

# Common fragile sites are preferential targets for HPV16 integrations in cervical tumors

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The development of cervical cancer is highly associated with human papillomavirus (HPV) infection. HPV integration into the genome of infected cervical cells is temporally associated with the acquisition of the malignant phenotype. A relationship between the sites of HPV integration in cervical cancer and the position of the common fragile sites (CFSs) has been observed at both the cytogenetic and molecular levels. To further explore this relationship at the molecular level, we used RS-PCR to rapidly isolate cellular sequences flanking the sites of HPV16 integration in 26 primary cervical tumors. Human bacterial artificial chromosome clones were isolated based on these flanking sequences and used as probes for fluorescence *in situ* hybridization on aphidicolin-stimulated metaphases. Our data demonstrate that 11/23 HPV16 integrations in cervical tumors occurred within CFSs ( $P < 0.001$ ). In addition, we show that deletions and complex rearrangements frequently occur in the cellular sequences targeted by the integrations and that integrations cluster in FRA13C (13q22), FRA3B (3p14.2), and FRA17B (17q23). Finally, our data suggest that cellular genes, such as *Notch 1*, are disrupted by the HPV16 integrations, which may contribute to the malignant phenotype.

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## Introduction

Fragile sites are specific chromosomal loci that non-randomly exhibit gaps or breaks in response to specific culture conditions or exposure to certain chemical agents. Fragile sites are divided into two classes. Rare fragile sites (RFSs) occur in less than 5% of the population and are associated with expanded CCG repeats or expanded AT-rich minisatellite repeats (Richards, 2001). Common fragile sites (CFSs) are

thought to be present in all individuals and the majority of them are induced in response to treatment with the DNA polymerase inhibitor, aphidicolin. The 87 CFSs are distributed throughout the genome. To date, six aphidicolin-sensitive CFSs have been cloned and characterized: FRA3B, FRA7G, FRA7H, FRA16D, FRAXB, and FRA6F (Wilke *et al.*, 1996a; Huang *et al.*, 1998; Mishmar *et al.*, 1998; Krummel *et al.*, 2000; Mangelsdorf *et al.*, 2000; Paige *et al.*, 2000; Arlt *et al.*, 2002; Morelli *et al.*, 2002). The mechanistic reason for the instability of these sites is still unclear, but it is likely different from that of the RFSs since no unstable repeat sequences have been shown to be associated with CFSs. Cytogenetic techniques have demonstrated that CFSs are very large regions (~150 kb to ~1 Mb) throughout which breakage and instability can occur (Richards, 2001). CFSs are highly unstable and recombinogenic regions of the genome. They are preferential sites of sister chromatid exchange, translocations, deletions, intrachromosomal gene amplification, and integration of plasmid DNA and tumor-associated viruses (reviewed in Smith *et al.*, 1998). For these reasons, CFSs, and the genes contained within them, have been proposed to play a mechanistic role in the initiation or progression of human tumors (Yunis and Soreng, 1984) (Smith *et al.*, 1998; Richards, 2001).

Infection with human papillomavirus (HPV) is the major risk factor for developing cervical cancer and essentially all cervical tumors contain DNA sequences from this virus (Walboomers *et al.*, 1999). Approximately 100 different HPV types have been identified. They are typically classified as ‘low’ risk, those that induce mostly benign lesions such as warts and papillomas, or ‘high’ risk, those that are associated with invasive carcinomas. HPV16 and HPV18 are the prototypical high-risk HPV types and are found in the majority of cervical tumors. These high-risk HPVs possess *in vitro* immortalization capabilities (Pecoraro *et al.*, 1989) and can transform established cell lines or primary cells in conjunction with other oncogenes (Crook *et al.*, 1988).

The oncogenic capabilities of high-risk HPVs are mainly due to the expression of the viral *E6* and *E7* genes that abrogate the functions of p53 and pRB, respectively (Dyson *et al.*, 1989; Werness *et al.*, 1990). However, several lines of evidence indicate that

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high-risk HPV infection alone is insufficient to cause cancer. Although high-risk HPVs can efficiently immortalize primary cells *in vitro*, transformation does not occur without the contribution of other cellular changes. Additionally, only a small percent of women infected with high-risk HPVs develop cancer and cancer develops only after long periods of infection. It is likely that other mutations are necessary to achieve a malignant phenotype and that the genomic instability that occurs via the loss of functional p53 and pRB may drive the selection of these additional changes that are necessary for the progression of the tumor.

Premalignant cervical lesions typically harbor HPV in its episomal form. However, the majority of cervical carcinomas contain HPV that has integrated into a single locus within the host cell genome (Cullen *et al.*, 1991). Thus, HPV integration is temporally associated with the acquisition of the malignant phenotype. During the process of integration, deletions and/or rearrangements typically occur within the HPV genome that selectively retain expression of the *E6* and *E7* genes, underscoring the importance of the continued expression of these genes in the maintenance of the malignant phenotype. This is consistent with the observation that cells with integrated HPV have a selective growth advantage and that E2 damage is associated with poor prognosis and shortened disease-free survival (Jeon *et al.*, 1995; Vernon *et al.*, 1997; Kalantari *et al.*, 1998).

It has been proposed that CFSs are preferential targets for HPV integration in cervical tumors. A correlation between the cytogenetic locations of CFSs and HPV integration has been suggested (Cannizzaro *et al.*, 1988; Popescu and DiPaolo, 1989; Popescu *et al.*, 1990). Molecular evidence of this relation was first observed when the sequence of the FRA3B region at 3p14.2 and cellular sequences flanking an HPV16 integration in a cervical tumor were shown to be identical (Wilke *et al.*, 1996). Additionally, we previously demonstrated that two of the three HPV16 integrations cloned from cervical tumors occurred within CFSs (Thorland *et al.*, 2000). Herein, we demonstrate that HPV16 integration preferentially occurs within CFSs. We also examine the structural rearrangements of genomic DNA that occur upon integration, clusters of HPV16 integration at specific cytogenetic locations, and the integration-related disruption of cellular genes that are known to be involved in tumorigenesis.

## Materials and methods

### Samples

HPV 16-positive cervical tumor specimens were obtained from 26 patients. DNA extraction and HPV typing were performed as previously described (Gostout *et al.*, 1998). All tumors were Grade 2 – Grade 4 carcinomas. 20 tumors were squamous cell carcinomas, three tumors were adenocarcinomas, two tumors were adenosquamous carcinomas, and one tumor had unknown histology. In total 19/26 tumors were Stage 1, so limited amounts of tumor material were available for analysis.

### PCR conditions

HPV16-specific primer sequences and the use of RS-PCR to clone cellular sequences flanking the sites of HPV integration were previously published (Thorland *et al.*, 2000). RS-PCR utilizes primers that recognize a given restriction enzyme recognition site, anneal to that site, and promote DNA synthesis in a PCR. Three restriction site oligos (RSOs) were utilized that were specific for the restriction enzymes *Bam*HI, *Eco*RI, and *Xba*I. A volume of 2 pmol of each specific primer and 20 pmol of the RSO primer were combined with 50 ng of cervical tumor DNA, Expand High Fidelity 10 × PCR buffer containing 15 mM MgCl<sub>2</sub>, 200 μM of each dNTP, and 0.7 U of Expand High Fidelity polymerase (Roche, Indianapolis, IN, USA) in a 20 μl reaction. Cycling conditions were as follows: 94°C for 3 min followed by 10 cycles of 94°C for 30 s, 45°C for 30 s, and 68°C for 3 min followed by 25 cycles of the same conditions, but adding 20 s each cycle to the extension time, followed by 10 min at 72°C. An amount of 1 μl of the product from the first round of PCR was used as template for the nested PCR. Nested RS-PCR conditions were identical to the first round except that a 55°C annealing temperature was used. Conditions for all other PCR were performed with 50 ng of template, 10 pmol of each primer, 10 × REDTaq PCR buffer containing 15 mM MgCl<sub>2</sub>, 200 μM dNTPs, and 1.25 U of REDTaq polymerase (Sigma, St Louis, MO, USA) in a 25 μl reaction. The cycling conditions were as follows: 30 s at 94°C, 30 s at 55°C, 30 s at 72°C for 30 cycles, followed by a 10 min extension at 72°C.

### Sequencing

An amount of 100 ng of PCR products were treated with 1 U of Exonuclease I (USB, Cleveland, OH, USA) for 15 min at 37°C followed by 15 min at 80°C. Treatment with 1 U of shrimp alkaline phosphatase (Roche, Indianapolis, IN, USA) was performed under identical conditions. The template was combined with 3.2 pmol of the appropriate sequencing primer. Sequencing was performed by the Mayo Clinic Molecular Core Facility.

### Computer analysis

BLASTN searches with the cellular sequences flanking the sites of HPV16 integration and contig analysis used to determine the sizes of deletions and complex rearrangements were performed on the National Center for Biotechnology Information web site (<http://www.ncbi.nlm.nih.gov/index.html>). The chromosomal locations of BAC clones and the identification of genes interrupted by the HPV16 integrations based on the 'Known Genes' or 'Spliced ESTs' tracks from the April 2001 freeze were obtained from the UCSC genome browser (<http://genome.ucsc.edu/>).

### BAC and radiation hybrid screening

All sequences flanking the site of HPV16 integration, except the flanking sequence from CC91, were homologous to BAC clones that were sequenced as part of the Human Genome Project. Computer searches using the 3' flanking sequence from tumor CC91 revealed that this sequence was derived from a portion of the human genome that had not yet been sequenced. To determine the chromosomal location and obtain a BAC clone for FISH analysis for CC91, primers were designed to the genomic sequence flanking the site of HPV16 integration. These primers were used to screen the Stanford G3 Radiation Hybrid Mapping Panels (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. The

chromosomal location based on linked markers and LOD scores was obtained from the Stanford University web site. These primers were also used to screen the Down-To-The-Well human BAC DNA pools (Incyte Genomics, Palo Alto, CA, USA) to obtain a BAC clone that represented the sequence flanking the HPV16 integration in this tumor.

#### *BAC isolation*

BAC clones that represented sequences flanking the sites of HPV16 integration were obtained (Invitrogen, Carlsbad, CA, USA). The BAC DNA isolation procedure was adapted from the Maxi-prep protocol (Qiagen, Valencia, CA, USA). Precise protocol available upon request. Primers designed within the sequences flanking the sites of HPV16 integration were used to check the isolated BAC DNA for the correct insert sequence by PCR. Of the isolated BAC DNAs, 20 contained the expected insert (data not shown). However, three of the clones (520F22, 81E19, and 964E11 – see Figure 2) did not contain the appropriate insert and were unusable for further study.

#### *FISH*

PHA-stimulated peripheral blood lymphocytes were cultured for 72 h, arrested with colcemid, and fixed according to standard procedures (Verma and Babu, 1989). Aphidicolin (0.4  $\mu$ M) (Sigma, St Louis, MO, USA) was added to the culture for the final 24 h to induce CFS expression. In some cases, 2.5 mM caffeine was added for the final 6 h to enhance CFS breakage (Glover *et al.*, 1986). BAC DNA (1  $\mu$ g) was labeled with biotin-16-dUTP by nick translation as recommended by the manufacturer (Roche, Indianapolis, IN, USA). Labeled BAC probes were hybridized to the metaphase spreads using standard procedures. Probe detection and amplification followed the manufacturer's protocols (Ventana Medical Systems, Tucson, AZ, USA). Hybridization signals were detected with a Zeiss Axioplan microscope and images were captured using PowerGene MacProbe software (Applied Imaging, Santa Clara, CA, USA).

Individual BAC clones are generally smaller than the regions of breakage within CFSs. Thus, it is necessary to look at the distribution of hybridization signals (proximal to, distal to, or crossing the site of aphidicolin-induced breakage). BACs were determined to be within a CFS if hybridization signals were observed both proximal and distal to the site of breakage in different metaphases. At least 20 metaphases with breakage at each CFS were examined.

#### *Statistical methods*

A Z-test was used to assess the statistical significance of the relation between CFSs and HPV16 integration. The following assumptions were made. The average size of a CFS is 1 Mb and the human genome is 3000 Mb in size.

## **Results**

### *Detection and chromosomal location of integrated HPV16 by RS-PCR*

RS-PCR is a PCR-based technique for amplification of unknown nucleotide sequences adjacent to known nucleotide sequences (Sarkar *et al.*, 1993). We have previously demonstrated that this technique can efficiently amplify the junctions between HPV16 and

flanking cellular sequences at viral integration sites in cervical tumors (Thorland *et al.*, 2000). RS-PCR was used to screen 26 HPV16-positive cervical tumors for viral-cellular junctions. A total of 33 viral-cellular junction sequences were detected from 17 tumors.

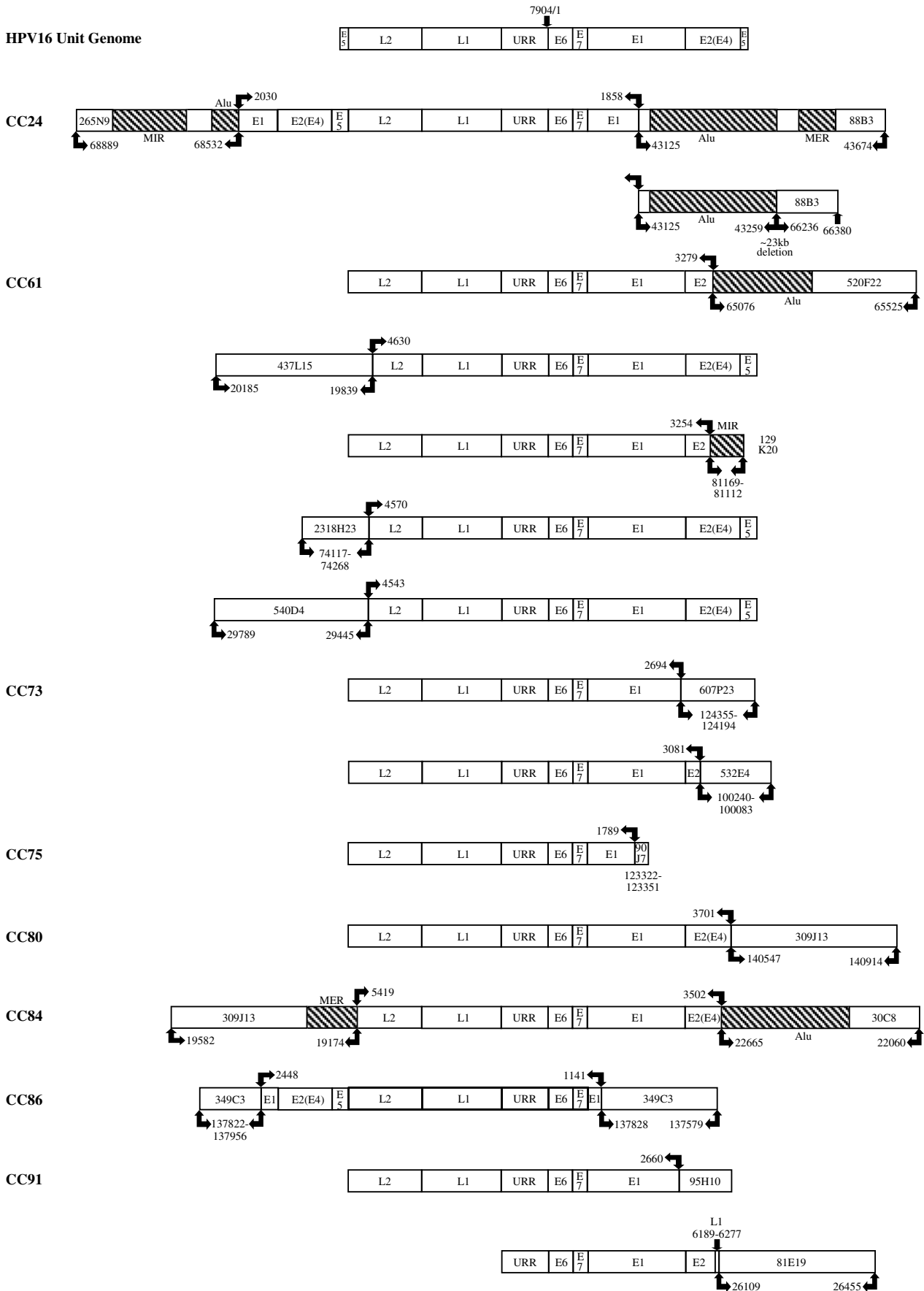
Chromosomal locations were assigned to 28/33 loci disrupted by HPV16 integration (Figure 1). A set of PCR primers was designed within the flanking cellular sequence at each of these loci. To verify the HPV16-cellular junction, PCR was performed using a primer derived from the identified cellular flanking sequence and an HPV16 primer near the junction. The resultant PCR products were sequenced to verify that the junctions were specific to each tumor. The remaining five loci disrupted by HPV16 integration (CC9 – duplicon sequence (Ji *et al.*, 2000) (3' junction at HPV16 bp 864); CC61 – LINE 1 (5' junction at bp 4865), Alu (5' junction at bp 4377), and repetitive DNA within a rDNA spacer (5' junction at bp 7254); CC73 – human alpha satellite DNA (3' junction at bp 3228)) could not be assigned to a specific genomic location due to their position within repetitive elements.

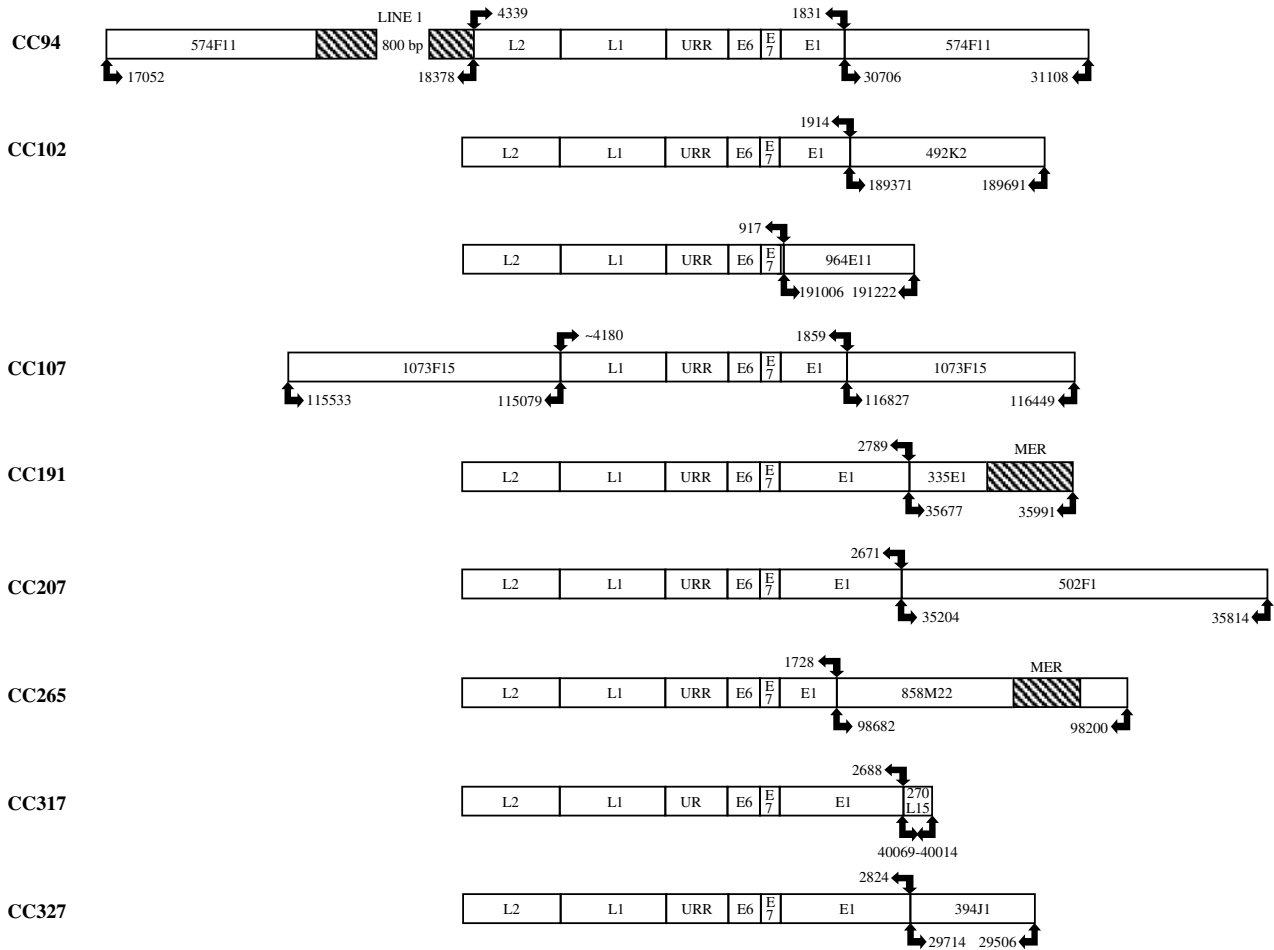
### *Multiple HPV16 integrations were detected in some tumors*

Most cervical tumors with integrated HPV16 have a single, clonal site of integration. However, tumors have been reported that contain more than one integration site (Choo *et al.*, 1987; Luft *et al.*, 2001). In this study, 4/26 tumors were found to contain multiple integration sites (Figure 1). Unless a complex rearrangement occurred within the HPV genome, one 5' and one 3' junction should exist for each integration site. In tumors CC91 and CC102, two 3', but no 5' junctions, were determined by RS-PCR. In both tumors, cellular sequences flanking the 3' integrations mapped to different chromosomes (Figure 1, legend), indicating that two independent integrations occurred in these tumors. Similarly, three 3' junctions were isolated from CC73. One of these integrations occurred within alpha-satellite DNA and the other two occurred on chromosomes 11 and 12, respectively, indicating that three independent integrations occurred. Finally, two 3' junctions and six 5' junctions were isolated from CC61. The sequences flanking three of the 5' junctions occurred within repetitive sequences so the chromosomal location is unknown. However, the other five junctions occurred on five different chromosomes. Thus, it is likely that at least six independent integrations occurred in this tumor. This tumor has an integration pattern that is quite different from any HPV16-positive cervical tumor reported previously.

### *HPV16 integration causes deletions and complex rearrangements in flanking cellular DNA*

Deletions and complex rearrangements of the cellular DNA at the sites of HPV integration in cervical tumors have been reported (Wilke *et al.*, 1996; Gallego *et al.*, 1997). 5' and 3' junctions for the same HPV16





**Figure 1** Loci disrupted by HPV16 integration in cervical tumors. Breakpoints within the HPV16 genome (drawn to scale) are denoted by arrows above the schematics (numbering system based on Genbank accession number K02718). The HPV16 unit genome is shown for comparison. In tumors where only the 3' integration junction was determined, it is unknown where within the HPV genome the 5' integration junction occurs, so both the L1 and L2 genes are shown on the schematic, although parts or all of these sequences may actually be deleted at the integration site. Similarly, in tumors where only the 5' integration junction was determined, the E1, E2, and E5 genes are shown. Flanking sequences (drawn to a different scale than the HPV genome) are denoted by arrows below the schematics. The numbering system for each flanking sequence is based on the sequence of the BAC clone in GenBank as of September 30, 2001 (some BAC sequences are still being assembled as part of the Human Genome Project so their final numbering system has not yet been established). Repetitive elements within the genomic flanking regions are denoted by hatched regions. Two alleles were detected for the same integration in CC24. One of the alleles contained a ~23 kb deletion of the flanking sequence. The HPV16 genome in CC91 contains a 2847 bp deletion that removes portions of the E2(E4) and L1 regions, and all of the E5 and L2 regions. Multiple integrations were found in tumors CC61, CC73, CC91, and CC102. Accession numbers and chromosomal locations for each sequence flanking the site of integration are as follows: 265N9, AC027312, 5q35; 88B3, AC008719, 5q35; 520F22, AL512452, 13q21; 437L15, AC004003, 8q21.3; 129K20, AC016900, 3p14.1; 2318H23, AC010634, 5p15.3; 540D4, AC025568, 12q21.31; 607P23, AC027750, 12q13; 532E4, AC061979, 11p15.5; 90J7, AC010163, 10q22; 309J13, AL162376, 13q22.2; 30C8, AL138964, 13q22.2; 349C3, AC010238, 5q31.1; 95H10, no accession number – BAC isolated from Genome Systems BAC pools and localized by radiation hybrid analysis (see Materials and methods), 15q15; 81E19, AC021100, 3q26.1; 574F11, AL162253, 9p24.1; 492K2, AL358973, 1q41; 964E11, AL079303, 14q13.3; 1073F15, AC004686, 17q23.2; 335E1, AL109920, 6q21; 502F1, AC006600; 17q23.2; 858M22, AL118510, 20p12.1; 270L15, AL603784, 9q34.3; 394J1, AC008149, 12q23.1

integration event were determined in 5 tumors – CC24, CC84, CC86, CC94, and CC107 (Figure 1). The cellular sequences derived at the 5' and 3' junctions of CC94 were contained within the same BAC clone. However, the junctions were separated by 12 328 bp of BAC sequence, indicating that the HPV16 integration event in this tumor caused a large deletion. Both junctions in CC86 also occurred within the same BAC. However, the sequence at the junctions indicates that a 7 bp duplication occurred along with the addition of an 8 bp orphan

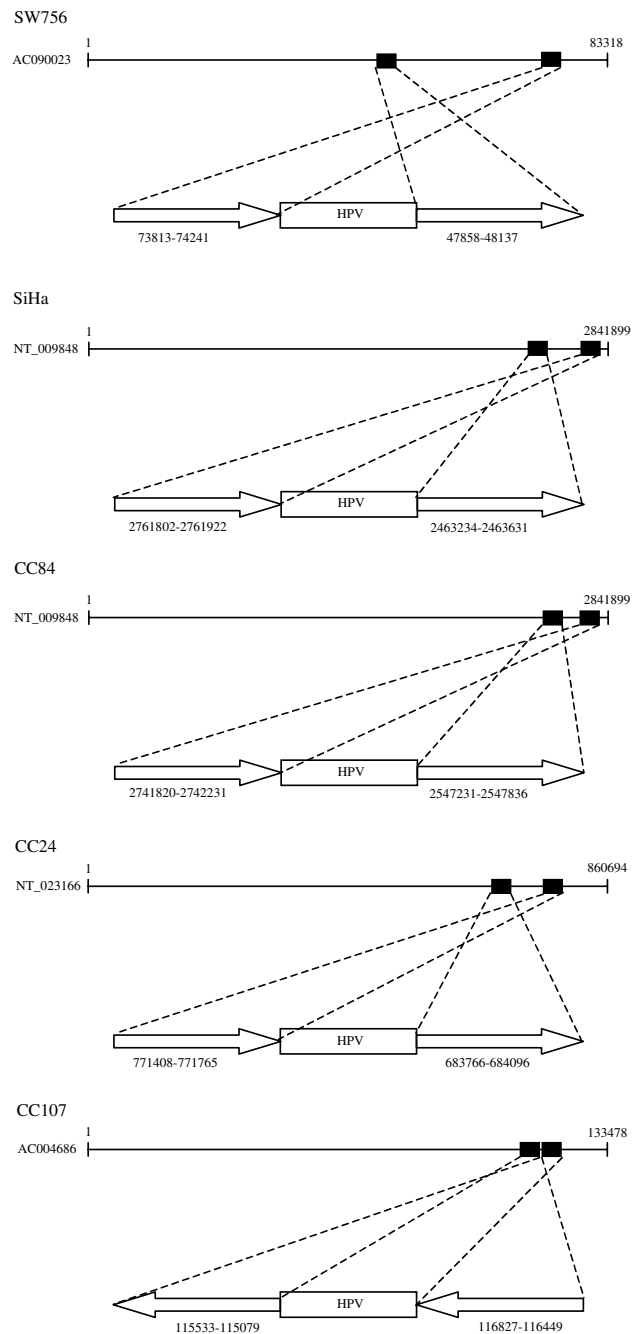
sequence, which causes the formation of a 10 bp direct repeat at the site of integration. Orphan nucleotides, direct and inverted repeats, very short regions of homology, and stretches of purines, pyrimidines, or alternating purines and pyrimidines have been observed at viral–cellular junctions in other cervical tumors (Choo *et al.*, 1990, 1996; Kahn *et al.*, 1994). An examination of the other 32 junctions sequenced in this study indicates that these types of sequence characteristics are common at the junctions (data not shown).

The cellular sequences derived at the 5' and 3' junctions of CC24 were homologous to two different sequenced BAC clones (Figure 1). However, these BACS are both located on 5q35 and overlap each other within the same sequence contig. Interestingly, the 3' junction was determined with two combinations of primers from the RS-PCR screening, and the flanking sequence from one of the products contained a 22 976 bp deletion relative to BAC clone 88B3 (Figure 1). In addition to the deletion that occurred in the flanking sequence, the cellular sequences at the 5' and 3' junctions were 88 003 bp away from each other. However, the orientation of the bases at each junction indicated that a complex rearrangement must have occurred in order to achieve the observed orientation of the flanking cellular regions (Figure 2). The HPV16 integrations in CC84 and CC107 also caused similar complex genomic rearrangements. The cellular sequences at the 5' and 3' junctions in CC84 were homologous to two overlapping BAC clones within the same sequence contig on chromosome 13q22. The junction sequences were 195 000 bp apart, but again the orientation was consistent with a complex rearrangement. Finally, the 5' and 3' junctions isolated in CC107 occurred within the same BAC clone, approximately 1748 bp apart, also in an orientation consistent with a complex rearrangement (Figure 2).

Based on the above data, a review of the literature was performed to locate other cervical tumors or cell lines in which both junctions had been determined and the genome sequence in the region of the integration had been completed. Five other tumors and cell lines fit this criteria: SW756 (Gallego *et al.*, 1997), SiHa (Baker *et al.*, 1987) H701 (Wagatsuma *et al.*, 1990; Wilke *et al.*, 1996), H901 (Wagatsuma *et al.*, 1990), and M50 (Choo *et al.*, 1990). The tumors H705, H901, and M50 all demonstrated deletions of 96 001, 63 832 and 1081 bp, respectively, of cellular sequence at the site of HPV16 integration (data not shown). However, both SW756 and SiHa cell lines had complex rearrangements similar to those observed in this study (Figure 2). In total, five of 10 tumors and cell lines demonstrated complex rearrangements at the site of HPV integration. This has implications for the disruption of expression of cellular genes within large genomic regions surrounding the sites of HPV integration in cervical tumors (see below).

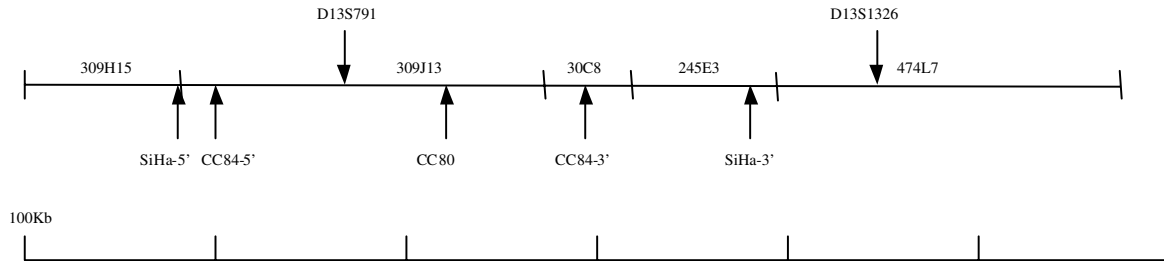
#### *Pattern of chromosomal integration sites reveal clusters of integration*

Previous studies have reported the chromosomal locations of HPV integration in multiple cervical tumors. A generally random distribution is apparent. However, two chromosomal regions, 8q24 and 12q14-15, have been reported to be hot spots for HPV integration (Lazo, 1999). In this study, the chromosomal locations of 22 HPV16 integrations in cervical tumors have been determined. Integrations occurred on 14 different chromosomes (Figure 1, legend). Six chromosomes were targeted more than once: Chr. 3 – 3p14.1



**Figure 2** Complex rearrangements at the sites of HPV16 integration. Completely sequenced genomic regions in which both ends of the HPV16 integrations occurred are represented by the upper line for each cervical tumor or cell line. The accession numbers and number of base pairs in each clone or contig are also shown. Black boxes indicate the regions within each clone or contig that are homologous to the sequences flanking the sites of HPV integration. Dashed lines and arrows indicate the direction and region of the clone or contig that the flanking sequence is derived from. Numbers under the arrows indicate the precise base pairs that are homologous to the flanking sequences

and 3q26.1; Chr. 5 – 5p15.3, 5q31.1 and 5q35; Chr. 9 – 9p24.1 and 9q34.3; Chr. 12 – 12q13, 12q21.31 and 12q23.1; Chr. 13 – 13q21 and 13q22.2 (twice);



**Figure 3** Sites of HPV16 integration into 13q22. The top line represents the genomic relationship between the BAC clones in the region (drawn to scale). Two markers are included for relative positioning of this genomic region. Three independent HPV16 integrations occurred within 300 kb of each other. Both junctions were cloned for SiHa and CC84. Only the 3' breakpoint could be isolated for CC80. HPV16 integrations in both SiHa and CC84 were involved with complex genomic rearrangements (see Figure 2)

**Table 1** FISH data for clones derived from sequences flanking the sites of HPV16 integration

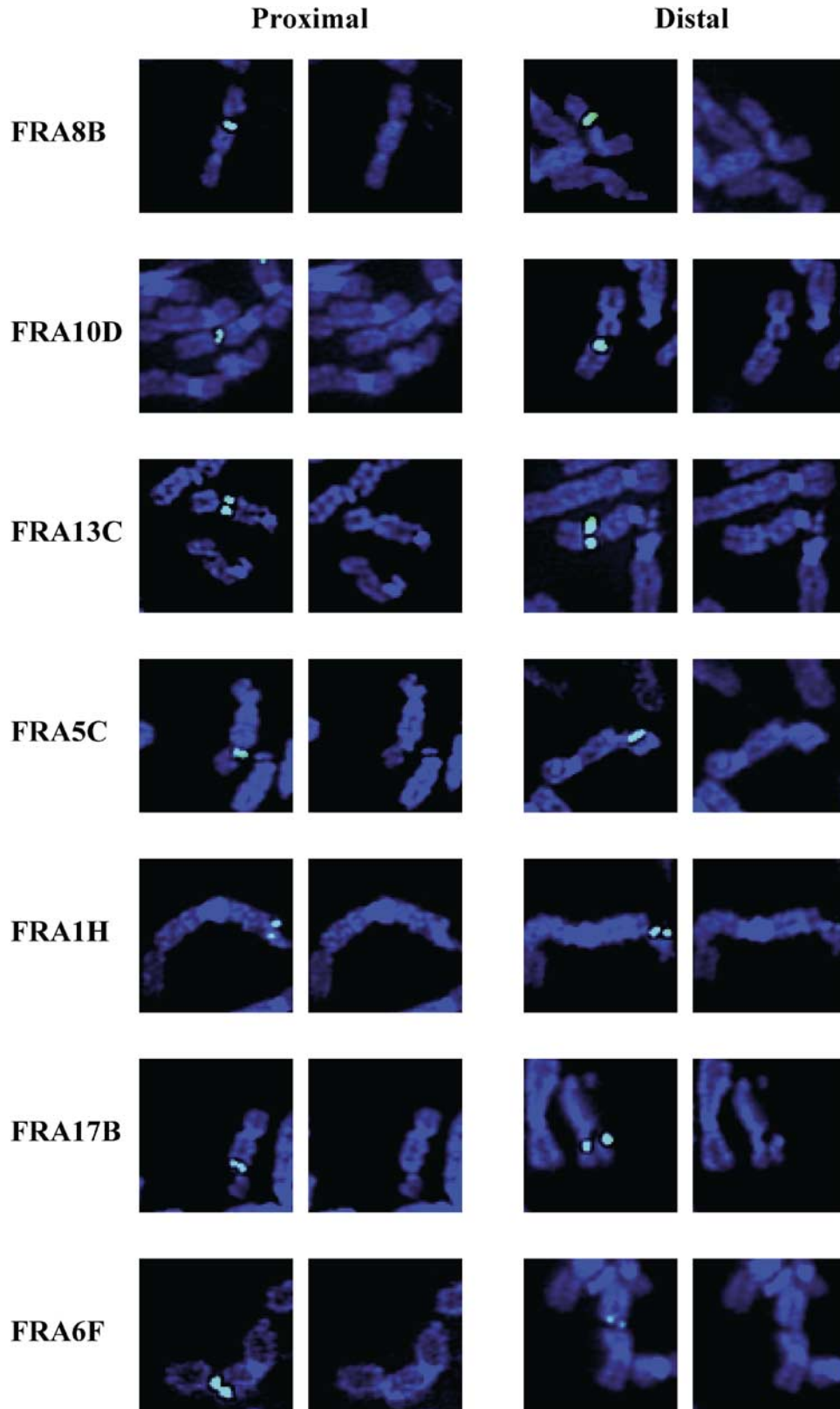
Tumor	BAC	Location	Nearest CFS	Proximal <sup>a</sup>	Crossing <sup>b</sup>	Distal <sup>c</sup>
24	88B3	5q35	5q31.1 (FRA5C)	0	0	20
61	437L15	8q21.3	<b>8q22.1 (FRA8B)<sup>d</sup></b>	20	2	2
	129K20	3p14.1	<b>3p14.2 (FRA3B)</b>	17	0	3
	540D4	12q21.31	12q21.32(FRA12B)	20	0	0
	2318H23	5p15.3	5p14(FRA5E)	0	0	20
73	607P23	12q13	12q21.32(FRA12B)	20	0	0
	532E4	11p15.5	11p15.1 (FRA11C)	0	0	20
75	90J7	10q22	<b>10q22.1 (FRA10D)</b>	7	0	13
80	309J13	13q22.2	<b>13q21.2 (FRA13C)</b>	4	1	16
84	309J13	13q22.2	<b>13q21.2 (FRA13C)</b>	4	1	16
86	349C3	5q31.1	<b>5q31.1 (FRA5C)</b>	8	0	12
91	95H10	15q15	15q22(FRA15A)	20	0	0
94	574F11	9p24.1	No CFS on 9p	NA	NA	NA
102	492K2	1q41	<b>1q42 (FRA1H)</b>	6	0	14
107	1073F15	17q23.2	<b>17q23.1 (FRA17B)</b>	14	0	6
191	335E1	6q21	<b>6q21 (FRA6F)</b>	6	0	14
207	502F1	17q23.2	17q23.1 (FRA17B)	20	0	0
265	858M22	20p12.1	20p12.2(FRA20B)	20	0	0
317	270L15	9q34.3	9q32 (FRA9E)	0	0	20
327	394J1	12q23.1	12q24(FRA12E)	20	0	0

<sup>a</sup>Proximal indicates the number of times the hybridization signal occurred on the centromeric side of breakage at the appropriate CFS. <sup>b</sup>Crossing indicates that the signal was split across both sides of the breakage indicating that the breakage occurred within the genomic region represented by the clone. <sup>c</sup>Distal indicates the number of times the signal occurred on the telomeric side of breakage at the appropriate CFS. <sup>d</sup>Bold type indicates integrations that occurred within a CFS. NA – not applicable

Chr.17 – 17q23.1 and 17q23.2. No integrations were observed in the previously identified clusters. However, a new cluster of integrations was observed on 13q22. Tumors CC80 and CC84 had HPV16 integrations that occurred within the same BAC clone, 309J13. In addition, a previously characterized HPV16 integration in the cervical tumor cell line SiHa also occurred on 13q21-31 (Bauer-Hofmann *et al.*, 1996). Analysis of the relative locations of these three integrations demonstrated that they all occurred within a <300kb region of the genome (Figure 3). This finding indicates that there may be selection for HPV16 integration in this region (see below). An additional cluster was also noted on 17q23. Two integrations from the present study and one previously reported (Thorland *et al.*, 2000) occurred in this region. However, these integrations, while close together at the cytogenetic level, are megabases apart at the molecular level.

#### HPV16 integrations preferentially occur within CFSs

We have previously shown that HPV16 integrations frequently occur in CFSs (Thorland *et al.*, 2000). In order to extend this analysis, BAC DNA from clones that represent the genomic DNA flanking 20 of the chromosomal sites of HPV16 integration was isolated. The chromosomal locations of 19/20 BAC clones were at or near chromosomal bands known to contain CFSs (Table 1). FISH was performed with each BAC clone and metaphases with breakage at the appropriate CFS were characterized. The distribution of hybridization signals for each BAC clone was determined (Table 1). The data indicate that 9/20 HPV16 integrations occurred within eight different CFSs (two independent integrations occurred within FRA13C). Representative FISH images demonstrating BAC clone hybridization, both proximal and distal to the aphidicolin-induced breakage at 7/8 CFSs, are shown (Figure 4). The BAC



**Figure 4** Representative FISH images at the CFSs. Clones derived from sequences flanking the sites of HPV16 integration were used as probes for hybridization. Metaphases with breaks both proximal and distal to each CFS are shown with and without hybridization signals

clone 129K20 hybridized to 3p14.1. This region was thought to be close to, but outside of, the CFS, FRA3B. However, upon further FISH analysis, it was demonstrated that this BAC was within the FRA3B region of instability. This indicates that FRA3B is considerably larger than previously reported and led us to further characterize this region. The FISH data for clone 129K20 and characterization of the entire FRA3B region are described elsewhere (Becker *et al.*, 2002). In combination with our previously published data (Thorland *et al.*, 2000), 11/23 HPV16 integrations cloned from cervical tumors occurred within CFSs. This finding is highly significant ( $P < 0.001$ ). However, it has been reported that most CFS reside in light G bands (Hecht, 1988). Thus, it was assumed that only half of the human genome (1500 Mbp) is the true target size for integration into CFSs. This more rigorous test was also highly significant ( $P < 0.001$ ).

*Cluster of HPV16 integrations on 13q22 are all contained within FRA13C*

The BAC clone 309J13 from the hot spot of HPV16 integration at 13q22.1 was determined to be within FRA13C (Table 1). However, the integration site in SiHa was outside of this BAC. To determine if the integration in SiHa also occurred within FRA13C, DNA from BAC clones 309H15 and 474L7 flanking this region (Figure 3) were also hybridized to aphidicolin-induced metaphases. Both of these clones were also contained within the CFS. 309H15 hybridization was proximal to breakage at FRA13C three times and distal 22 times. 474L7 hybridization was found to be proximal three times, distal 13 times, and crossed the region of breakage four times. These data indicate that all three HPV integrations in this region occurred within FRA13C.

*HPV16 integrations occur within cellular genes*

The genomic regions at the sites of HPV16 integration were examined for known genes. Table 2 lists the genes

that are interrupted as a result of the HPV16 integrations in our series of tumors. KCNIP1 is a protein that interacts with potassium channels and has mainly been studied in the brain (An *et al.*, 2000). AA626712 is a spliced EST sequence of unknown function. RIPK2 is a proapoptotic kinase that is involved in both FAS and TNFR1-mediated signaling (Inohara *et al.*, 1998; McCarthy *et al.*, 1998; Thome *et al.*, 1998). MYF5 is involved in skeletal muscle development. MUC5B is a highly expressed mucin that is the major component of cervical mucus. Interestingly, the integration occurred within exon 30 of this gene, which is highly repetitive and, at 10.7 kb, is the largest exon known in the human genome (Desseyn *et al.*, 1997). FLJ23312 is a protein of unknown function. The sucrose-isomaltase gene is involved in hydrolysis of intestinal sugars. ODC1 is a mitochondria oxodicarboxylate carrier. DKFZP566I133 is a protein of unknown function. Notch1 is a signaling protein altered in several tumor types. Finally, ELK3 is known to be a member of the ETS family of transcription factors that negatively regulate *c-fos* expression.

**Discussion**

We have previously demonstrated that RS-PCR is an efficient method for isolating the sequences flanking the sites of HPV16 integrations in cervical tumors. In this study, RS-PCR was utilized to screen 26 cervical tumors. Flanking cellular sequences were isolated from 17 of these tumors. Previous studies have determined the precise molecular locations of HPV integrations in relatively small numbers of tumor samples. This study considerably raises the total number of tumors reported and should facilitate the analysis of the mechanism and importance of HPV integration in the future. The determination of these molecular locations has allowed us to examine the relation between the sites HPV16 integration and the locations of CFS and cellular genes.

**Table 2** Known genes in which HPV16 integrations occurred

Tumor	Chromosomal integration site	Gene	Position in gene	Gene function
24	5q35	KCNIP1	Complete gene deletion	Kv channel interacting Protein
	5q35	AA626712	5' of last two exons	Unknown
61	8q21.3	RIPK2	Intron 4	Kinase involved in proapoptotic signaling
	12q21.31	MYF5	Intron 1	Skeletal muscle development
73	11p15.5	MUC5B	Exon 30	Major component of cervical mucus secretion
86	5q31.1	FLJ23312	Intron 2	Unknown
91	3q26	Sucrose-Isomaltase	Intron <sup>a</sup>	Hydrolysis of intestinal sugars
102	14q13.3	ODC1	Intron 3	Mitochondria oxodicarboxylate carrier
107	17q23.2	DKFZP566I133	Exon 12	Unknown
317	9q34.3	Notch1	Exon <sup>a</sup>	Transmembrane signaling protein involved in differentiation, proliferation, and apoptotic decisions
327	12q23.1	ELK3	Intron 3	ETS family of transcription factors; negatively regulates <i>c-fos</i> expression

<sup>a</sup>The complete exon-intron structure for the sucrose-isomaltase and *Notch 1* genes have not been delineated. However, it is known that the integration took place within an intron of the sucrose-isomaltase gene and within an exon of the *Notch 1* gene

HPV integration observed in cervical tumors has generally been thought of as a random event that is necessary only for the continued expression of the *E6* and *E7* oncogenes. Previous studies have demonstrated that HPV integrations may occur within CFSs (Thorland *et al.*, 2000; Wilke *et al.*, 1996). However, an analysis on a much larger scale was necessary to demonstrate that integrations preferentially occur in CFS. The analysis of the locations of these BAC clones relative to the sites of CFSs, in conjunction with our previous data (Thorland *et al.*, 2000), demonstrates that 11/23 HPV16 integrations occur within CFSs. This finding provides conclusive evidence that CFSs are preferentially targeted by HPV16 integrations ( $P < 0.001$ ). It remains to be seen whether the preferential integration into CFSs is solely due to the inherent instability of these regions, or if integration into some or all of these regions confer an advantage to HPV-infected cells and are subsequently selected.

To date, the molecular location and analysis of six CFSs have been reported in the literature (Wilke *et al.*, 1996a; Huang *et al.*, 1998; Mishmar *et al.*, 1998; Krummel *et al.*, 2000; Mangelsdorf *et al.*, 2000; Paige *et al.*, 2000; Arlt *et al.*, 2002; Morelli *et al.*, 2002). This analysis along with our initial report (Thorland *et al.*, 2000) has determined the molecular localization of eight additional CFSs. The molecular localization of these additional CFSs will provide a basis for the continued study of the mechanism of CFS induction as well as the role they play in cancer. Interestingly, the integration in CC102 was determined to occur within FRA1H, which has previously been characterized as a 5-azacytidine-sensitive CFS. However, FRA1H was also efficiently induced with aphidicolin, indicating that an individual CFS may be induced by more than one treatment condition. This phenomenon has been observed previously (Yunis *et al.*, 1987). The localization of this CFS will allow the analysis of the first 5-azacytidine-sensitive CFS.

The cumulative evidence in both *in vitro* cell culture systems and *in vivo* in cervical tumors has demonstrated that infection with a high-risk HPV alone is insufficient for conversion to a malignant phenotype. This indicates that additional alterations are necessary for cervical tumors to arise and progress. We propose that in at least a subset of tumors, the HPV integration event itself is a mutation that contributes to the malignant phenotype by altering the expression of cellular genes. It is possible that integration events occur multiple times in a population of premalignant HPV-infected cells. However, if integration into a specific locus confers a selective growth advantage to a particular cell, then the clonal HPV integration observed in that tumor may be a molecular marker for genes involved in cervical carcinogenesis.

HPV integration may alter the expression of cellular genes in several ways. Previous studies have shown that HPV integration into the host cell genome may cause large deletions (Wilke *et al.*, 1996), amplification (Wagatsuma *et al.*, 1990), and complex rearrangements (Gallego *et al.*, 1997) of cellular DNA. Thus, the

expression of cellular genes at or near the integration site may be affected. Clusters of HPV integration have been observed previously at 8q24 and 12q15 (Lazo, 1999). Multiple integrations into specific chromosomal loci may be indicative of genes that are common mutational targets in cervical carcinomas. In addition, HPV integration within or near genes that are known to be involved in carcinogenesis has been observed in cervical tumors and cell lines (Choo *et al.*, 1995; Wilke *et al.*, 1996; Sastre-Garau *et al.*, 2000). Indeed, one HPV integration has been shown to occur directly within a gene that has tumor-suppressive properties in cervical cell lines (Reuter *et al.*, 1998). In this paper, we have demonstrated that many of the above observations also occur in our set of cervical tumors. Each of these will be discussed in turn.

One apparently simple deletion and three complex rearrangements were shown to occur in 4/5 tumors in which both ends of the same integration were available for analysis. Thus, these types of large-scale mutations seem to be very common at integration sites. The deletions we observed in both our samples and those from the literature range in size from approximately 1 to 100 kb. These deletions could affect the expression of cellular genes either directly, from deletion of portions of genes, or indirectly, through changes in gene expression owing to altered chromatin structure or the loss of regulatory elements. However, the scenario for complex rearrangements is not as clear since the precise genomic structures resulting from these rearrangements are not known in these cases. The 5' and 3' sequences flanking the HPV16 integration in SiHa cells are nearly 300 kb away from each other and in the opposite orientation from what would be expected, indicating that complex rearrangements may have effect on genes over very large genomic distances. Library construction and analysis of relevant clones of the samples with complex rearrangements will likely be necessary to reveal the structure and extent of these mutations.

We have identified three novel clusters of HPV integration. Integration at chromosome 13q22 was observed in two primary tumors and in the cervical tumor cell line SiHa. All three of these integrations occurred in a very small (<300 kb) region within the CFS, FRA13C. It is unclear why this region of the genome is a frequent target of HPV16 integration. At least two of these integrations were involved in complex genomic rearrangements that likely involve significantly larger areas surrounding the integration sites. There are no known genes within this region. However, several genes are predicted by the GENSCAN program (Burge and Karlin, 1997) and spliced ESTs exist in this region. This narrow region of chromosome 13 has also been implicated in prostate, breast, and other cancers (Dong *et al.*, 2000; Kainu *et al.*, 2000; Chen *et al.*, 2001). We are continuing our analysis in this region to isolate genes that may be involved in cervical carcinogenesis.

The second cluster of integrations occurs on chromosome 17q23. Three HPV16 integration events (two from

this study and one from our prior study (Thorland *et al.*, 2000) were localized to this cytogenetic band. Two of these three integrations were shown to occur within FRA17B. However, these integrations are spread over roughly 4 Mb of genomic sequence. The third cluster of integrations occurs within the most active aphidicolin-induced CFS, FRA3B. The first molecular evidence that HPV integrations targeted CFSs was obtained when an HPV16 integration from a cervical tumor was observed within this CFS (Wilke *et al.*, 1996). One of the HPV16 integration sites in this study was initially thought to be close to, but outside of, the presumed boundaries of FRA3B. Subsequent analysis has demonstrated that FRA3B is much larger than previously thought and that this integration also occurred within this CFS (Becker *et al.*, 2002). While it is unclear whether the previously identified hot spots of integration at 8q24 and 12q15 occur within CFS regions, it is interesting that all three of the hotspots identified in this study occur at CFSs. The question remains whether these are hotspots solely due to the inherent instability at CFSs or whether these regions are hotspots because integration within these regions has functional implications for the initiation or progression of the tumors. The former is likely an explanation for the hotspots of integration at FRA3B and FRA17C because of the large distances between the integrations, while the latter is a possible explanation for the very tight cluster of integrations at FRA13C. Future analysis of the genes in these regions will determine their significance as mutational targets in cervical tumors.

Several of the chromosomal loci identified through the cloning of HPV16 integration sites in this study contain known genes (Table 2). Many of these genes have no known function or have not been fully characterized. Additionally, several of these genes have no known relationship to pathways that are activated or disrupted in cancer cells. However, at least three of these genes can reasonably be postulated to have roles in cervical tumor development.

Loss of apoptotic responses is a general phenotype thought to be necessary for most, if not all, tumors. RIPK2 is a proapoptotic kinase that is involved in both FAS and TNFR1-mediated signaling (Inohara *et al.*, 1998; McCarthy *et al.*, 1998). Overexpression of this protein promotes activation of caspase-8 and signals cell death. A kinase-mutant form of RIPK2 was also shown to inhibit FAS-mediated apoptosis. Loss of this protein because of HPV integration could confer an advantage to the tumor cell through loss of apoptotic responses.

The Notch family of transmembrane proteins are involved in cell fate decisions through cell-cell interactions. These cell fate decisions may affect differentiation, proliferation, and apoptotic pathways. Notch1 was originally identified as a target of translocation in T-cell acute lymphoblastic leukemia (Ellisen *et al.*, 1991). This 7:9 translocation resulted in the overexpression of a truncated form of Notch1 in approximately 10% of T-ALL cases. Since then, several lines of evidence have determined that expression of truncated forms of Notch1 are oncogenic in human tumors and animal

models (Girard *et al.*, 1996; Capobianco *et al.*, 1997). Increased expression and nuclear staining of Notch1 have been shown to be present in all cervical tumors examined, indicating that perturbation of this pathway occurs in most, if not all, cervical tumors (Zagouras *et al.*, 1995). Recently, it was demonstrated that coexpression of truncated Notch1 with HPV16 E6 and E7 transforms an immortalized human epithelial cell line through resistance to apoptosis (Rangarajan *et al.*, 2001). The HPV16 integration into Notch1 in CC317 occurs within an exon between the transmembrane domain and the ankyrin repeats. This integration likely produces a protein truncation very similar to that shown to be oncogenic in the studies mentioned above, indicating that the HPV16 integration event in this tumor may have contributed to the malignant phenotype through truncated Notch1 expression.

ELK3 is known to be a member of the ETS family of transcription factors that regulate cell proliferation, differentiation, and oncogenic transformation. ETS family members integrate signals from the Ras-MAPK signaling pathway to immediate-early genes that contain serum response elements in their promoters (Yordy and Muise-Helmericks, 2000). In unstimulated cells, ELK3 is a negative regulator of *c-fos* expression through its serum response element. However, upon stimulation of the Ras signaling pathway by growth signals, ELK3 becomes an activator of *c-fos* expression. It is possible that disruption of expression of ELK3 due to HPV integration results in *c-fos* overexpression. High-risk HPV subtypes can cooperate with *v-fos* to transform baby mouse kidney cells and form tumors in nude mice (Crook *et al.*, 1988). In addition, Jun-B has also been shown to be mutated through HPV16 integration (Choo *et al.*, 1995). Jun-B is a negative regulator of AP-1 complexes that are composed of homo- and heterodimers of *c-fos* and *c-jun* family members and is itself regulated by ETS transcription factors (Shaulian and Karin, 2001). Taken together, these data indicate that deregulation of growth signals transmitted along the Ras-MAPK pathway may be a necessary step in the formation of cervical tumors, and in some tumors HPV integration events may contribute to this loss.

In summary, we have cloned and localized 20 sites of HPV16 integration from 17 cervical tumors. We demonstrate that these integration events preferentially occur within CFSs ( $P < 0.001$ ) and that the locations of HPV integration cluster at specific chromosomal loci. These clusters may indicate highly unstable regions that are particularly susceptible to HPV integration or may be indicative of loci that contain genes that are important in the development of cervical tumors. In addition, we demonstrate that deletions and complex rearrangements of the cellular DNA are frequent at the sites of HPV integration. These mutations may affect the expression of cellular genes. Thus, integrations into specific loci may be selected for and may be markers for genes that contribute to cervical carcinogenesis. We have shown that integrations occur in genes that are

known to be mutated in, or are members of pathways that are commonly disrupted in, human tumors. Finally, we plan to critically examine the loci at the sites of integration to determine the effects on local gene expression and to determine whether altered expression of these genes are important in the development of cervical cancer.

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