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Deletion in Human Chromosome Region 12q13–15 by Integration of Human Papillomavirus DNA in a Cervical Carcinoma Cell Line*

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In human cervical carcinomas papillomavirus DNA is frequently integrated in the cell genome. We have cloned the integration site of human papillomavirus-18 DNA in human chromosome region 12q13–15 present in the SW756 cervical carcinoma cell line. Viral DNA is broken from nucleotides 2643 to 3418 in the E1 and E2 open reading frames, resulting in a deletion of 775 bases of viral DNA. Cloning and sequence analysis of the rearranged and germline alleles shows that there is no homology between the target cellular and viral DNA, suggesting it is a nonhomologous recombination. The target cellular region is called papillomavirus associated locus 2 (*PAL2*). The 5′- and 3′-flanking probes derived from the hybrid viral-cellular clone detect completely different germline restriction fragments in DNA from cells with normal chromosome 12. There is no overlap between the restriction maps of the target germline clones obtained with 5′- and 3′-flanking probes. Probes from these germline clones beyond the breakpoint position do not detect any DNA rearrangement in SW756 cells DNA. These data prove that there is a deletion of cellular DNA as consequence of the integration, with an estimated minimum size of 14 kilobases. Both cellular flanking probes are outside the amplicon of this chromosome region identified in the OSA and RMS13 sarcoma cell lines, comprising *SAS-CHOP-CDK4-MDM2* genes and where translocation breakpoints are located in liposarcomas. The integration at 12q13–15 might have been selected by its contribution to the tumor phenotype.

Recurrent chromosomal alterations are a hallmark of many types of tumors, and their cloning and characterization in lymphoid tumors has been very instrumental in the identification of oncogenic loci (1–3). In solid tumors, the characterization of chromosome aberrations lags further behind. However, specific translocations associated to specific types of solid tumors are often identified (4–7) and are also likely to be related to some aspect of tumor phenotype (8). These chromosomal alterations are usually manifested as translocations, amplifications, deletions, or losses of heterozygosity. In virus-associated tumors, such as cervical carcinoma linked to papillomaviruses and hepatocellular carcinoma linked to hepatitis B virus, the integration of viral DNA represents an additional type of DNA damage. This viral DNA integration can be considered an alternative to translocations but with similar biological consequences (9–11). Furthermore the viral DNA in these integration sites will provide a tag for their identification, cloning, and characterization.

Human papillomaviruses have been associated with tumors of genital and skin origin (12). There are more than 70 viral DNA types, but two of them, HPV16[1] and HPV18, are linked to over 70% of the cervical cancers (12). The mechanism by which these viruses are implicated in oncogenesis is very complex and has two main components. First of all, the oncogenic early viral genes E7 and E6 are capable of interacting with the products of *RB* (13) and *P53* (14, 15) genes respectively, thus achieving a functional inactivation of two cellular tumor suppressor genes. However, E6 and E7 viral genes can also be expressed from viral DNA in an episomal form (16). Second, most malignant tumors of the genital tract have viral DNA integrated in the cellular genome causing a genomic rearrangement (11, 12). Nevertheless, these integrations are usually studied from the consequences on viral genes (16) rather than on the cellular genome; this latter aspect is the object of the present work.

Integration of HPV DNA does not form part of the viral life cycle. The integration mechanism appears to be the result of illegitimate recombination near DNase I hypersensitive sites (17–19). The integrated viral DNA confers growth advantage to the cell due to viral gene deregulation (20). Cytogenetic analysis of cervical carcinomas (21) shows that integrations are clonal and that the viral DNA is located in some chromosomal regions that have already been associated with other tumor phenotypes (9, 10). The observation of clonal population is likely to be the result of a strong selection for the biological consequences of some integration events, thus their detection in regions linked to the tumor phenotype (9, 10). These findings are very similar to those already described for the role of nontransforming retroviruses in oncogenesis (21), where the most characteristic observation is the detection of common sites of provirus insertion, an event known as insertional mutagenesis, which are linked to a tumor phenotype (22).

Two human chromosomal regions, 8q24 and 12q13–15, are common integration regions for papillomavirus DNA in genital cancers. Both HPV-16 and HPV-18 DNA have been found in the two regions (21–27). The cervical carcinoma SW756 cell line has HPV type 18 DNA integrated in 12q13–15 (26, 28), and this locus, called papillomavirus-associated locus 2 (*PAL2*), is the object of the present report.

Human chromosomal region 12q13–15 undergoes several

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X88931 and X88932.

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[1] The abbreviations used are: HPV, human papillomavirus; ORF, open reading frame; kb, kilobase(s); PAL, papillomavirus associated locus.

types of genetic alterations. Translocations and amplifications have been found in melanoma (7), lipoma (29), liposarcomas (6, 30–32), gliomas (33), leiomyosarcomas (29, 34–36), pleomorphic adenomas (29), and lymphomas (37, 38). Integration of hepatitis B virus DNA in this region has been reported in one case of hepatocellular carcinoma (39), and integration of HPV-16 DNA has been found in another cervical carcinoma cell line, SK-v (27, 28).

The detection of such a variety of gross 12q13–15 chromosomal alterations suggests that this is a region likely to be rich in genes related to the tumor phenotype (40), most of them related to cell cycle control (41). Among them there are membrane receptors and proteins like *WNT1* and two members of the transmembrane four superfamily of proteins, *SAS* and *ME491*, which are implicated in the control of cell proliferation (42); the antigen *ME491/CD63* has been correlated with the prognosis of melanoma (43). There are several genes coding for proteins implicated in cell cycle regulation like *CDK2* and *CDK4* (44) and for oncogenes like *MDM2*, which interacts with p53 (45). Genes coding for signal transduction molecules like *RAP1A* and *RAB5b* and for transcription factors like *GLI1*, *CHOP*, and *ATF1*. However, in most cases these genes have not been linked to any specific tumor with the exception of translocations affecting *CHOP* in liposarcomas resulting in a gene fusion (6, 32, 47). Furthermore the 12q13–15 region is also likely to contain a tumor suppressor gene based on data from the detection of loss of heterozygosity in seminomas (48, 49), gliomas (50), and prostate cancer, where the presence or absence of a fragment derived from region 12q13–15 determined its oncogenic potential (51).

We report the cloning and characterization of *PAL2*, the integration site of HPV-18 DNA in chromosome region 12q13–15 present in the cervical carcinoma cell line SW756 (26) as well as of the corresponding germline target sequences. This viral-cellular DNA recombination is nonhomologous and causes the deletion of cellular DNA and a small region of viral DNA. The integration of HPV18 is not located in the area of DNA amplification described in 12q13–15 in some sarcoma cell lines. This is a genomic region likely to be implicated in the tumor phenotype.

EXPERIMENTAL PROCEDURES

Cell Culture—The SW756 cell line was obtained from Dr. J. A. DiPaolo, National Cancer Institute, Bethesda, MD. The cervical carcinoma SW756, the colon carcinoma Colo320, and the rabdomyosarcoma RMS13 cell lines were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. The MOLT4 T-cell lymphoma, the OSA osteosarcoma (from O. Mykeblost, Oslo, Norway), and the Burkitt lymphoma cell lines (from G. Klein, Stockholm) were grown in RPMI 1640 supplemented with 10% fetal calf serum. All the other cell lines were obtained from ATCC (Rockville, MD).

Probes—HPV18 genomic clone was provided by H. zur Hausen and E. M. De Villiers (Heidelberg, Germany). CHOP probe, plasmid WT10, was obtained from P. Aman (Lund University, Sweden). CDK2 and CDK4 probes, plasmids pCDK2 and pCDK4, were from D. Beach (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). MDM2 probe, plasmid MDM2-FL5, was from B. Vogelstein (John Hopkins University, Baltimore, MD). SAS probe, plasmid pSJP2, was from P. Meltzer (National Center for Human Genome Research, National Institutes of Health, Bethesda, MD). SP1 probe, plasmid pPacSP1, was from R. Tjian (University of California, Berkeley, CA). RARγ probe was from P. Chambon (INSERM U184, Faculté de Medicine, Strasbourg, France). WNT1 probe was obtained from the ATCC (Rockville, MD). The DNA insert to be used as probe was isolated by restriction digestion and purified with a Geneclean II kit (Bio 101, Inc., La Jolla, CA). The probes were labeled using a commercial random priming kit from Amersham Corp.

DNA and RNA Preparation and Analysis—DNA was prepared following standard procedures based on cell lysis and treatment with proteinase K and ribonuclease A (52). Afterward, the DNA was cleaned by repeated phenol and phenol-chloroform extractions (53). Digestions with restriction enzymes were performed following manufacturer's instructions (Promega or Boehringer Manheim) (52, 53). The digested DNA was fractionated in an 0.8% agarose gel in Tris/sodium acetate/EDTA buffer (52) with phage λ DNA digested with *Hin*dIII or *Bst*E2 as size markers. The DNA was transferred to Hybond-N^+ nylon membranes (Amersham Corp.) and fixed by alkali treatment. RNA was prepared by cell treatment like guanidinum thyocianate followed by centrifugation in cesium chloride according to a standard protocol (54).

Hybridization of DNA to different probes was carried out in 5 × SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, at 65 °C for 18 h. The filters were washed in 2 × SSC, 0.1% SDS at 62 °C for 15 min followed by three washes in 0.2 × SSC, 0.1% SDS at 62 °C for 20 min. Northern blots were hybridized under the same conditions, except that the high stringency washes were done at 55 °C (55). Quantitation of the blots were performed using a densitometer from Bio-Rad.

Genomic Cloning—To clone the rearranged allele from SW756 cells with integrated HPV18 DNA, 75 μg of cellular DNA were digested to completion with *Eco*RI and fractionated in a sucrose gradient by centrifugation (52). DNA fractions comprising the 10–15-kilobase pair range were pooled and ligated to *Eco*RI-digested λDASH (Stratagene, La Jolla, CA) arms phage vector purified by sucrose gradient centrifugation.

To obtain human germline clones, we used a commercially available human genomic library made by partial *Sau*3A digestion of DNA from a 3-year-old Caucasian male and addition of *Eco*RI linkers, followed by cloning in λFIXII vector (Stratagene, La Jolla, CA). This library was screened with cellular DNA sequences flanking the integrated viral DNA. Positive phage clones were partially digested with different restriction enzymes and mapped by hybridization to end-labeled T7 and T3 primers. DNA fragments free of repetitive sequences were subcloned in pBluescript SKII(−) plasmid and used for different studies as probes.

DNA Sequencing—The different clones in pBluescript SKII(-) vectors were sequenced by the chain termination method using dideoxynucleotides with T7 DNA polymerase (Sequenase) from Amersham Corp. The sequences have the following GenBank accession numbers: X88931 for *PAL2A* and X88932 for *PAL2B*.

RESULTS

Cloning of HPV-18 DNA Integrated in the Genome of SW756 Cells—The SW756 cell line is derived from a cervical carcinoma and contains HPV18 DNA integrated in the chromosome region 12q13–15 (26, 28). The integrated HPV18 viral DNA can be identified as bands hybridizing to viral DNA generated by enzymes that do not cut within the viral genome, like *Sal*I, *Xho*I, *Eco*RI, and *Hin*dIII. By restriction enzyme analysis and hybridization to subgenomic viral probes, we first determined that viral DNA breakpoint must be located between the *Hin*cII sites at positions 2472 and 3605 (not shown). To clone the integrated viral DNA, we made a library of *Eco*RI-digested DNA from SW756 cells screened with a complete HPV18 DNA probe. We obtained a 12.5-kb clone, λ12QHP, that was mapped with several restriction enzymes (Fig. 1) and hybridized to subgenomic viral probes, confirming that the breakpoint of the circular and extrachromosomal viral DNA before integration is between the *Hin*cII restriction sites at the end of the E1 and beginning of E2 ORFs. This clone lacks the internal *Eco*RI site at nucleotide 2440 within the E1 ORF. The phage clone, λ12QHP, has cellular DNA in both flanks. From the λ12QHP clone, we prepared three cellular flanking probes. Two probes were from the 5′-flank; the pS3 probe is a 1.6-kb *Eco*RI-*Spe*I fragment, and pHH probe is a 0.7-kb *Hin*dIII-*Hin*cII fragment with some viral nucleotides near the *Hin*cII site. From the 3′-end we prepared probe p500, which is an 0.5-kb *Hin*cII-*Hin*cII fragment (Fig. 1) also with some viral nucleotides at one end.

Flanking Cellular Probes Detect Different Genomic Regions Suggesting a Deletion at the Integration Site—As a consequence of the integration, there might be complex cellular DNA rearrangements or deletions. To ascertain if both flanking probes detected the same cellular genomic region, we determined the germline restriction pattern with both probes in DNA from several cell lines. We used probe pS3 from the

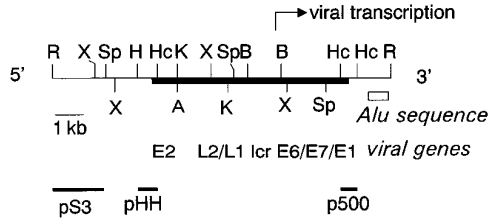

FIG. 1. **Structure of the phage clone containing the integrated human papillomavirus type 18 DNA in the genome of SW756 cells surrounded by cellular sequences.** The λ12QHP clone was isolated from a genomic library of SW756 cells and screened with HPV-18 DNA. The *thick line* represents the region where DNA of viral origin is located within the clone. The *arrow* indicates the start and direction of transcription of retain viral early genes. Under the map, the locations of several probes used in this work are indicated, *pS3*, *pHH* from the 5′-flank, and *p500* from the 3′-flank. *R*, *Eco*RI; *Sp*, *Spe*I; *X*, *Xba*I; *H*, *Hin*dIII; *Hc*, *Hin*cII; *B*, *Bam*HI; *K*, *Kpn*I; *A*, *Apa*I. The restriction sites were determined by partial digestion of the clone followed by hybridization to labeled T3 and T7 oligonucleotide primers. The clone was hybridized to viral subgenomic probes and to total human DNA to detect fragments with repetitive sequences. The retained viral open reading frames are indicated as well as the relative position of viral genes. The breakpoints affect the E2 and E1 ORFs. The *lcr* is the viral long control region where the origin of replication and several transcription control sequences are located.

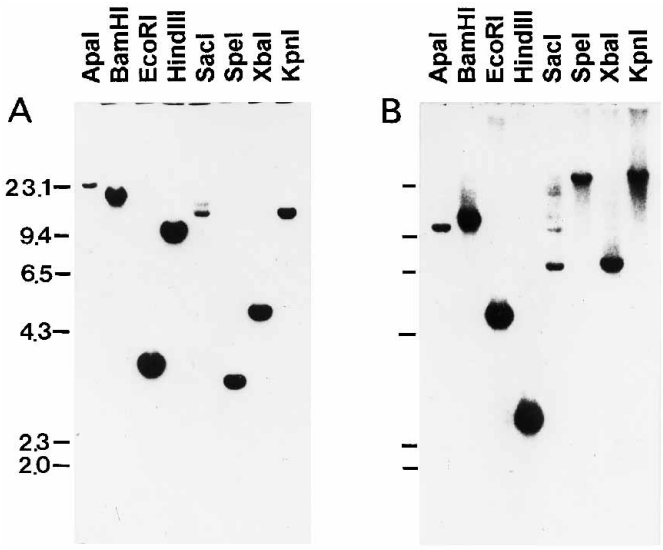

FIG. 2. **Detection of different genomic cellular sequences (*PAL2*) with flanking probes derived from the two ends of the integrated viral DNA.** Southern blots of cellular DNA from the Ramos cell line with normal chromosome 12 digested with several restriction enzymes and hybridized to cellular probe pS3 derived flanking the 5′ breakpoint (*A*) or to cellular probe p500 flanking the 3′ breakpoint (*B*).

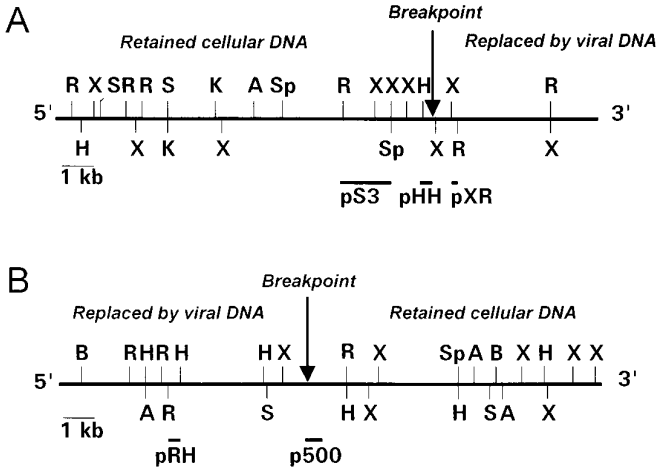

FIG. 3. **Structure of germline clones corresponding to flanks of the integrated viral DNA.** *A*, germinal clone, λh12/8HH, corresponding to the 5′ breakpoint and isolated with probe pS3. *B*, germinal clone, λh12/1–500, derived from the 3′ breakpoint obtained with probe p500. The location of different probes used in this work are indicated. The restriction map of the two overlapping clones is shown, with the relative position of the probe used for their isolation, the cellular flanking probe from the rearranged allele. In these clones, we have also determined the presence of repetitive DNA sequences. The *arrow* represents the location where the recombination between cellular and viral DNA took place. *R*, *Eco*RI; *X*, *Xba*I; *S*, *Sac*I; *K*, *Kpn*I; *A*, *Apa*I; *Sp*, *Spe*I; *H*, *Hin*dIII. The *arrow* indicate the location of the breakpoint and the separation between retained and lost DNA.

5′-flank and probe p500 from the 3′-flank. To develop this pattern, we used a cell line, Ramos, that does not have any alteration on chromosome 12. The pS3 (5′) probe detects the following bands, *Eco*RI (3.2 kb), *Hin*dIII (9.6 kb), *Xba*I (5.8 kb), *Sac*I (15 kb), *Apa*I (24 kb), *Bam*HI (19 kb), *Kpn*I (15 kb), and *Spe*I (3 kb). The probe derived from the 3′-flank, p500, detects the following fragments: *Eco*RI (4.5 kb), *Hin*dIII (2.8 kb), *Xba*I (6.9 kb), *Sac*I (6.7 kb), *Apa*I (1.2 kb), *Bam*HI (14 kb), *Kpn*I (35 kb), and *Spe*I (30 kb). Probes pS3 (Fig. 2*A*) and p500 (Fig. 2*B*) detected a completely different genomic region in all restriction digestions performed. This observation suggests that the region detected by each probe is not overlapping and therefore there is a deletion of cellular DNA as a consequence of the integration of viral DNA in SW756 cells. The size of the deletion is likely to be at least larger than 14 kb based on the size of the observed restriction fragments.

Cloning the Target Germline Alleles—The genomic target region corresponding to both flanks of the integration site were obtained by screening a genomic library made from a healthy individual. One clone, λh12/8HH, was obtained with probe S3 from the 5′-flank. The clone is 18 kilobase pairs long and spans the 5′ breakpoint, of which approximately 6 kb were replaced as result of HPV 18 integration (Fig. 3*A*). This part of the target region is called *PAL2A*.

From the 3′-end, two clones were isolated with probe p500, λh12/32 and λh12/1–500, of 13 (not shown) and 17 kb, respectively (Fig. 3*B*). These two clones are overlapping and cover a total genomic region of 23 kb, of which 8 kb were lost as a result of the integration. This side of the target region is called *PAL2B*.

The comparison of restriction maps from clones λh12/8HH, λh12/32, and λh12/1–500 shows no overlap between *PAL2A* (5′) or *PAL2B* (3′) regions. These data support the conclusion of a deletion of genomic DNA at the integration site, which has a minimum size of 14 kb, and the cloned genomic region, which has been replaced by viral DNA in the rearranged allele.

Internal Genomic Probes Detect Germline But Not Rearranged Alleles—The use of cellular probes derived from the germline clones corresponding to the region that has been replaced by viral DNA should detect only the germline allele, but not the rearranged one, if there is a deletion as a result of HPV18 DNA integration. To confirm this interpretation, we hybridized DNA from three cell lines, RMS13, OSA, and SW 756, with probes derived from the two phage germline clones, λh12/8HH and λh12/1–500, in such a way that they are to both sides of the breakpoints. The SW756 cell line has a 17-kb *Eco*RI band that is detected with an HPV18 probe (Fig. 4*A*). The 5′ breakpoint was examined with the pS3 probe and with probe pXR, a 0.25-kb *Xba*I-*Eco*RI fragment (Fig. 3*A*) from the region replaced by the viral DNA. The results for *PAL2A*, containing the 5′ breakpoint, are shown in Fig. 4, *B* and *C*. The 3′ breakpoint was studied with probes p500 and pRH, a 0.5-kb *Eco*RI-*Hin*dIII fragment, from the region replaced by viral DNA (Fig. 3*B*). The results for the *PAL2B* region, containing the 3′ breakpoint, are shown in Fig. 4, *D* and *E*. The two probes retained in

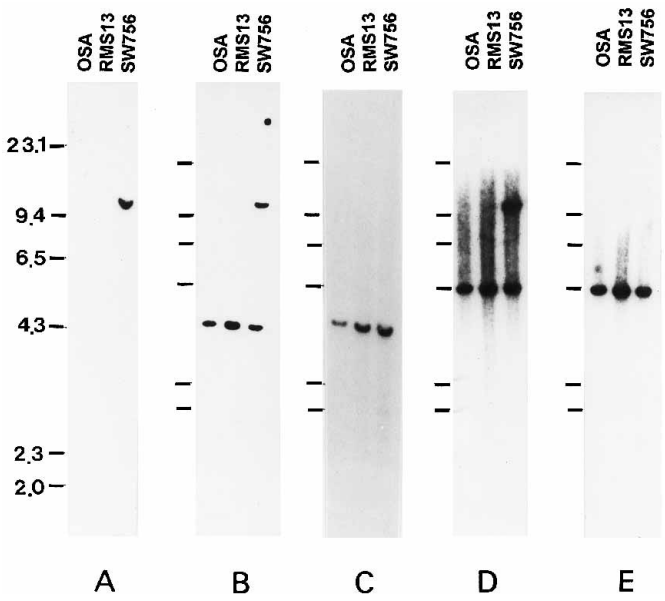

FIG. 4. **Deletion of internal genomic probes in SW756 cells DNA.** 10 μg of DNA from OSA and RMS13 cell lines as reference for not rearranged alleles and from SW756 cells were digested to completion with *Eco*RI and fractionated in an 0.8% agarose gel. The DNA was transferred to Hybond-N$^+$ membranes and hybridized to different probes. *A*, detection of rearranged allele with viral DNA detected by hybridization to HPV-18 DNA. *B*, detection of rearranged and germline alleles from the 5′ breakpoint with flanking probe pS3. *C*, lack of detection of 5′ rearrangement of genomic DNA from the 5′ genomic clone with probe pXR, derived from the region replaced by viral DNA. *D*, detection of rearranged and germline alleles with a probe from the 3′-flank, p500. *E*, lack of detection of rearrangement of genomic DNA replaced by viral DNA with probe, pRH, derived from the 3′ genomic clone.

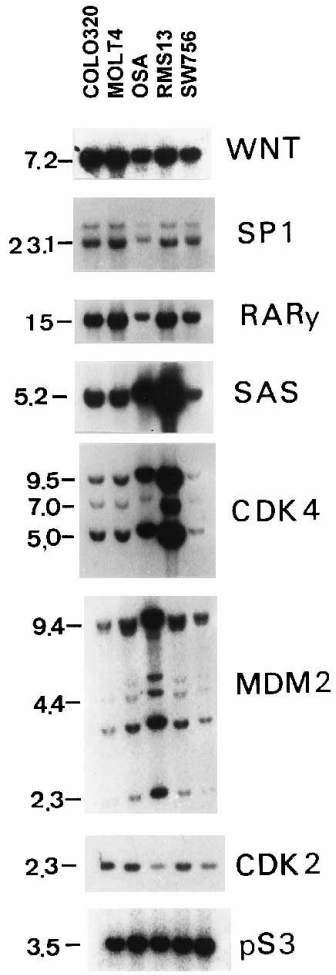

FIG. 5. **Relationship of integration site to two amplicons of 12q13 region.** Both ends of the integrated viral DNA are outside the amplicon of 12q13. *Eco*RI-digested DNA from five cell lines, Colo320, Daudi, OSA, RMS13, and SW756 was hybridized to several probes from genes located in 12q13, some of which, *SAS* and *CDK4*, are included in both amplicons, and one, *MDM2*, is amplified only in the OSA cell line. From the *PAL2A* locus, probe S3 was used. The probe used in each case is indicated to the *right* side of the corresponding blot. In some blots, the detection of several bands is due to the use of cDNA probes that contain several exons.

the hybrid clone, pS3 and p500, detect germline and rearranged alleles (Fig. 4, *B* and *D*). The internal probes, from replaced cellular DNA, pXR and pRH, only detect germline alleles (Fig. 4, *C* and *E*), ruling out their retention as part of a more complex rearrangement. These observations confirm the interpretation of a cellular DNA deletion at the integration site of HPV18 DNA in 12q13 between the regions defined by the *PAL2A* and *PAL2B* loci.

HPV-18 DNA Integration Site Is Outside Known Amplicons of Human 12q13–15 Region—There are two cell lines, OSA and RMS13 with an amplicon in the 12q13–15 region (45). This amplicon contains the region where most translocations occur (29–33, 46). We determined if the cellular flanking probes are located within these amplicons. For this purpose, we digested DNA from five cell lines, Colo 320, MOLT4, OSA, RMS13, and SW756 and hybridized them to the breakpoint flanking probes; to probes from genes within the amplicon like CDK4 and SAS; to probes from genes outside the amplicon like SP1, WNT1, RARγ, and CDK2; and to MDM2, which is within the OSA amplicon but outside the RMS13 amplicon. Densitometric analysis of these Southern blots detected a 5-fold amplification of the genes included within the amplicon. The results with these cellular gene probes and with flanking probe pS3 are shown in Fig. 5. The results with the other three *PAL2* probes are shown in Fig. 4, but only data for three cell lines is included in this figure. These data demonstrate that both ends of the integration site in SW756 cell line, the *PAL2A* and *PAL2B* loci, are outside the genomic region defined by the amplicons of both cell lines, OSA and RMS13.

Sequence of the Cellular-Viral Junctions and Target Cellular Sequences—The sequence of the viral cellular junctions in the rearranged allele were determined and compared with the cellular and viral sequences. We sequenced 761 nucleotides from the germline *PAL2A* region (Fig. 6*A*). The cellular sequence spanning the 5′ breakpoint does not contain repetitive elements and is AT-rich. There is no DNA homology between the viral and cellular target sequences. The 5′ recombinant breakpoint was sequenced from the cellular *Hin*dIII to the most proximal viral *Hin*cII (probe pHH in Fig. 1) and occurs within the early region E2 gene at nucleotides 3418 (not shown).

The germline sequence spanning the 3′ breakpoint was obtained from clone p500 and is shown in Fig. 6*B*. The sequence comprising the 3′ breakpoint was determined from the viral *Hin*cII site (nucleotides 2643) to the most proximal cellular *Hin*cII site in the hybrid clone (Fig. 1). The breakpoint of viral DNA is located at nucleotide 2643 within the E1 ORF.

There was no significant homology between the viral DNA and the sequence of the target alleles, *PAL2A* and *PAL2B*. A search did not reveal any homology of the target sequences to any known sequence in the GenBank data base.

The retained viral DNA has several point mutations in the region proximal to the breakpoints with respect to the reference HPV18 DNA. In the 5′ breakpoint 190 viral nucleotides were sequenced, and we detected viral mutations at positions 3462 (A→T), 3533 (T→C), 3557 (C→A), 3577 (C→T), 3584

A

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          10         20         30         40         50
          |          |          |          |          |
   1 CCATCTTACT CCTGATTAGT ATAATAGGCT CCTAACCTGT CTACCTGCTG
  51 CTCCCTGGCC CTTGCAGTCT ATTCTCTACA CAGCAGCCAG AATGATGCAA
 101 CACCCCCGCC AACCTGTTAT TTGAAGCTTT TCGAGCATAC AGAAAACTTG
 151 AAAGAACAAT ACAATATAAA ATGTCTTTTG CAGCTTCTTT TTCTGATCCA
 201 AGATCCAATC AAATGCAATT TGATCACATT TGATTGTTGT ATATTTTTAG
 251 TCTTTCTTAA TCTAGAATAA TCTTTCTTCT TTTTAAATGA CATGTACTTT
 301 TTCATATAGA ATATCCCACA CTTTCTGAAT TTGTCCGTTT CCTCATATAT
 351 TATGTTGTAT AATTCATATT GCATCAGATC AAGAGGCACA TAATGTCAGG
 401 TTGTCCTAAT GCTTATGATG CTGTTTGATC ACCTGACTAA TGAATCAACT
 451 CACCAGAGTT CTCCATTGGG AAGATAAATT TTCCTCTTTG TAATTAGTAA
 501 GAAATTCATG GGGTGATACT TTGATCACCT TTCAAATATC CACGATCTTT
 551 TACCCAATGG TTTTGCTATC TACTAATAAC CTTTTCTAGA TATTCCCGTA
        Replaced by viral DNA
 601 ATATTGTTCC TTATCATTTT CAAATTTTTT CTTAAACCCA CCTGACTAGC
 651 TATTTTTAAA TTTTTTAAGT CAATACTCCC CATCCTTCTT ACTATACTAC
 701 TTTATTTTCT CCACCAATTT TATCCATTTC TTACATTATT TCCTGTGTTT
 751 ATTGTCTGCC TCCAATTAGA ATTAAGCTCC GAGAGTGTAG G
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B

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          10         20         30         40         50
          |          |          |          |          |
   1 CCATATCATT ATGTAAAGAG AGTGGCAAAG AATTTCAGAC ACCTTTAATC
     Replaced by viral DNA
  51 TACCACAACC ACAATTACAT GGTCAGGTCT TGTGGGGTAA AGGAGCATCT
 101 AGTTGACTTG CCCAAACTTC TCCCCTGCCA CAGACTTCCC CTTGCCACAG
 151 ACTTCCCCTT GTTCCTCCTT GGATGCTCCC CTCTTACAAT GCAGCCTTCA
 201 CTCCAGGGAC TCTGGGCCTG TTGCATTTTA CTTTTCTGTC ACGACCAGCC
 251 AACTTCTCTC TACTGACTTT CTCTCCACCT CATAGATTTT TGATTATTCA
 301 CAGGCTCTGC TGCTCCATGG CATTATTGTC ATATGGCTAC TGCTATAAAA
 351 ATTAGTAGTG TACTGAAAAG GTT
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FIG. 6. **Nucleotide sequence of germline target sequence at both ends of integration site of HPV18 DNA in SW756 cells.** *A*, nucleotide sequence of the *PAL2A* germline spanning the 5′ cellular-viral recombination breakpoint in the hybrid clone described in Fig. 1. *B*, nucleotide sequence of the germline *PAL2B* region spanning the viral-cellular recombination breakpoint corresponding to the 3′ in the integration site described in Fig. 1. The location of each of the breakpoints is indicated by an *arrow*. The deleted cellular DNA that is replaced by viral DNA in the recombinant clone is indicated in *boldface* characters. The accession numbers are X88931 and X88932 for *PAL2A* and *PAL2B* respectively.

(A→C), and 3951 (T→G). In the 3′ breakpoint, the small region sequenced has mutations at positions 2482 (T→C), 2509 (G→A), and 2551 (G→T) and also in the *Eco*RI site within E1 ORF that is lost, but this mutation was only identified by the loss of the restriction site and not by sequencing.

DISCUSSION

The detection of integrated HPV DNA in the host genome is a common characteristic in cases of advanced cervical carcinoma. This observation can therefore be considered a recurrent type of genetic damage that could be selected for and thus contribute to the tumor phenotype. This integration has two different aspects. First, it can be looked at from the point of view of the viral DNA. There might be a selection for or against certain viral genes that will determine which viral genes are retained and the position where the recombination will occur in the viral genome. Thus, it is important to retain the genes that would favor cell proliferation, like E6 and E7, and delete or damage its repressor, E2, and that is what actually is observed in SW756 (Fig. 1) and in other cervical carcinoma cell lines (17, 28, 53). Most of the work on the role of HPV integration has been focused from this perspective (11, 28, 56). Furthermore, the integrated viral genes might have an altered regulation as a consequence of the cellular flanking sequences. Thus in SW756 cells, the regulation by glucocorticoids of E6 and E7 gene expression is affected by the integration site (57). The expression of the viral oncogenes might favor additional chromosome instability that could be the cause of other genetic defects not directly linked to the integration itself (58). Secondly, the integration chromosomal location might also be selected for the effects on the host genome either by gene damage or by a *cis* effect of viral sequences on nearby cellular genes that might lead to a deregulated expression. Therefore, the cellular genetic alteration resulting from viral DNA integration is a potentially oncogenic event. In most of the cases, where the chromosomal location has been mapped, like 8q24 or 12q13–15 (9), it is a genomic region already associated with the tumor phenotype suggesting a possible role that has not yet been demonstrated in cancer cases associated to DNA tumor viruses. The limited evidence available is consistent with this view (9, 10), although that is a common observation in oncogenesis by nontransforming retroviruses (22).

In this report, we have shown in the SW756 cervical carcinoma cell line that the viral integration has resulted in a deletion of viral and cellular DNA, at chromosome region 12q13–14. Three types of evidence support this interpretation. There are completely different restriction patterns in germline *PAL2A* and *PAL2B* DNA hybridized to cellular probes derived from both flanks on the integrated viral DNA (Fig. 2); there is no overlap between *PAL2A* and *PAL2B* genomic clones derived from both ends (Fig. 3), and there is lack of detection of rearranged allele by cellular probes from the cellular region replaced by viral DNA (Fig. 4). This integration is a likely consequence of nonhomologous recombination because of the lack of any significant homology between viral and cellular sequences. The location of the break within the viral genome and, probably, the chromosomal location are likely to be the result of strong biological selection for them. It is interesting to note the large differences in deleted DNA, 0.775 kb for viral DNA and more than 14 kb of cellular DNA. Although the deleted cellular DNA is likely to be much larger based on cytogenetic data, partial loss of a band occurs in the 12q13–15 region (25). The discrepancy between the sizes of cellular and viral deleted DNA might be explained as the result of illegitimate recombination between viral DNA and a loop of chromosomal DNA. The cellular DNA, due to its highly organized superstructure, might have in close proximity two distant regions; in this DNA, the viral DNA recombines by forming a bridge between the two distal ends of the loop and results in deletion of the intervening cellular DNA. The DNA sequence at the viral-cellular junctions (Fig. 6) shows no significant homology, suggesting that it is a nonhomologous recombination (59) in agreement with other cases of recombination between HPV and cellular DNA (60).

The lack of any significant homology between viral DNA and the two target, *PAL2A* and *PAL2B*, sequences at the integration site and the subsequent observation of its localization in chromosome regions associated to the tumor phenotype, like the case of *PAL1* in 8q24 (55) strongly suggest that there is a strong biological selection for some chromosomal locations. This selection is more likely to be related to the properties of this chromosomal region than to the retention of some viral genes, like E6 and E7. The expression of E6 and E7 could be achieved from almost any position where they might integrate in so far as they are structurally intact. If that was the case, there would be no reason to expect the integrations to be located in oncogenic regions.

The location of the integration in the SW756 cell line maps to a genomic region implicated in several oncologic phenotypes. The type of alteration includes both translocations and amplifications. Most recurrent chromosomal alterations contribute to the tumor phenotype by altering a gene. This gene modification can be very heterogeneous, ranging from its overexpression to changes in regulation, mutations, or deletions. The type of modification depends on the biological function of the gene. Thus the so called oncogenes are deregulated or overexpressed, or their products are altered while tumor suppressor genes undergo inactivating alterations, like mutations or deletions.

Deletion of cellular sequences is a characteristic observation in genetic alterations of tumor suppressor genes. There are three lines of evidence pointing to the existence of a tumor

suppressor gene in the 12q13–15 region. The first is loss of heterozygosity in 40% of male germ cell tumors (48) and in glioblastomas (50). Furthermore, in a prostate cancer cell line containing a deletion in this region, the malignant phenotype could be reversed after introduction of a normal chromosome 12, and the malignant phenotype reappeared following the loss of the introduced chromosome (51). The deletion detected by HPV-18 integration might be an indicator of the location of this putative suppressor gene, which is also known to be outside the OSA and RMS13 amplicons in the 12q13–15 (49).

This human region is syntenic with regions of mouse chromosomes 10 and 15 (40). The amplified genes in OSA and RMS13 cell line are all located in the region that is syntenic with mouse chromosome 10 (40). The data on the amplicons in different cell lines combined with data from translocation breakpoints in different types of tumors suggests the following gene order in this region: *COL2A1-WNT1-ATF1-GPDH-SP1-RARγ-A2MR-GLI* (40). The region amplified in OSA and RMS13 comprises *A2MR-GLI-MDM2-ERB3-GST3* and *CHOP*, but it does not include *RARγ, SP1*, or *WNT1* (33, 45). Thus, the lack of amplification of *PAL2* places it in a region outside this gene cluster (25). The precise location of *PAL2* will be determined when the yeast artificial chromosome containing it is isolated and placed within the existing map of the region.

HPV integration was postulated to be a late event in the process of oncogenesis by human papillomaviruses (61). During the early steps of the oncogenic process by HPV, the extrachromosomal location of the E6 and E7 viral genes is enough to generate an expanding population, because of cell cycle deregulation, where additional genetic alterations might occur. In cervical carcinoma, there are frequent rearrangements of region 11q13 not linked to HPV (5). Some of these additional genetic alterations might be the consequence of viral DNA integration. If the integration happens in an oncogenic region, it is likely to be selected for its contribution to some aspects of the tumor phenotype, which are unlikely to be related to the initial steps of transformation. In this case, the viral E6 and E7 contribute to expand the cells carrying important oncogenic mutation (5). The detection of *PAL*1 in 8q24 (55) and *PAL2* in 12q13–15 is consistent with this view. But their role in the tumor phenotype will only be known when the implicated genes are identified.

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Deletion in Human Chromosome Region 12q13-15 by Integration of Human Papillomavirus DNA in a Cervical Carcinoma Cell Line

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