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

(Received for publication, June 21, 1995, and in revised form, August 9, 1995)

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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 ampiclon 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 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...
types of genetic alterations. Translocations and amplifications have been found in melanoma (7), lipoma (29), liposarcomas (6, 30–32), gliomas (33), leiomyosarcomas (25, 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 alteration 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 the location 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 rhabdomyosarcoma DiPaolo, National Cancer Institute, Bethesda, MD. The cervical carcinoma SW756, the colon carcinoma Colo320, and the rhabdomyosarcoma are obtained from ATCC (Rockville, MD).

**Probes—**HPV18 genomic clone was provided by H. zur Hausen and the cervical carcinoma SW756, the colon carcinoma Colo320, and the rhabdomyosarcoma are obtained from ATCC (Rockville, MD).

**Northern Blot Analysis—**The filters were washed in 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 was digested with EcoRI and fractionated in a sucrose gradient by centrifugation (52). DNA fractions comprising the 10–15-kilobase pair range were pooled and ligated to EcoRI-digested ADASH (Stratagene, La Jolla, CA) arms phage vector purified by sucrose gradient centrifugation.

**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 SalI, Xhol, EcoRI, and HindII. By restriction enzyme analysis and hybridization to subgenomic viral probes, we first determined that viral DNA breakpoint must be located between the HindII sites at positions 2472 and 3605 (not shown). To clone the integrated viral DNA, we made a library of EcoRI-digested DNA from SW756 cells screened with a complete HPV18 DNA probe. We obtained a 2.5-kb clone, l12QHP, 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 HindII restriction sites at the end of the E1 and beginning of E2 ORFs. This clone lacks the internal EcoRI site at nucleotide 2440 within the E1 ORF. The phage clone, l12QHP, has cellular DNA in both flanks. From the l12QHP clone, we prepared three cellular flanking probes. Two probes were from the 5′-flank; the p53 probe is a 1.6-kb EcoRI-SpeI fragment, and pH7 probe is a 0.7-kb HindII-HindII fragment with some viral nucleotides near the HindII site. From the 3′-end we prepared probe p500, which is an 0.5-kb HindIII-HindII 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 p53 from the
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 p53 (5') probe detects the following bands: EcoRI (3.2 kb), HindIII (9.6 kb), XbaI (5.8 kb), SacI (15 kb), ApaI (24 kb), BamHI (19 kb), KpnI (15 kb), and SpeI (3 kb). The probe derived from the 3'-flank, p500, detects the following fragments: EcoRI (4.5 kb), HindIII (2.8 kb), XbaI (6.9 kb), SacI (6.7 kb), ApaI (1.2 kb), BamHI (14 kb), KpnI (35 kb), and SpeI (30 kb). Probes p53 (Fig. 2A) and p500 (Fig. 2B) 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 region corresponding to both flanks of the integration site were obtained by screening a genomic library made from a healthy region 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, p53, pH1 from the 5'-flank, and p500 from the 3'-flank. R, EcoRI; Sp, SpeI; X, XbaI; H; HindIII; C, HindI; B, BamHI; K, KpnI; A, ApaI. 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 reading frames are indicated as well as the relative position of viral genes. The breakpoints affect the E2 and E1 ORFs. The reading frames are indicated as well as the relative position of viral genes.

The use of cellular probes derived from the integrated HPV18 DNA integration. To confirm this interpretation, we hybridized DNA from three cell lines, RMS13, OSA, and SW 756, with probes derived from 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 EcoRI band that is detected with an HPV18 probe (Fig. 4A). The 5' breakpoint was examined with the p53 probe and with probes pXR, a 0.25-kb XbaI-EcoRI fragment (Fig. 3A) from the region replaced by viral DNA. The results for the PAL2A region, 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 EcoRI-HindII fragment, from the region replaced by viral DNA (Fig. 3B). The results for the PAL2B region, containing the 3' breakpoint, are shown in Fig. 4, D and E. The two probes retained in
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**Fig. 4.** Deletion of internal genomic probes in SW756 cells.

A. Detection 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 EcoRI and fractionated in an 0.8% agarose gel. The DNA was transferred to Hybond-N Plus 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. EcoRI-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 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. 6A). 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 HindII to the most proximal viral HindII (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. 6B. The sequence comprising the 3' breakpoint was determined from the viral HindII site (nucleotides 2643) to the most proximal cellular HindII 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
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**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. This is the first 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 amplicons in the 12q13–15 (49). The 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).

REFERENCES

1. Haluska, F. G., Tsujimoto, Y., and Croce, C. M. (1987) Annu. Rev. Genet. 21, 321–345
2. Rabbitts, T. H. (1994) Nature 372, 143–149
3. Korsmeyer, J. I. (1992) Annu. Rev. Immunol. 10, 785–807
4. Mietz, J. A., M. (1994) Catalog of Human Chromosomal Aberrations in Cancer, 4th ed., Wiley-Liss, New York
5. J. H. S. N. R. A. R. A. M. E. N. A. (1995) Am. J. Hum. Genet. 57, 705–715
6. Rabbitts, T. H., Forster, A., Larson, R., and Nathan, P. (1983) Nature Genet. 341–345
7. Zucman, J., Delattre, O., Desmaze, C., Epstein, A. L., Steenman, G., Spelman, F., Fletcher, C. M. D., Aurias, A., and Thomas, G. (1993) Nature Genet. 4, 335–340
8. Karp, J. E., and Broder, S. (1995) Nature Med. 1, 309–320
9. Papescu, N. C., Zimonjic, D., and DiPaolo, J. A. (1990) Hum. Genet. 84, 383–386
10. Lazo, P. A., Gallego, M. I., Ballester, S., and Feduchi, E. (1992) FEBS Lett. 296, 2955–2962
11. Chung, I. J., Park, Y. C., and Thomas, G. (1993) Cell Growth & Differ. 4, 243–260
12. DeMeulemeester, T., and Winge, D. R. (1993) Mol. Cancer Res. 15, 589–594
13. Hotta, H., Ross, A. H., Huetner, I., Isobe, M., Wendeboe, S., Chao, M. Y., Ricciardi, R. P., Tsujimoto, Y., Croce, C. M., and Koprowski, H. (1988) J. Virol. 62, 731–734
14. Demetrick, D. J., Zhang, H., and Beach, D. H. (1994) Oncogene 9, 278–285
15. Forus, A., Florenes, V. A., Maelandsmo, G. M., Meltzer, P. S., Fodstad, O., and Knuutila, S. (1993) Cell Growth & Differ. 4, 1050–1065
16. van de Ven, W. J. M., Schepenakers, M. F. P. M., Vos, S. A., Kuijper, J., and Smith, G. R., eds.) pp. 621–653, American Society for Microbiology, Washington, DC