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Integration of Human Papillomavirus Type 16 into the Human Genome Correlates with a Selective Growth Advantage of Cells†

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Integration of human papillomavirus type 16 (HPV-16) DNA into a host chromosome has been hypothesized to result in altered expression of two viral transforming genes, E6 and E7, in cervical cancers. In order to investigate the role that changes in viral genomic state and gene expression play in cervical carcinogenesis, we have derived clonal populations of human cervical epithelial cells which harbor multiple copies of either extrachromosomal or integrated viral DNA. The clonal populations harboring extrachromosomal HPV-16 DNA stably maintained approximately 1,000 viral copies for at least 15 passages (~100 cell doublings), which contrasted with the unstable HPV-16 replicons in the parental counterpart. In the clonal populations harboring integrated viral DNA, 3 to 60 copies of HPV-16 DNA were found integrated in either of two forms: type 1, in which all the copies of HPV-16 DNA were disrupted in the E2 open reading frame upon integration, and type 2, in which intact viral copies were flanked by disrupted viral copies and cellular sequences. Despite the lower HPV-16 DNA copy number, the clonal populations with integrated viral DNA had levels of E7 protein that were in most cases higher than those found in the clonal populations harboring extrachromosomal viral DNA. Irrespective of viral genomic state, the clonal populations were capable of undergoing terminal differentiation and unable to form colonies in soft agar, which is indicative of the nontumorigenic nature of these cells. Importantly, a cell population with integrated viral DNA was found to outgrow another with extrachromosomal DNA when these cells were cocultured over a period of time. Thus, integration of human papillomaviral DNA correlates with increased viral gene expression and cellular growth advantage. These observations are consistent with the hypothesis that integration provides a selective advantage to cervical epithelial precursors of cervical carcinoma.

Human papillomaviruses (HPVs) are small DNA tumor viruses that have a stringent tropism for epithelial cells. Specific genotypes of HPVs (e.g., HPV type 16 [HPV-16], HPV-18, HPV-31, and HPV-33) that infect the anogenital tract are also involved in the etiology of malignant lesions, most notably cervical carcinoma (for a review, see reference 49). They are collectively referred to as the high-risk anogenital HPVs. Cervical intraepithelial neoplasia (CIN), benign cervical lesions that are the progenitors of cervical carcinomas, are frequently detected in women infected with high-risk HPVs (32). CIN is graded I through III depending on the extent to which epithelial differentiation is curtailed. In contrast to the extrachromosomal states in normally infected tissues, high-risk HPV DNAs, especially HPV-16 and HPV-18 DNA, are often found to be integrated into the human genome in high-grade CIN lesions (CIN III) as well as in cervical cancers and derived cell lines (4, 39, 48). These integration events have been hypothesized to cause the increased expression of two viral transforming genes, E6 and E7 (11, 45). These genes are thought to play an important role in the development of HPV-associated cervical cancers, given their oncogenic properties in tissue culture (9, 18, 26, 30) and transgenic mice (1, 16, 23, 25) and their capacity to functionally inactivate the cellular tumor suppressors p53 and pRb (5, 29, 31, 36). Thus, increased expression of E6 and E7 might provide a selective growth advantage to the affected epithelial cell.

It has been hypothesized that the integration events commonly seen in cervical cancers and derived cell lines lead to the derepression of P_{97}, the papillomaviral promoter that directs expression of the viral oncogenes E6 and E7. This transcriptional derepression is predicted by the fact that gene products encoded by the E2 open reading frame (ORF), commonly disrupted by integration, can inhibit the P_{97} promoter (45). Mutational inactivation of E2 or E1, another gene commonly disrupted by integration, has been demonstrated to increase the transforming potential of various papillomaviruses, including HPV-16 (24, 35, 37). Recently, derepression of the P_{97} promoter has likewise been implicated in cervical cancers in which the viral genomes persist in the extrachromosomal state. Here, derepression is argued to occur as a result of mutational deletion of binding sites for the cellular transcription factor YY1 (27). These YY1 sites, positioned upstream of the P_{97} promoter, mediate YY1-dependent repression of this promoter. To date, the difficulty in addressing these hypotheses has been the lack of a suitable in vitro system to study the role that changes in viral genomic state and gene expression play in tumorigenicity. Recently, two human cervical epithelial cell populations established from CIN I lesions have been reported: W12 harboring HPV-16 DNA (42) and CIN612 harboring HPV-31 DNA (3). These cells are unique in that the viral genomes are maintained in an extrachromosomal state, which can provide a system with which to study the role of integration in cervical cancer development. In this study, we have utilized the W12 cell population to test the validity of the hypothesis that viral DNA integration results in augmented expression of the HPV oncogenes E6 and E7. Furthermore, we tested a corollary of this hypothesis that integration and its consequent induction of E6 and E7 gene expression provide a selective

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† Dedicated to the memory of Rex Risser.

growth advantage to the cell. We chose to use the W12 cell population, since HPV-16 is the most commonly found genotype in cervical cancers and integration of HPV-16 DNA in cervical cancers has been well documented (49). Our approach involved the isolation of clonal populations of W12 cells harboring extrachromosomal or integrated HPV-16 DNA. Using these cell populations, we demonstrate that integration correlates with increased synthesis of E7 protein and that cells harboring integrated viral DNA have growth advantages over those harboring extrachromosomal viral DNA.

MATERIALS AND METHODS

Cell culture. W12 parental cells at passage 12 (p12) were obtained from Margaret Stanley (Cambridge, England). Cell culture was performed as previously described, with some modification (40). Cells were grown on mitomycin-treated Swiss mouse 3T3 cells in F medium (0.66 mM Ca^{2+}) that contains three parts F12 medium and one part Dulbecco modified Eagle medium (DMEM) supplemented with 0.4 μg of hydrocortisone per ml, 8.4 ng of cholera toxin per ml, 5 μg of insulin per ml, 24 μg of adenine per ml, 10 ng of epidermal growth factor per ml, and 5% fetal bovine serum. Cells (2×10^5) were plated on freshly prepared feeders and grown to subconfluency for a week, with three feedings. For total RNA preparation, cells were grown without feeders in low-Ca^{2+} (0.05 mM) F medium that contains one part Ca^{2+}-free F12 and three parts Ca^{2+}-free DMEM with 5% Chelex-treated fetal bovine serum, $CaCl_2$, and the supplements described above. In order to derive clonal cell populations, 100 or 1,000 W12 cells from p14, p15, or p17 were plated on a 3T3 feeder layer and grown in F medium for 2 weeks. Individual colonies were picked up by using either cloning rings or 3-mm-diameter paper disks (Whatman). Scc13ya cells were grown on a feeder layer in DMEM containing 5% fetal bovine serum and 0.4 μg of hydrocortisone per ml. SiHa and CaSki cells were grown in DMEM containing 10% fetal bovine serum.

Southern and Northern (RNA) hybridizations. For Southern analysis of W12 cell populations, feeder cells were removed with 0.02% EDTA, and plates were washed with phosphate-buffered saline solution. Total genomic DNA was isolated by standard methods (17). Prior to electrophoresis, these DNAs were sheared by passage through a 22-gauge needle or digested with restriction enzymes. DNA (3 to 5 μg) was loaded on 0.7 or 1.0% agarose gels, electrophoresed, and transferred to a nylon membrane (DuPont). Alternatively, low-molecular-weight enriched genomic DNA isolated by the Hirt method (19) was used for reverse-field gel electrophoresis (RFGE). For positive controls in RFGE, DNAs isolated from bovine papillomavirus type 1 (BPV-1)-transformed mouse C127 cells (ID13 and C127/BPV clone b) were used (15). RFGE was performed as previously described (47). Two-dimensional gel electrophoresis was performed as described by Cullen et al. (10), with some modification. Total genomic DNA (3 μg) was loaded on a 0.5% agarose gel and electrophoresed in 1× Tris-borate-EDTA at 15 V for 20 h. The lane of interest was cut out of the gel, around which 1% molten agarose containing 0.5 μg of ethidium bromide per ml was poured. The resulting gel was run in a perpendicular direction to the first dimension in 1× Tris-borate-EDTA containing 0.5 μg of EtBr per ml at 50 V for 6 h. Following RFGE and two-dimensional gel electrophoresis, denatured DNAs were transferred to nylon membranes. Southern hybridization was carried out as previously described (13). The HPV-16 full-length probe that was generated by cutting pHPV-16 with *Bam*HI was used. The BPV-1 probe was generated by cutting p142.6 with *Bam*HI. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was generated by cleaving the GAPDH cDNA clone with *Pst*I.

RNA PCR. Total cellular RNA was isolated by standard methods (6). RNA PCR was performed as previously described (41), with some modification. RNA (2 μg) was reverse transcribed with a $d(T)_{20}$ primer tagged with an *Eco*RI restriction enzyme site to generate cDNA from polyadenylated RNA. A *Hin*dIII-tagged sense primer that spans HPV-16 nucleotides (nt) 831 to 850 together with the above $d(T)_{20}$ primer was used for PCR of the cDNA. The resulting products were subcloned into pUC19 and sequenced with Sequenase (Boehringer Mannheim Biochemicals).

Cellular properties. To assess the plating efficiency of cells, 100 or 1,000 viable cells were plated onto a 3T3 feeder layer in F medium. After 2 weeks, the cells were fixed in 37% formaldehyde for 30 min and stained with 0.14% methylene blue staining solution overnight. Colonies were counted; colony-forming efficiency (CFE) was calculated by dividing the number of colonies formed by the number of cells plated. Resistance to terminal differentiation was assessed by suspending cells in growth medium made semisolid with 1.68% methylcellulose as previously described (40). Cells were removed from tissue culture plates with 0.5 mM EDTA–0.1% trypsin, washed with serum-containing medium to inactivate trypsin and then with serum-free medium, and finally suspended in F medium at a density of 10^6 cells per ml in sterile 50-ml polypropylene tubes. Suspended cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells were recovered from suspension at various times by repeated dilution of the medium with serum-free medium and subsequent centrifugation at 1,000 × *g*. To assess survival, cells recovered from the methylcellulose were plated on 60-mm-diameter dishes containing 3T3 feeder cells and cultured for 2 weeks, after which they were fixed in 37% formaldehyde and stained with 0.14% methylene blue, and colonies were scored.

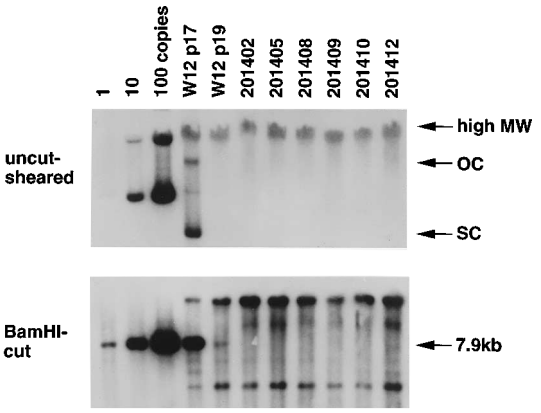

FIG. 1. Southern analyses of the 2014 series of W12 clonal populations as well as W12 parental populations at p17 and p19. Shown are autoradiographs of Southern blots containing sheared (top panel) or *Bam*HI-restricted (bottom panel) DNAs from six clonal W12 cell populations and two parental W12 populations at p17 and p19, hybridized to a full-length HPV-16-specific DNA probe. A copy number reconstruction series (1, 10, and 100 copies per cell) is included (see Materials and Methods). The arrows indicate high-molecular-weight (high MW), open-circular (OC), supercoiled (SC), and linearized (7.9-kb) forms of HPV-16-specific DNA.

Soft agar assay. A total of 1×10^5 or 5×10^5 cells was seeded in F medium containing 0.35% Noble agar (16). The cultures were supplemented every 3 days with 0.5 ml of F medium for 4 weeks, after which they were stained overnight in 0.05% iodonitrotetrazolium. Colonies larger than 0.1 mm in diameter were counted under a dissecting microscope.

Selective growth advantage. The clonal populations 20861 and 20863 were combined at ratios of 1:1, 1:10, and 1:100. The resulting mixed populations were cultured and passaged on 3T3 feeders in F medium as described above. At each passage, total genomic DNA was extracted and subjected to Southern analysis after shearing or *Bam*HI digestion. Bands corresponding to supercoiled HPV-16 DNA (sheared DNA) and junction fragments (*Bam*HI-digested DNA) were quantitated on a Molecular Dynamics PhosphorImager.

Radioimmunoprecipitation of E7 protein. Immunoprecipitation of ^{35}S-labeled lysates of W12 clonal populations and cervical cancer cell lines was performed with a rabbit polyclonal antibody raised against bacterially synthesized, purified E7 protein (33) as previously detailed (34). For each immunoprecipitation, the same amount of protein lysate, determined by the Bradford protein assay (Bio-Rad), was used.

RESULTS

DNA analysis of the W12 parental population. The W12 cell population was established from a cervical biopsy of an HPV-16-positive patient and was described previously to harbor, on average, 100 copies of HPV-16 viral DNA as a monomeric plasmid (42). For the purpose of our studies, we set out to identify subpopulations of W12 cells that contain the viral DNA integrated into the host genome, as is found in high-grade CIN lesions and in cervical cancers. To this end, W12 cells (referred to herein as the parental W12 cell population) were cultured for multiple passages, after which total genomic DNAs were isolated and subjected to Southern analysis. Approximately 1,000 copies of extrachromosomal HPV-16 DNA were present (on the basis of the presence of a 7.9-kb supercoiled HPV-16-specific band in Southern analyses performed on sheared total genomic DNA) at p14, the earliest passage analyzed (data not shown); however, there was a rapid loss of this extrachromosomal DNA by p19 (Fig. 1). Depending on the particular culture, complete loss of detectable (less than one copy per cell) extrachromosomal viral DNA occurred between p16 and p19; this variation likely reflected subtle differences in cell culture conditions in our hands. These results demon-

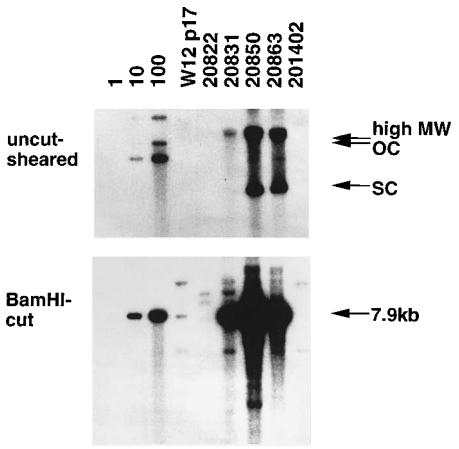

FIG. 2. Southern analysis of extrachromosomal and integrated W12 clonal populations. Shown are autoradiographs of Southern blots containing sheared or *Bam*HI-restricted DNAs isolated from two extrachromosomal (20850 and 20863) and three integrated (20822, 20831, and 201402) clonal populations. For comparison, DNA from the p17 W12 parental population was included. Viral copy number in different W12 clonal populations is summarized in Table 1.

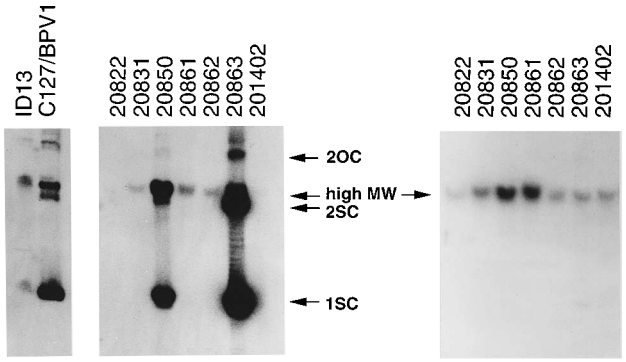

FIG. 3. RFGE and subsequent Southern analysis. Shown are autoradiographs of Southern blots containing Hirt DNAs resolved by RFGE. (Left panel) As a control, Hirt DNAs from two BPV-1-transformed C127 cell lines, ID13 and C127/BPV1, known to contain monomeric and multimeric BPV-1 replicons were resolved by RFGE, and the resulting Southern blots were hybridized to a BPV-1-specific probe. (Middle panel) RFGE analysis of Hirt DNA isolated from multiple W12 clonal populations. The Southern blot was hybridized to a full-length HPV-16 probe. (Right panel) The same blot was rehybridized with a GAPDH probe to identify the position at which contaminating high-molecular-weight DNA migrated. This control demonstrates that high-molecular-weight DNA contaminating the Hirt DNA preparation comigrates with open-circular monomeric DNA (see control, BPV-1-specific bands in the left panel). Therefore, only the presence of monomeric and multimeric supercoiled forms of HPV-16-specific DNA was taken as evidence for extrachromosomal HPV-16 DNA in the clonal populations. Indicated by arrows are open-circular dimer (2OC), high-molecular-weight DNA/open-circular monomer (high MW), and supercoiled dimer (2SC) and monomer (1SC).

strated that, at least in our hands, the parental W12 population did not stably maintain HPV-16 replicons. Interestingly, integrated copies of HPV-16 were detected (on the basis of the presence of HPV-16-specific bands of sizes distinct from the 7.9-kb HPV-16-specific band in *Bam*HI-digested DNAs) in the parental W12 population (Fig. 1). In contrast to the extrachromosomal viral DNAs, these integrated viral DNAs persisted, apparently at the same copy number, throughout the multiple passages analyzed (data not shown).

Generation of clonal populations containing extrachromosomal versus integrated HPV-16 DNA. Given the apparent heterogeneity of the W12 parental population, we clonally expanded W12 cells that had been plated at a low density to generate homogeneous populations harboring either extrachromosomal or integrated HPV-16 DNA. In the first attempt, 10 clonal populations were derived from p17 parental W12 cells. All of these clonal populations contained the same integration event, as judged initially by the identity in hybridization patterns generated by Southern analysis of *Bam*HI-digested DNAs (Fig. 1). Furthermore, this hybridization pattern was indistinguishable from that seen in the parental population of cells, indicating that within the W12 parental cells there had occurred, by p17, a clonal expansion of cells harboring a particular integrated HPV-16 DNA of this kind. Detailed restriction enzyme digestion and Southern analyses were performed on several of these clonal populations and are schematically summarized in Fig. 5. These data verified that the multiple clones analyzed had a structure identical to that of integrated viral DNA and that the integration event led to the disruption of the viral genome within the E2 ORF, as observed in cervical cancers. This result is significant, as it establishes that the integration event that led to outgrowth within the W12 cell population is similar to the integration events seen in cervical cancers (7, 38).

To identify alternative integration events and to isolate clonal populations that contain extrachromosomal HPV-16 DNA, an additional 65 colonies were picked from parental W12 cells at earlier passages, p14 and p15. Only six clonal populations of these 65 colonies were successfully established; four clones were analyzed on the particular blots shown in Fig. 2. Among these clonal populations, two clones were found to harbor HPV-16 replicons at a high copy number on the basis of the presence of monomeric and dimeric supercoiled DNA (Fig. 2, lanes 20850 and 20863). The presence or absence of extrachromosomal DNA in these clonal populations was verified by RFGE (Fig. 3) and two-dimensional gel electrophoresis (Fig. 4) analyses, two methodologies that permit the better separation of topologically constrained, circular DNAs from linear DNAs. Other clonal populations did not show any detectable supercoiled DNA species by these multiple analyses

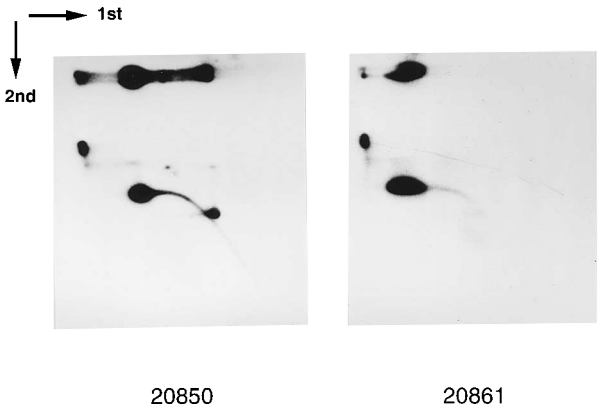

FIG. 4. Two-dimensional gel electrophoresis and subsequent Southern analysis. Shown are autoradiographs of Southern blots of total genomic DNAs isolated from the 20850 (extrachromosomal) and 20861 (integrated) clonal populations. At the top of each panel can be seen the Southern hybridization pattern of HPV-16-specific DNA resolved only on the first dimension (0.5% agarose gel). Below in each panel is seen the Southern hybridization pattern resulting from resolution of the HPV-16-specific DNA on the second dimension. Under these conditions, linearized DNAs of various length run as an arc, while topologically constrained circular forms of DNA migrate more slowly, forming distinct spots above the arc. Note the presence of those spots on the 20850 blot but not on the 20861 blot.

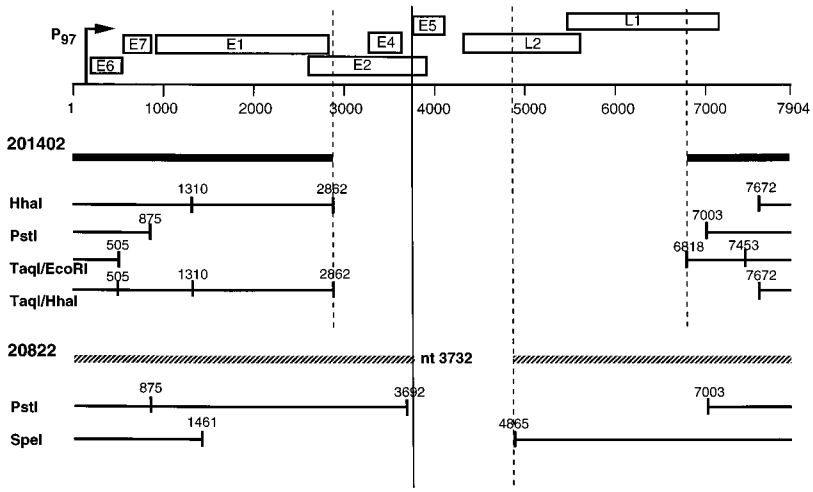

FIG. 5. Schematic illustration of the structure of integrated HPV-16 DNAs in 201402 (upper) and 20822 (lower). The line at the top depicts the HPV-16 DNA circular genome linearized at nt 1. Above it are indicated the relative positions of the early (E) and late (L) translational ORFs. The solid and the hatched boxes represent the integrated HPV-16 DNAs in 201402 and in 20822, respectively. The lines underneath these boxes show the HPV-16 genomic DNA fragments that were detected by Southern analyses after digestion with the various restriction enzymes indicated on the left. On the basis of minor DNA fragments hybridized to an HPV-16-specific probe, 201402 might contain HPV-16 sequences resulting from a potential second integration. The dashed lines show potential intervals between the viral-cellular junctions. In the case of 20822, the exact position of E2 disruption was mapped at nt 3732 by the sequencing of a cloned RNA PCR product, which is depicted as a solid line.

but, interestingly, had hybridization patterns that were distinguishable from those present in the clones derived from p17 parental W12 cells (Fig. 2, lanes 20822 and 20831, and Fig. 7, lanes 20861 and 20862). This result indicates that multiple, independent integration events had occurred in earlier passages (p14 and p15) of the parental W12 cell population, though only one such integration led predominantly to the outgrowth in later passages (p17). Importantly, all the clones established were found to contain HPV-16 DNA in one form or another.

Patterns of integration in W12 cells compared with those in cervical cancers. The hybridization patterns generated by *Bam*HI digestion of the different W12 clonal populations containing exclusively integrated viral DNA indicated that two fundamentally different types of integration events had occurred. In one type, type 1, all copies of the viral DNA were disrupted by the integration event, as judged by the absence of unit-length, 7.9-kb, viral genomes (Fig. 1, 2014 series; Fig. 2, lane 20822). Of the two type 1 clones isolated, both had integration events that led to the disruption of the E2 ORF (schematically summarized in Fig. 5). This type of integration event is analogous to that found in a cervical cancer cell line, SiHa (2, 14), and in primary cervical carcinoma tissue (46). The viral-cellular junction in the E2 ORF in clone 20822 was precisely mapped to nt 3732 by the sequencing of a cloned RNA PCR product. The flanking cellular sequence was demonstrated to bear no homology to any known unique sequences (performed with the BLAST algorithm—homology was found only to CA microsatellite sequences) as in CaSki (41) (Fig. 6). In the second type of integration event, type 2, multiple copies of unit-length HPV-16 are present in the host genome (as judged by the presence of the 7.9-kb band in Southern analyses of *Bam*HI-digested DNAs) and are flanked by a partial copy of the viral genome disrupted by the integration event (as judged by the presence of HPV-16-specific bands in addition to the unit-length band). The presence of unit-length viral DNA in the type 2 clones (Fig. 2, lane 20831, and Fig. 7, lanes 20861 and 20862) limited us from clearly establishing by Southern analyses the position of the disruption within the junction copies of the viral DNA; however, S1 nuclease analyses of mRNAs derived from these type 2 integration events suggest that integration in these clones likewise led to disruption of the E2 ORF (22). The type 2 integration pattern described here is similar to that seen in the cervical cancer cell line CaSki (2, 48) and in primary cervical carcinoma tissue (46). Irrespective of the type of integration event, amplification of both viral and flanking cellular sequences was noted in all integrated clonal populations on the basis of the intensity of hybridization to the junction-derived HPV-16-specific bands compared with copy number standards. These data indicate that integration must have occurred prior to amplification, which agrees with that seen in cervical cancers (46). The state of viral DNA present in these multiple clones is summarized in Table 1.

Stability of extrachromosomal HPV-16 DNA in clonal populations. As noted in Fig. 1, the parental W12 populations failed to stably harbor extrachromosomal HPV-16 DNA. To address whether HPV-16 replicons are stably maintained in the clonal populations 20850 and 20863, cells were cultured over multiple passages and the state of HPV-16 DNA over the time course was determined by Southern hybridization (Fig. 7). As expected, the HPV-16 DNAs present in the integrated clonal populations (20861 and 20862) were also stably maintained over a similar time course in culture. Importantly, 20850 and 20863 were shown to stably maintain extrachromosomal HPV-16 DNA at least through the 15 passages (~100 cell

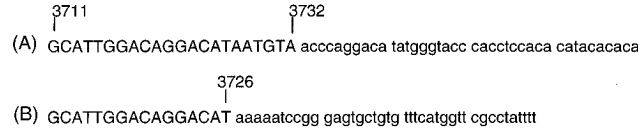

FIG. 6. Nucleotide sequences of sense strand at viral-cellular junctions in 20822 (A) and CaSki (B). The data from the sequencing of the cloned RNA PCR products are shown. For CaSki, our sequence data were consistent with those from Smits et al. (41). Sequences of ~10 nt upstream and 40 nt downstream of viral-cellular junctions are shown. The junctions in 20822 and CaSki were mapped to nt 3732 and 3726, respectively. Uppercase letters represent HPV-16 nucleotides, and lowercase letters represent cellular sequences.

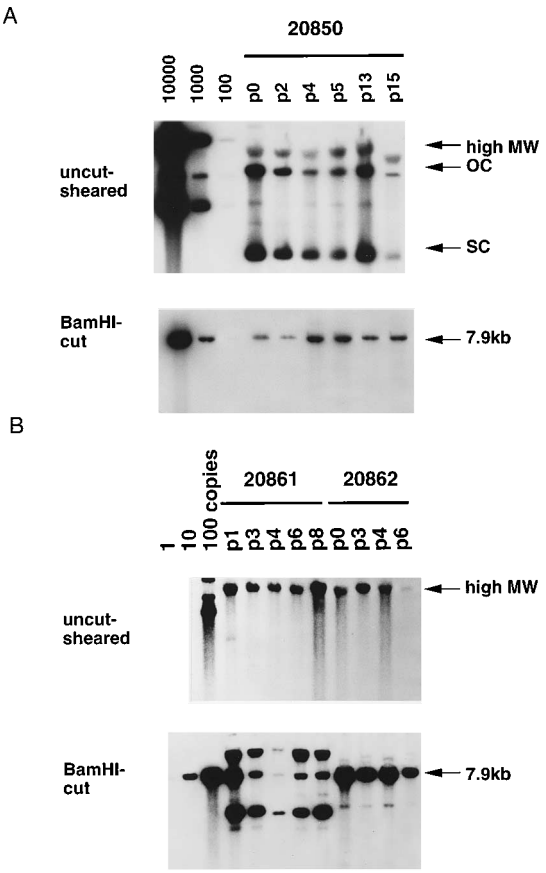

FIG. 7. Southern analyses of DNAs isolated from W12 clonal populations at different passages. Shown are autoradiographs of Southern blots of both sheared (top panels) and *Bam*HI-digested (bottom panels) total genomic DNAs from extrachromosomal clone 20850 (A) and integrated clones 20861 and 20862 (B) at different cell passages. Low levels of supercoiled HPV-16 DNA were detected in p1 20861 cells; however, this supercoiled DNA could not be detected in subsequent passages of DNAs. Arrows indicate the positions of high-molecular-weight (high MW), open-circular (OC), supercoiled (SC), and unit-length (7.9-kb) linear HPV-16 DNA.

TABLE 1. Properties of W12 clonal populations and cervical tumor cell lines[a]

Cell population	Viral copy no.[b]	E7 protein level[c]	% Plating efficiency[d]	
			100 cells	1,000 cells
Extrachromosomal (20850, 20863)	1,000	0.017, 0.12	13.7 ± 1.5	12.2 ± 0.1
Integrated				
Type 1				
20822	3	1.1	45.7 ± 4.5	37.9 ± 5.8
201402	5	0.034	33.0 ± 6.0	ND
SiHa	2	ND	ND	ND
Type 2				
20831, 20862	60	0.21, 0.48	ND	ND
20861	30	1.3	49.0 ± 4.0	28.4 ± 4.9
CaSki	600	1.0	ND	ND

[a] All quantitations were performed on a PhosphorImager (Molecular Dynamics). ND, not done.
[b] Viral genome copy number was assessed in reference to the reconstruction series on each Southern blot.
[c] Levels were corrected to equivalent trichloroacetic acid-precipitable counts in the ^{35}S-labeled lysate used for immunoprecipitations.
[d] Indicated is the percentage of the number of live cells plated (either 100 or 1,000 cells) that gave rise to colonies 2 weeks after plating. Values shown are the average values ± standard deviations of at least three independent platings for each clonal population.

doublings) analyzed. This result raised a question as to the fundamental difference between the parental and the clonal populations.

Relative growth properties of integrated versus extrachromosomal clonal populations. Collectively, the difference in stability of extrachromosomal viral DNA in the parental versus clonal W12 cell populations, and the apparently selective outgrowth of cells exclusively harboring integrated viral DNA in the parental W12 population, led us to hypothesize that cells harboring integrated viral DNA have growth advantages over those harboring extrachromosomal viral DNA. In order to test this hypothesis, a clonal population harboring integrated HPV-16 DNA (20861) was cocultured with one harboring extrachromosomal viral DNA (20863). These two populations were combined at ratios of 1:1, 1:10, and 1:100 (ratio of 20861 to 20863) and cultured for various periods, after which DNAs isolated from these cultures were subjected to Southern hybridization to assess what proportion of cells contain integrated viral DNA. The percentage of cells with integrated versus extrachromosomal viral DNA was measured by quantitating levels of junction-specific bands (on the blot with *Bam*HI-cut DNA) compared with levels of supercoiled form DNA (on the blot with uncut sheared DNA) (Fig. 8A). When the two populations were combined at a 1:1 ratio, a 25-fold increase in the ratio of integrated to extrachromosomal cells was observed within three passages (Fig. 8B). Similarly, the 1:10 ratio that more closely mimics the parental population at p15 showed a 50-fold outgrowth of cells with integrated viral DNA. With a 1:100 ratio, within the passages looked at, a delay in the reduction of extrachromosomal copies of HPV-16 DNA was observed. These results indicate that cells with integrated HPV-16 DNA have a selective growth advantage over those harboring extrachromosomal HPV-16 DNA and that the extent of the selective outgrowth depends on the starting ratio of the mixed populations.

Further analyses were performed to assess differences between clonal populations harboring extrachromosomal versus integrated viral DNA that could contribute to the growth advantage of the latter. The plating efficiency of cells harboring extrachromosomal HPV-16 DNA was ~13%, two- to threefold lower than that of cells with integrated viral DNA (Table 1). Plating efficiencies would likely contribute to differences in the above outgrowth experiment (Fig. 8A and B), given that cells were passaged several times during the time course study. In the same context, we also noted that the growth rate of colonies over time was visibly reduced for clones harboring extrachromosomal DNA (20850 or 20863 [data not shown]). These results are in concordance with those obtained from the mixed-culture experiment.

Levels of HPV-16 E7 expression in clonal populations. As indicated previously, integration has been hypothesized to cause an increased expression of the viral oncogenes E6 and E7 (11, 45). To test this hypothesis, levels of E7 protein produced in our different clonal W12 populations were measured in a radioimmunoprecipitation experiment (Fig. 9). The levels of E7 protein varied among the clonal populations. With the exception of 201402, E7 protein synthesis was greater in cell populations harboring integrated HPV-16 DNA than in those harboring extrachromosomal viral DNA. The E7 protein levels in the integrated clones, in some cases, approximated that seen in the cervical cancer cell line CaSki (Fig. 9; Table 1). Thus,

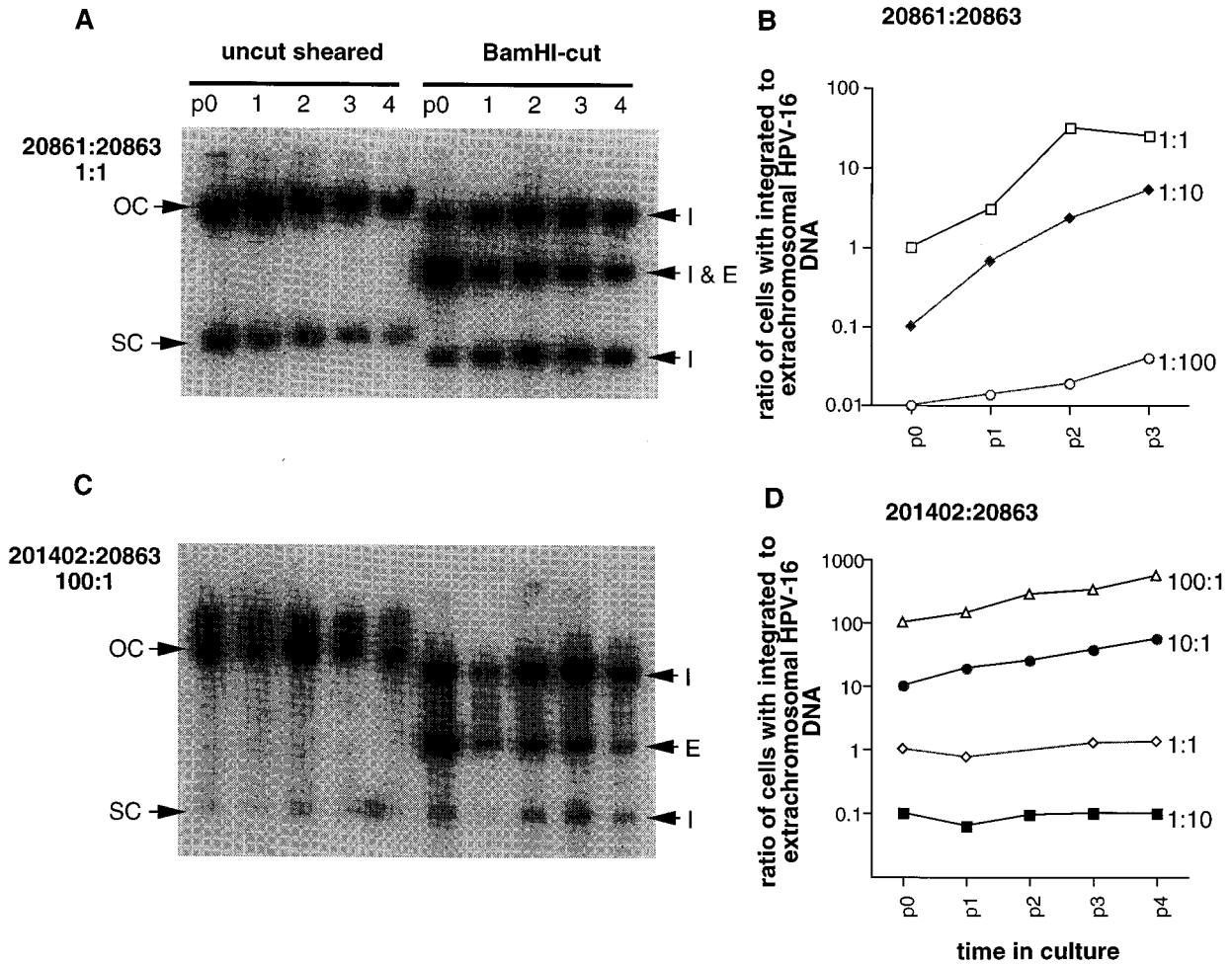

FIG. 8. Selective growth advantage of cells harboring integrated HPV-16 DNA over those harboring extrachromosomal viral DNA. (A and C) Autoradiograph of a Southern blot of DNAs isolated from cocultures of 20861 and 20863 (1:1) (A) or 201402 and 20863 (100:1) (C) at different passages. Both sheared and *Bam*HI-cut total DNAs were analyzed. Arrows indicate the positions of different *Bam*HI-generated HPV-16-specific bands: bands labeled I arise from integrated DNA, bands labeled E arise from extrachromosomal DNA, and bands labeled I & E arise from both. (B and D) Quantitation of Southern analyses performed on multiple mixed population outgrowth experiments on 20861 and 20863, in which the datum points were calculated by establishing the relative ratio of intensity (band intensities were quantitated with a Molecular Dynamics PhosphorImager) of the HPV-16-specific bands corresponding to extrachromosomal (supercoiled band on Southern blot of uncut sheared DNA) to integrated (lower I band on Southern blot of *Bam*HI-cut DNA) viral DNAs present at each passage compared with that in the initial mixed-cell population (p0) (B), or 201402 and 20863, in which datum points were calculated by comparing intensities of bands corresponding to integrated (upper I band) and to extrachromosomal (E band) viral DNAs (D). The ratios of cells with integrated to those with extrachromosomal viral DNA are graphed as a function of time in culture and are indicated on the right.

integration was highly correlative with increased levels of E7 protein synthesis.

Expression of E7 protein has been demonstrated to confer growth advantages to cells (30). In the growth advantage experiment described above (Fig. 8A and B), we found an integrated cell population (20861) to outgrow an extrachromosomal population (20863). The E7-specific radioimmunoprecipitation data (Fig. 9) raise the possibility that this growth advantage reflects the high E7 protein level in this integrated clone. Were E7 levels primarily responsible for the observed growth advantage, it would be predicted that an integrated clonal population that expresses E7 protein at a level lower than that of an extrachromosomal clone might not have such a selective growth advantage. In order to address this prediction, clone 201402, an integrated clonal population with low-level E7 expression, was combined with the extrachromosomal clonal population 20863 at various ratios and cocultured, and DNAs from various passages were analyzed as described above. Given the nature of type 1 integration that had occurred in 201402, quantitation was done by comparing the intensities of hybridization to bands corresponding to the integrated junction DNA (12 kb in size) and the unit-length viral DNA bands that arose totally from extrachromosomal viral genomes (7.9 kb in size) (Fig. 8C). At the ratios of 1:1 and 1:10 (201402 to 20863), the proportion of cells harboring integrated viral DNA appears to remain constant over a period of four passages (Fig. 8D). Thus, we did not see any growth advantage of the 201402 cells at the ratios used in the previous studies on 20861 (Fig. 8B). At higher ratios of 201402 to 20863 (100:1 and 10:1), however, a small fivefold increase in the ratio of integrated to extrachromosomal viral DNA was observed within the four passages. In a similar selective outgrowth experiment involving the coculturing of the type 1 integrant clone, 20822, which expresses high levels of E7 protein (Fig. 9), and the same extrachromosomal clone, 20863, a strong outgrowth of the 20822 cells occurred (data not shown), as seen with the other high-level E7 expression clone, 20861 (Fig. 8A and B). Collectively, these results demonstrate that E7 protein levels may contribute ex-

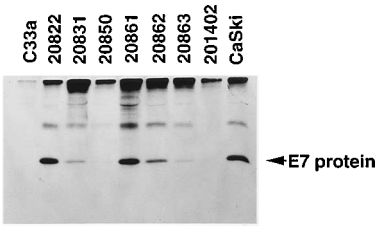

FIG. 9. Radioimmunoprecipitation of E7 protein. Shown is a fluorograph of a sodium dodecyl sulfate-polyacrylamide gel on which were resolved ^{35}S-radiolabeled proteins immunoprecipitated with an E7-specific polyclonal rabbit antibody. The same amount of protein lysate as judged by the Bradford protein assay (Bio-Rad) was loaded on the gel. C33A and CaSki are HPV-negative and -positive cervical cancer cell lines, respectively. The quantitation of E7 protein level is summarized in Table 1.

tensively to the selective growth advantage; however, other properties of integrated clones may contribute to a lesser degree. These results indicate a strong correlation between increased expression of E7 protein as a result of integration and cellular growth advantages.

Transformation and the differentiative state of clonal cell populations. While integration appears to be important in the etiology of some HPV-associated cancers, it is predicted to be insufficient for malignant transformation. This hypothesis was tested by performing the following assay. The parental population of W12 cells was demonstrated, in mouse xenografts (44) and in organotypic culture (21), to maintain the differentiative properties of a CIN I lesion. To compare the transformation state of our integrated versus extrachromosomal W12 clonal populations, cells were assayed for their abilities to form colonies when cultured in soft agar. Neither the integrated nor the extrachromosomal clonal populations were able to form colonies in soft agar (Table 2). A differentiation-defective phenotype is a common characteristic of high-grade preneoplastic and neoplastic cells. We therefore tested the abilities of clonal populations to retain replicative potential following induction of differentiation. For this purpose, cells were induced to undergo terminal differentiation by suspension for various periods in medium made semisolid with methylcellulose. The cells' capacity to maintain proliferative potential was then assessed quantitatively by recovering cells from suspension and replating them in a surface culture. Outgrowth of colonies was then quantified (Fig. 10). As demonstrated previously (34a), the HPV-negative squamous carcinoma cells, Scc13ya, were resistant to suspension-induced differentiation, whereas normal human foreskin keratinocytes (HFK) were sensitive, showing a 500-fold reduction in CFE within 24 h. A 30- to 50-fold reduction in CFE was observed with the W12 clonal populations irrespective of the genomic state of HPV-16 DNA. These data

TABLE 2. Ability of cells to form colonies in soft agar

Cell population	No. of colonies formed[a]		
	1st trial	2nd trial	3rd trial
HFK	0 (p2)	0 (p3)	0 (p3)
20831	0 (p1)	16 (p3)	0 (p16)
20850	ND	57 (p3)	0 (p4)
20861	3 (p4)	26 (p6)	4 (p13)
20862	ND	55 (p4)	4 (p10)
201402	1 (p2)	47 (p4)	0 (p15)
CaSki	3,600	4,800	TMTC[b]

[a] Indicated are the numbers of colonies formed in 0.35% soft agar 4 weeks after 10^5 cells were plated. Numbers in parentheses represent passage numbers at which cells were assayed. ND, not done.

[b] TMTC, too many to count ($>10^4$ colonies).

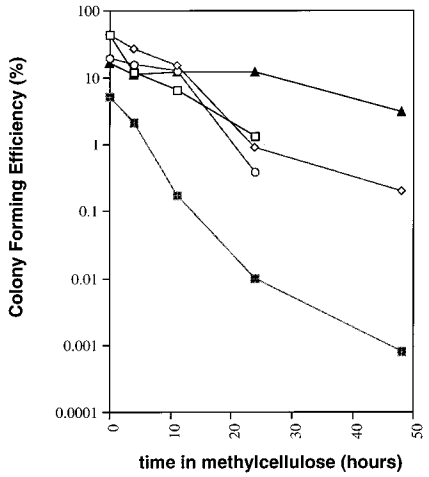

FIG. 10. Capacity of cells to proliferate upon methylcellulose-induced terminal differentiation. The graph shows CFEs of different W12 clonal populations (201402, open boxes; 20861, open diamonds; 20863, open circles) as a function of time in which cells were incubated with 1.68% methylcellulose. A squamous carcinoma cell line, Scc13ya (closed triangle), was used as a positive control, and HFK (closed boxes) served as a negative control. For 201402 and 20863, time point values after 24 h were not quantitated. Each datum point represents the average CFE value from two sets of triplicate samples (mean $\sigma = 25.1\%$) within a single experiment. The >3 orders of magnitude reduction in CFE for HFKs cultured in methylcellulose was reproducibly seen in multiple independent experiments, although the absolute CFE varied depending on the passage number of the HFKs used in a given experiment.

were similar to the fold reduction observed with parental W12 cell populations (data not shown). The absolute initial CFEs for the integrated clonal populations were threefold higher than for the extrachromosomal clonal populations; however, this likely reflects the difference in plating efficiency (Table 1). These results suggest that integration per se is not sufficient to alter the terminal differentiative properties or to induce transformation of cervical epithelial cells.

DISCUSSION

In this study, we have derived clonal populations of human cervical epithelial cells that harbor either extrachromosomal or integrated HPV-16 DNA. The genomic structure of the integrated viral DNA in these clonal populations appears very similar to those seen in cervical cancers. By comparing cellular properties of these clonal populations, we were able to demonstrate that integration of HPV-16 DNA into the human genome is highly correlative with the increased expression of the viral oncoprotein E7 and the selective outgrowth over cells harboring extrachromosomal viral DNA. We conclude that specific integration events such as those identified in this study and in cervical cancers likely provide a selective advantage to the host cells, as in cervical cancers.

Integrative disruption of the E2 gene in the clonal populations and the cervical cancer cell lines. A striking similarity was found in the patterns of viral DNA integration between our W12 clonal populations and cervical cancers. As in cervical cancers (46), two patterns of viral DNA integration were identified: in type 1, integration of a single copy of viral DNA occurred, with subsequent amplification; in type 2, integration of concatemerized HPV-16 genomes occurred, with subsequent amplification. In both our W12 clones and cervical cancers, copies of the integrated viral genome were disrupted within the E2 gene. No W12 clones containing integrated viral genomes disrupted in other regions of HPV-16 were isolated,

which suggests either that the E2 gene represents a recombinational hot spot or that a selective pressure leads to the outgrowth of cells harboring integrated, E2-disrupted viral genomes. Multiple observations made in this study support the latter hypothesis. First, we found an outgrowth of cells harboring integrated viral DNA disrupted in the E2 gene within the parental population of W12 cells (Fig. 1). Second, we found an integrated W12 clone possessing a selective growth advantage over an extrachromosomal W12 clone in a mixed-culture experiment (Fig. 8). Last, we found a strong correlation between integration and increased levels of viral E7 oncoprotein expression. Therefore, it is likely that cells harboring disruption of viral genomes disrupted in the E2 gene are selected for, at least in this tissue culture system. In other studies, we demonstrated that the increased expression of E7 in integrated clones is, at least in part, due to altered mRNA stability (22).

The mixed-culture experiment demonstrating outgrowth of cells harboring integrated DNA over those harboring extrachromosomal DNA utilized the integrated cell clone 20861. This cell population expresses E7 protein at levels 11 times greater than that of the extrachromosomal cell clone, 20863, used in this mixed-culture assay. Conversely, little growth advantage was observed when another integrated cell clone (201402) with E7 protein at levels three times lower than those of 20863 was employed in place of 20861. Thus, these observations reinforce the prediction that there is a strong correlation between E7 protein level and the observed growth advantage. Furthermore, the similar plating efficiencies of cells harboring integrated viral DNA in both 20861 and 201402 (Table 1) led us to believe that E7 protein levels play a major role in such selective growth advantages. The experiments to date do not allow us to determine the contribution of other factors to the growth advantage.

On the basis of our collective studies reported herein, we conclude that the original W12 population must have been heterogeneous in nature, having cells harboring extrachromosomal, integrated, or perhaps both forms of viral DNA. Consistent with this hypothesis, the rapid loss of extrachromosomal DNA in our Southern analyses of multiple passages of parental W12 cells (Fig. 1) was similar to that observed in our selective outgrowth experiments using mixtures of clonal populations (Fig. 8). This suggests that cells within the parental W12 population harboring extrachromosomal DNA have a selective growth disadvantage. As pointed out above, this may reflect primarily the fact that extrachromosomal clones express low levels of E7 compared with most integrated clones (Fig. 9). An alternative but less plausible hypothesis is that the parental W12 population was homogeneous in nature, but specific events in which those cells were collectively cured of large numbers of copies of extrachromosomal DNA while simultaneously integrating viral genomes occurred. This hypothesis would invoke the need for novel mechanisms for selective DNA expulsion or selective DNA degradation.

Absence of a specific integration site in the host genome. For some oncogenic viruses such as certain retroviruses and hepatitis B viruses, integration of viral genomes can provide a selective growth advantage due to altered expression of flanking cellular genes. Often such viruses are found to integrate close to specific cellular proto-oncogenes or tumor suppressor genes. In some HPV-associated cervical cancers, it has been shown that papillomaviral DNA integration occurred near the c-*myc* locus, resulting in overexpression of c-*myc* transcripts (8). Our clonal populations, however, showed neither amplification nor overexpression of the c-*myc* gene (data not shown). The flanking cellular sequences for multiple integration events in cervical cancers have been identified (46). In this study, we have identified the flanking sequences in one clonal population, 20822 (Fig. 6). No similarity or homology could be detected among these different flanking sequences or between these flanking sequences and other sequences present in the DNA sequence databases (other than highly repetitive sequences), in agreement with previous reports (12, 38, 46). Thus, our data provide further evidence that HPV integration does not occur at any specific locus on the host genome.

Requirement for high copy numbers of extrachromosomal HPV-16 genomes. The W12 clonal populations harboring extrachromosomal DNA contained extremely high copy numbers of the viral genome (Fig. 2), and these high copy numbers were maintained stably within the clonal populations for at least 100 cell generations (Fig. 7). These results suggest either that the viral extrachromosomal DNA is under a strict copy number control or that the high copy number is necessary to provide continued proliferation of these epithelial cells. Despite these high copy numbers, only low levels of E7 protein could be detected in these cell populations compared with those in the clonal populations containing a few copies of integrated viral DNA. Continued expression of E7 has been argued to be critical for the growth of cervical carcinoma cells (20, 43). Consistent with this hypothesis, we failed to isolate any clonal populations not harboring HPV-16 DNA. We therefore propose that it is necessary for extrachromosomal clones to maintain a high copy number to express E7 protein at a level sufficient to support continued cell proliferation. In the same context, the deletion of putative binding sites for the cellular transcription factor YY1 in the papillomavirus genome has been argued to cause heightened expression of E6 and E7 in cervical cancer cells that harbor extrachromosomal HPV-16 DNA genomes (27).

Integration of HPV-16 DNA does not grossly alter the properties of cervical epithelial cells. We were unable to detect differences in the cellular properties of integrated cell populations over extrachromosomal cell populations, other than the capacity for the integrated cell populations to outgrow extrachromosomal cell populations. For example, both cell populations were indistinguishable in their resistance to the induction of terminal differentiation. It is possible, however, that the integrated clones might display subtle differences in their differentiation program that are not apparent in the assay performed. Like the parental W12 cell population, both integrated and extrachromosomal clonal populations failed to form colonies in soft agar, suggesting that these cells are nontumorigenic. The parental W12 population has been demonstrated to generate virus-like particles when these cells were permitted to undergo differentiation in vivo with transplant chambers in mice (44). Likewise, when clonal populations of W12 cells were grown in organotypic cultures in vitro under the permissive culture conditions described elsewhere (28), we detected, by electron microscopy, virus-like particles in the terminally differentiating layers (21). The karyotype of these W12 clonal populations (21) also appeared to be similar to that of the parental W12 population (42); the clonal populations were near tetraploid. Thus, these clonal populations have features similar to those of the parental W12 cells, irrespective of the state of HPV-16 DNA in the cells.

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