prototype. The increased LCR activity of SiHa appears to be ascribable to a 38-bp deletion including silencer YY1-motifs #4 and #5, two of the five such motifs (#4, #5, #7, #8, and #9) shown to bind YY1 by a gel-shift assay.[17]

If the high-level expression of *E6* and *E7* genes is required for progression and maintenance of cancer cells,[1] the changes that occurred in SiHa-LCR would be one of the possible mechanisms involved in the progression of precancerous to cancer cells, especially in cells harboring a few viral DNA copies per cells. The effects of a strong promoter for expression of *E7* genes of HPV-16, -18, and -33 on transforming functions for rat 3Y1 cells have been

examined.[29] Use of the strong SRα promoter in place of the SV40 promoter markedly enhanced expression of the *E7* gene placed under its control and increased the efficiency of focus formation of transformed cells. Thus, it is conceivable that the increased viral oncoprotein expression resulting from the mutated SiHa-LCR may contribute to the malignantly transformed phenotype of the cells.

To search for the changes like that of SiHa HPV-16 LCR in clinical materials, we isolated and characterized LCRs from cancer biopsies. Among the LCRs from 51 patients with cervical cancer, two LCRs, one with a nucleotide substitution of G at nt 7799 for C within YY1-motif #5 (SA108) and the other with a deletion of YY1-binding sites #5, #7, and #8 (#2166), were found to induce significantly higher luciferase activities (Fig. 5). Like the one in the SiHa-LCR, the mutations in these two LCRs involves some of the motifs for silencer YY1. In agreement with previous studies,[19, 20] our results show that, despite the low prevalence, mutations in LCR capable of augmenting the enhancer-promoter activity indeed occur in the course of cancer progression and that the genetic changes of cells may be related to the silencer.

Among naturally occurring nucleotide substitutions in the LCR, so far only the replacement of G at nt 7799 with A (390 in the study by Dong *et al.*[19]) or C (SA108 in this study) (Fig. 5) clearly enhanced the transcription from the P97. The G at nt 7799 is within the YY1-binding motif #5, suggesting that binding of YY1 to motif #5 is important for the suppression of transcription under the authentic LCR. However, not all the changes in YY1-motifs affect the LCR function. Substitutions within YY1-binding motif #8 (#3341- and #1411-LCRs in this study) and #9 (432 in the study by Dong *et al.*[19]) did not affect the activity of the LCR. YY1-motifs #8 and #9 may not play a role in suppressing the transcription by binding with YY1.

Deletions in the LCR have a complex effect on the activity of the LCR. The enhancement of the transcription under the SiHa-LCR may be mainly ascribed to removal of YY1-binding sites #4 and #5 (Fig. 3 and Table I). However, the deletion of the 10-bp region (nt 7757 to 7766, the 5′-region of the SiHa-deletion, upstream region of the YY1-binding motifs #4 and #5), which by itself had no enhancing effect on the transcription, was important for the maximum enhancer-promoter activity of the SiHa-LCR. Disturbance of the optimum distance between *cis*-elements in the LCR may affect the activity of the LCR. Despite the similarity of deletion between #2166 and #216 (Fig. 5), #216-LCR did not enhance the transcription much (Fig. 5). The reason why the deletion including YY1-binding motifs #5, #7, #8, and #9 in #216 did not show any marked effect remains to be investigated.

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