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Random-Choice Replication of Extrachromosomal Bovine Papillomavirus (BPV) Molecules in Heterogeneous, Clonally Derived BPV-Infected Cell Lines

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Using fluorescence in situ hybridization and Southern blot analysis, we show that three clonally derived cell lines transformed with bovine papillomavirus (BPV), including ID13, the cell line commonly employed for BPV replication studies, are heterogeneous populations having extensive cell-to-cell variation in both the distribution and amount of BPV DNA. Different subclones of ID13 were found to differ in the form and amount of BPV DNA they contain. Most subclones showed no detectable BPV sequences; some contained either extrachromosomal BPV molecules distributed throughout the nucleus or BPV sequences integrated at discrete chromosomal sites, while others contained both integrated and plasmid forms. The results of density gradient analysis of BPV DNA from individual homogeneous subclones showed replication of the extrachromosomal BPV plasmids in a random-choice mode. In all cell lines studied, the presence after one round of chromosomal DNA replication of unreplicated BPV DNA and of BPV DNA having two postreplicative strands was independent of the presence of high-BPV-copy-number ("jackpot") cells. Our results substantiate the earlier conclusion that extrachromosomal BPV molecules replicate randomly and not according to a once-per-cell-cycle mechanism.

Bovine papillomavirus type 1 (BPV-1) normally infects and transforms fibroblasts and epithelial cells, causing warts in cattle, its natural host (18), but it can also replicate in and transform mouse C127 fibroblasts. Because BPV can be maintained as an extrachromosomal plasmid in C127 cells (12), it has been used extensively as a cloning vector and for studies of DNA replication in mammalian cells (for reviews, see references 11, 16, and 26). The mode of replication of extrachromosomal DNA molecules in eukaryotic cells can be either once per cell cycle in parallel with the chromosome, as is observed for Epstein-Barr virus (1, 29), or by a random-choice mechanism, as is seen for some bacterial plasmids (23). If replication is once per cell cycle, postreplicative DNA must somehow be marked to ensure that it does not replicate again until after cell division has occurred. Replication by random choice is independent of whether a particular molecule already has undergone a round of replication in that cell cycle.

These two mechanisms of replication can be distinguished by analysis of DNA that has been density labeled with the thymidine analog bromodeoxyuridine (BrdU) during replication and then centrifuged in a gradient that separates nonreplicated DNA (both strands unsubstituted; light-light [LL] density) from DNA that has replicated once (one strand substituted; heavy-light [HL] density) or more than once (both strands substituted; heavy-heavy [HH] density). After cells have passed through one S phase in the presence of BrdU, DNA species that replicate once per cell cycle will band at the HL density, whereas molecules that replicate by a random-choice mechanism will be distributed among the fractions with a theoretical distribution of 25% LL, 50% HL, and 25% HH.

Botchan et al. (4) have reported that the density labeling pattern resulting from continuous labeling of BPV in a nonsynchronous culture of the cell line ID13, which is derived from BPV-transformed mouse fibroblasts, parallels that of chromosomal DNA, which is known to replicate once per cell cycle, and have thus concluded that BPV also replicates in a once-per-cell-cycle mode. However, using ID13 and two additional BPV-transformed cell lines, Gilbert and Cohen (9) found by pulse-labeling and density gradient analysis that half of the BPV DNA isolated from mitotic cells that had been labeled for one S phase banded at the HL density, while up to 25% banded at the HH density and a similar amount banded at the LL density, as predicted by theoretical calculations for random-choice replication. In the same gradients, chromosomal DNA sequences banded at the HL density, characteristic of one round of replication.

To account for these differing results, Roberts and Weintraub (22) noted that cultures of BPV-transformed cells include rare "jackpot" cells (0.25 to 0.5% of the population), which contain a higher-than-average number of BPV copies (also seen in cell lines infected with BPV-2 [17]). They hypothesized that the BPV molecules in these jackpot cells may be undergoing runaway replication analogous to that occurring during lytic growth of simian virus 40 (SV40) and suggested that such replication in a small fraction of the cell population may account for the presence of HH DNA after just one S phase. Jackpot cells that had died prior to the period of BrdU labeling could, it was proposed, account for the LL DNA observed in density gradients. Together these events could give the appearance of random replication in a population in which once-per-cell-cycle replication of BPV is the normal and predominant mechanism.

Since the suitability of BPV as a model for chromosomal DNA replication depends upon the assumption of once-per-

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cell-cycle replication, we undertook to determine experimentally the effect of jackpot cells on density labeling analyses of the mode of replication. In the course of these experiments, we discovered that the location and copy number of BPV molecules vary widely among individual cells of the extensively employed clonal mouse cell line ID13. We further show that jackpot cells do not account for the random-mode density labeling patterns observed in the overall population of BPV-transformed cells and confirm that the normal mode of replication of extrachromosomal BPV molecules is in fact by a random-choice mechanism.

MATERIALS AND METHODS

Cell culture. Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin, and streptomycin at 37°C and 10% CO_2. Density labeling was achieved by addition of 30 μg of BrdU per ml to the cell cultures for 12 h. Subcloning was by limiting dilution to 0.2 cells per well in 96-well plates in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum, penicillin, and streptomycin.

Mitotic selection. Mitotic selection was performed by firmly tapping T-175 flasks containing a small amount of medium (10 ml) to dislodge loosely attached mitotic cells. At least 70% mitotic figures were present in each selection. The percent mitotic cells was determined by suspending a small aliquot of cells in 0.05 M KCl at 37°C for 10 min, fixing them in three washes of 3:1 methanol-acetic acid, dropping the cells on microscope slides, and counting the mitotic figures. Preshaking was done similarly to mitotic selection, but with firmer tapping to dislodge all cells that were loosely attached.

Density gradients. DNA was isolated from cells by resuspending the cell pellet in 1 ml of DNA extraction buffer (1% sodium dodecyl sulfate [SDS]–100 mM Tris-HCl [pH 8.0]–200 mM EDTA–100 μg of proteinase K per ml) and incubating the suspension at 56°C for 2 h. Samples were extracted with equal volumes of phenol, 1:1 phenol-chloroform, and 24:1 chloroform-isoamyl alcohol. A 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol were added, and the DNA was spooled with a glass rod, rinsed in 70% ethanol, air dried, and resuspended in Tris-EDTA with 10 μg of RNase A per ml. After incubation for at least 1 h at 37°C, the DNA was reprecipitated in ethanol and resuspended in Tris-EDTA. Replicated DNA was separated from unreplicated DNA on Cs_2SO_4 gradients as previously described (9). Briefly, DNA from approximately 10^7 cells was cut with *Eco*RV, loaded on a Cs_2SO_4 gradient with a refractive index of 1.3715, and centrifuged at 30,000 rpm in a Beckman VTi80 rotor at 19°C for 72 h. Eight-drop fractions were collected from the bottom of the centrifuge tube with a peristaltic pump and analyzed by the slot-blot methods of Brown et al. (5). Hybridizing sequences were detected by nonradioactive methods (PhotoGene System from Life Technologies, Inc.). Probes were labeled with biotin-14-dATP by using the Bio Nick labeling system (Life Technologies). The probes used were pdBPV-1 (24), which contains the entire BPV genome cloned into pML2d, and pCHOR32 (6), which contains a hamster *Alu*-like sequence that is homologous to a mouse repetitive sequence dispersed throughout the mouse genome. The amount of hybridization was measured with a Helena Laboratories Quick Scan R&D densitometer.

FISH. Fluorescence in situ hybridization (FISH) combining the techniques of Pinkel et al. (19) and Lawrence et al. (13) was performed. For metaphase analysis, colcemid was added to the cell culture at 0.015 μg/ml for 2 h before harvesting. Cells were fixed in 3:1 methanol-acetic acid, dropped on slides, air dried overnight, and then baked in a 65°C oven for 3 to 4 h. The slides were incubated in 100 μg of RNase A per ml in 2× SSC (1× SSC = 0.15 M NaCl–0.015 M sodium citrate) for 1 h at 37°C, rinsed in 2× SSC, incubated in 0.1 M triethanolamine–0.25% acetic anhydride for 10 min at room temperature, rinsed in 2× SSC, denatured in 70% formamide–2× SSC at 70°C for 2 min, dehydrated in an ice-cold ethanol series (70, 90, and 100%) for 5 min each, and air dried.

Probes were prepared by nick translation with the Bio Nick labeling system. Biotinylated probe and sonicated salmon sperm DNA were added to a hybridization mix to give final concentrations of 50% formamide, 10% dextran sulfate, 2× SSC, and 4 μg of probe and 50 μg of sonicated salmon sperm DNA per ml. The hybridization mix was denatured by incubation at 70°C for 10 min followed by rapid cooling on ice. Hybridization mix (20 μl) was placed on each slide and incubated at 37°C overnight in a humidified chamber. Washes were for 15 min each in 50% formamide–2× SSC at 45°C, 2× SSC, and 1× SSC at room temperature.

Hybridized sequences were detected by incubating the slides in 5 μg of fluorescein-avidin DN isothiocyanate (Vector Laboratories) per ml in 4× SSC–1% bovine serum albumin for 30 min at 37°C and rinsing them at room temperature for 10 min each in 4× SSC, 4× SSC–0.1% Triton X-100, and 4× SSC. Slides were stained for 5 min in 0.1 μg of propidium iodide per ml in 4× SSC and mounted in antibleach mounting medium (10). Fluorescence was detected with a Zeiss Photomicroscope III.

Southern blots. BPV DNA was analyzed by isolating total DNA as described above. Uncut DNA (1 μg) was run on a 0.7% agarose gel, blotted to nitrocellulose, and hybridized to nick-translated pdBPV-1 by standard procedures (15). Hybridization was carried out at 68°C in 2× SSC with the final wash at 68°C in 1× SSC–0.1% SDS.

Cell sorting. Dead cells were removed from mitotic cell preparations by resuspending the cells in medium with 50 μg of propidium iodide per ml, which is taken up by dead cells, and collecting live (unstained) cells on a Coulter Epics V dual laser cell sorter.

RESULTS

Jackpot cells do not affect density labeling results. It has been suggested that jackpot cells, as a consequence of runaway BPV replication, are loosely attached dying cells that contaminate preparations obtained by the mitotic shake-off procedure (22). It was further proposed that jackpot cells that have died before the addition of BrdU cannot incorporate the density label; the BPV from these cells consequently would appear in the LL fraction of the gradient, whereas rapidly replicating BPV molecules in the still-living jackpot cells would incorporate the density label and thus appear in the HH fraction. We have carried out two separate types of experiments to determine whether dead cells in actively growing ID13 populations significantly affect density labeling results (Table 1). First, we found that removal of dead and loosely attached cells by vigorous preshaking of the plates prior to addition of BrdU did not alter the density distribution of BPV DNA; second, removal of dead cells by cell sorting after BrdU labeling plus mitotic shaking had no effect on the distribution of BPV DNA. These results do not support the notion that the LL BPV DNA observed in

TABLE 1. Quantitation of HH, HL, and LL DNA from density gradients

Cells	% Hybridization to DNA from[a]:					
	BPV			Chromosome		
	HH	HL	LL	HH	HL	LL
Preshaken[b]	12	54	34	0	75	25
Not preshaken	12	57	31	0	73	27
Total mitotic ID13	19	62	18	0	76	24
Live mitotic ID13[c]	10	63	26	0	76	24
Total mitotic clone B	14	57	29	0	71	29
Live mitotic clone B	16	48	36	0	84	16

[a] Numbers are from densitometric quantitation and represent the hybridization to each DNA peak as a percentage of total hybridization to the gradient.
[b] Flasks of clone B cells were shaken vigorously to remove dead and loosely attached cells prior to addition of BrdU and mitotic shaking.
[c] Mitotic preparations of ID13 and clone B were stained with propidium iodide, and live (unstained) cells were collected by cell sorting.

density gradients after labeling with BrdU throughout one S phase is a consequence of dead cells in the population.

To further investigate the possible effect of jackpot cells on the density labeling results, the three cell lines shown by Gilbert and Cohen (9) to have identical replication patterns were studied by FISH. The cell line ID13 was clonally derived from mouse C127 cells infected with wild-type BPV-1 virus (7). Clones B and D are relatively recent isolates of BPV-1-infected C127 cells (9).

Extensive variability in both the intensity and the distribution of the fluorescence signal was observed by FISH analysis of interphase nuclei of all three cell lines (ID13 and clone B shown in Fig. 1A and B); the patterns of fluorescence included no detectable signal, a faint to bright diffuse signal, one to many distinct fluorescent spots ranging in size from small to very large, and a combination of distinct spots plus a diffuse fluorescence signal in the same cell. Most cells with two or more distinct spots also contained a diffuse signal. The extent and nature of cell-to-cell variability in BPV content and distribution observed by FISH analysis were characteristic of a continuum and made the grouping of cells into distinct subpopulations impractical. An upper limit for the copy number of BPV in ID13 cells was estimated by comparison of the hybridization signal of BPV in the brightest cells seen in the ID13 population with that of lytically replicating SV40 in COS7 cells (Fig. 1D and E). The brightest signal observed for BPV was much less intense than the signal observed for SV40, suggesting a BPV copy number substantially lower than the 10,000 to 100,000 copies estimated for SV40 (22). To evaluate most rigorously the possible role of jackpot cells in the previously observed density labeling results, we defined jackpot cells liberally as all cells that exhibited any diffuse fluorescence over the nucleus whether or not distinct spots were present. Using this definition, we found that cell lines ID13 and clones B and D, all of which show identical patterns of density labeling consistent with a random-choice mode of replication (9), differ markedly in their numbers of jackpot cells (Table 2).

ID13 is a heterogeneous cell line. Cell lines transformed with wild-type BPV-1 previously have been found to contain some combination of supercoiled monomers and multimers as well as integrated forms of BPV DNA (2, 12, 25). The FISH results described above demonstrate that in addition to this size heterogeneity, there is considerable variability in spatial localization and amount of BPV DNA from cell to cell within three different clonally derived cell lines. Consistent with the evidence that BPV sequences can integrate into genomic DNA, 63% of metaphase spreads of ID13 examined by FISH showed the BPV hybridization signal associated with a mouse chromosome (Fig. 1C). Many different mouse chromosomes appeared to be involved, including one metacentric chromosome that was present in about half of the cells. Although these BPV insertions were not mapped cytogenetically, the association of BPV DNA sequences with chromosomes was proportional to the chromosomal size distribution in the ID13 cell line (data not shown), implying that the association is random.

To investigate further the characteristics of BPV heterogeneity, subclones of ID13 were isolated and the location of the BPV hybridization signal in individual subclones was determined by both FISH and Southern blotting. Southern blots performed on uncut DNA from 119 randomly selected subclones revealed four types of BPV hybridization patterns: 60 had no detectable BPV-hybridizing sequences under conditions estimated conservatively to be able to detect five or fewer copies of BPV per cell, 9 had predominantly autonomous BPV, 33 showed hybridization only to high-molecular-weight (MW) DNA corresponding to the gel position of genomic DNA, and 17 had both autonomous and higher-MW forms of BPV. Examples of each type of pattern are shown in Fig. 2.

While FISH analysis of randomly selected subclones showed essentially no cell-to-cell variability of the BPV signal within 24 of 27 subclones, great differences between different subclones were observed. Three general types of subclones were seen: those showing no detectable signal by FISH analysis (20 subclones), those showing a constant number of hybridization loci per cell within the subclone (4 subclones), and those showing a variable signal from cell to cell within the subclone (3 subclones). Cells previously described as jackpot cells were found only in the last category of subclones. The four subclones that showed a constant number of hybridization loci per cell uniformly showed an association of the BPV signal with a specific chromosome in metaphase spreads. The site of the BPV-specific hybridization signal in cells within a particular subclone was always the same, whereas different subclones showed BPV signals associated with different chromosomal sites.

Comparison of Southern blot data with the FISH characteristics of selected individual subclones showed a strong and consistent correlation in the type of signal observed (Table 3). Ten of 12 subclones containing only high-MW BPV-hybridizable sequences by Southern blotting showed a single area of fluorescence associated with a particular chromosome by FISH analysis. All subclones having predominantly autonomous BPV DNA or no detectable BPV by Southern blotting showed no FISH signal, and 8 of 11 subclones containing both autonomous and high-MW BPV DNA showed variable FISH signals. The observed association of high-MW BPV DNA with chromosomal DNA and the appearance of BPV hybridization with the same chromosome in all of the cells within a subclone together suggest that the BPV sequences in these cells are chromosomally integrated. Because the copy number of autonomous BPV on Southern blots is not significantly lower than that of the integrated sequences in some subclones visualized by FISH, the absence of a FISH signal in subclones containing only supercoiled BPV DNA suggests that the BPV molecules in these cells are distributed throughout the nucleus without significant clumping. Although the threshold of detection of

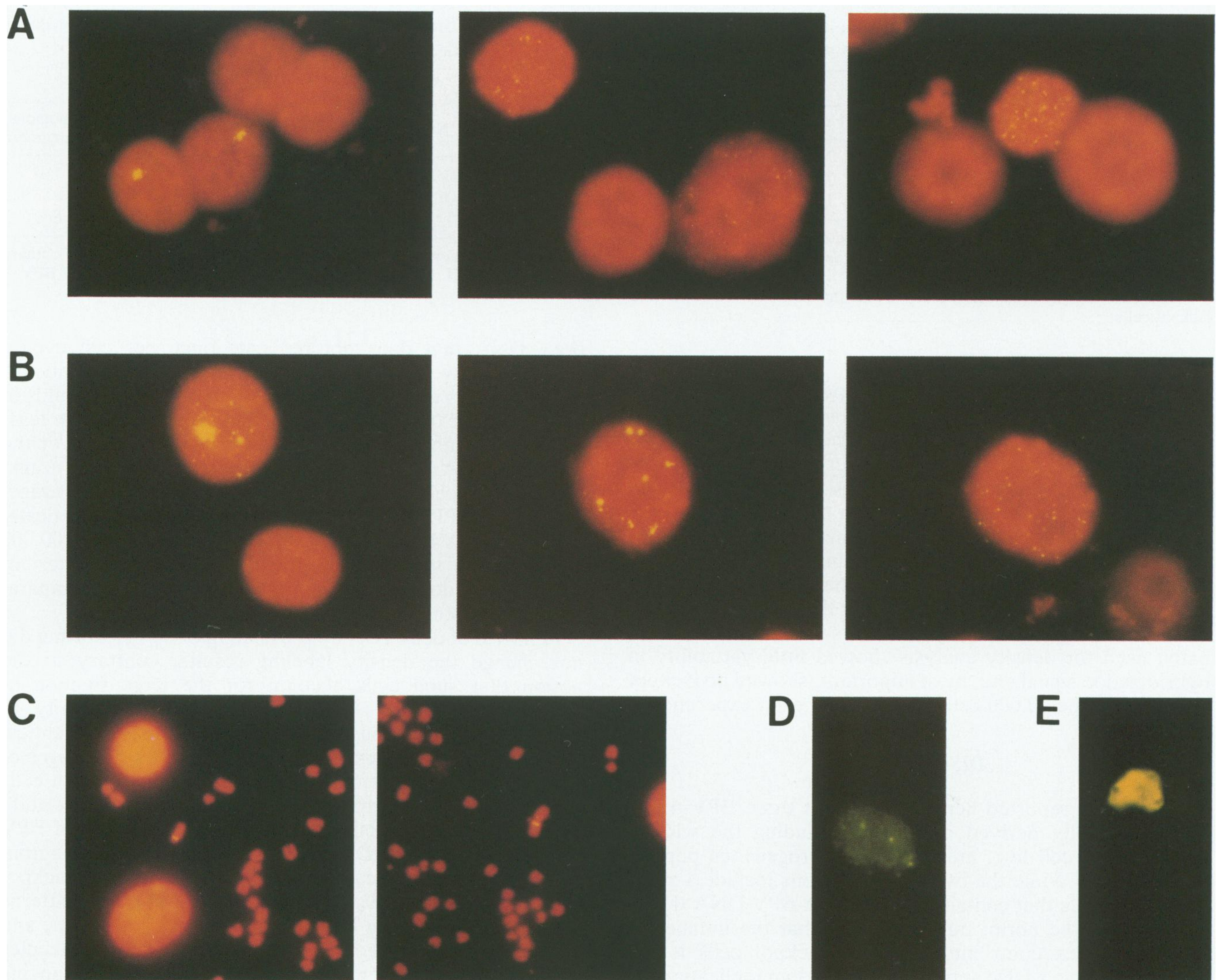

FIG. 1. Copy number and distribution of BPV in ID13 and clone B cells. Logarithmically growing cells were fixed, and BPV sequences were detected with FISH. (A and B) ID13 and clone B interphase nuclei, respectively, demonstrating the range of hybridization signals observed including a single locus of fluorescence, faint and bright diffuse fluorescence, and a combination of spots and a diffuse signal. (C) Mitotic spreads from ID13 showing association of BPV DNA with different mouse chromosomes. (D) Bright ID13 jackpot cell. Photographic exposure was for 2 min. (E) Lytically replicating SV40. Photographic exposure was for 15 s. Nuclei in panels A through C were stained with propidium iodide; cellular DNA in panels D and E was unstained.

FISH with the conditions used has not been precisely determined, an approximately 100-kb alpha satellite repeat on human chromosome 7 (27) is easily seen under the same conditions, suggesting that any aggregate of 13 or more of the 7.9-kb BPV plasmids together would have been detected.

The extent of variability in the BPV hybridization signal within individual ID13 subclones was assessed further by FISH analysis of at least 20,000 cells from each of several subclones. The following results were obtained: three subclones containing only supercoiled monomer BPV molecules showed almost no variability of the fluorescence signal; no cells resembling jackpot cells (i.e., cells showing diffuse fluorescence over the nucleus) were detected in the more than 60,000 individual cells from the three subclones observed. Jackpot cells also were not detected in a total of 40,000 cells observed from two subclones containing only integrated BPV molecules. However, jackpot cells were found at a relatively high frequency (about 1%) in two of the seven subclones that contained both autonomous and integrated BPV DNA. These subclones, which showed great cell-to-cell variability in the FISH signal, had multiple small and large fluorescent spots as well as bright diffuse hybridization over the nucleus.

Replication of autonomous BPV is by a random-choice mechanism. To investigate the possible effect of the observed heterogeneity of BPV location and amount within the cell line ID13 on the analysis of BPV replication mode, the experiments carried out by Gilbert and Cohen (9) using the heterogeneous cell lines ID13, clone B, and clone D were repeated with two ID13 subclones that showed BPV homogeneity by both Southern blotting and FISH analysis. In both of the subclones chosen for study, BPV was present in only the extrachromosomal form; additionally, no jackpot cells were detected during examination of a total of 40,000

TABLE 2. Number of jackpot cells found by FISH in three separate cell lines transformed with wild-type BPV-1

Cell line	No. of cells counted[a]	Jackpot cells	
		No.[b]	% of total
ID13	40,500	74	0.2
Clone B	36,500	22	0.06
Clone D	33,000	17	0.05

[a] The number of total cells counted was estimated by counting the cells in every fifth scan across the slide and multiplying the average by the number of scans done.

[b] Cells showing any diffuse fluorescence over the nucleus were counted as jackpot cells.

TABLE 3. Correlation of FISH results with Southern blot data for selected subclones

FISH result	Southern blot result[a]			
	No signal	Integrated	Autonomous	Autonomous and integrated
No signal	13	2	3	1
Single locus	0	10	0	2
Variable	0	0	0	8

[a] The values shown represent the number of subclones found by Southern blotting and FISH analysis to contain each of the indicated forms of BPV.

cells each (see above). In each experiment, the freshly thawed cells subjected to density labeling were examined concurrently by Southern blotting and FISH in order to verify that only supercoiled monomer (i.e., extrachromosomal) BPV molecules were present. As shown in Fig. 3 and Table 4, about one-fifth to one-quarter of the BPV-hybridizable sequences in each of the subclones banded at the HH density following density labeling during a single cell cycle, indicating that some of the extrachromosomal BPV molecules replicated more than once during the cell cycle used for density labeling; FISH analysis of cells taken from the same batch used for density analysis showed little variability in hybridization signal and, most important, showed no jackpot cells among the 20,000 cells examined in each experiment.

DISCUSSION

The results reported here indicate that three BPV-transformed clonally derived cell lines, including the widely-studied ID13 cell line, are actually heterogeneous populations of cells. While the overall populations include a small number of cells that contain an amount of BPV DNA that is greater than the norm, our data show that the number of BPV molecules contributed by such jackpot cells is not sufficient to give a false appearance of random replication, as has been suggested by Roberts and Weintraub (22). To account for the nearly 50% of the DNA in the density shift experiments that bands at either the HH or LL densities (i.e., DNA that does not replicate once per cell cycle), jackpot cells would have to contain, on average, a number of BPV plasmids equivalent to the total number of plasmids in the remainder of the population. This would require at least a 1,000-fold amplification of BPV in jackpot cells over the average copy number of BPV (about 100 in BPV-transformed C127 cells [12]) or 100,000 copies per cell on average. Side-by-side FISH comparison of jackpot cells with lytically replicating SV40, estimated to contain 10,000 to 100,000 copies of the virus (22), shows that even the brightest of jackpot cells does not yield a hybridization signal comparable to that seen for lytically replicating SV40.

Removal of dead cells by preshaking or by cell sorting did not change the density labeling results, contrary to the notion that such cells account for the large fraction of (nonreplicating) BPV DNA that bands at the LL density. In addition, three previously studied cell lines having identical BPV replication modes (9) showed different percentages of jackpot cells, consistent with the view that the jackpot cells present in normally growing cell populations do not significantly affect density labeling results during studies of BPV replication. Finally, ID13 subclones containing only autonomous BPV sequences and lacking any detectable jackpot cells whatsoever still yielded density labeling patterns closely approximating the ratio of 25% LL, 50% HL, and 25% HH that is theoretically predicted from molecules replicating by a random-choice mechanism. Thus, changing the fraction of jackpot cells or removing them entirely from

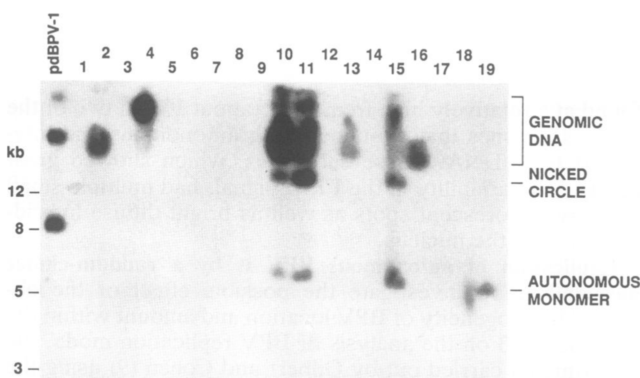

FIG. 2. ID13 subclones containing various forms of BPV DNA. Total DNA was isolated from subclones of ID13 and analyzed on a Southern blot. The DNA was uncut, and BPV sequences were detected with pdBPV-1. Autonomous and integrated BPV sequences are indicated. The marker lane contains 1 ng of uncut pdBPV-1; lane numbers represent individual subclones.

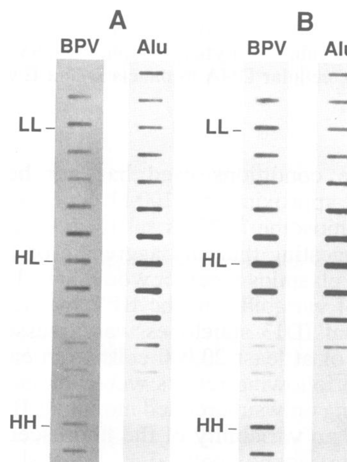

FIG. 3. Density labeling of autonomous BPV plasmids. Subclones ID13-3 (A) and ID13-70 (B) were density labeled as described in Materials and Methods. Cs_2SO_4 density gradient fractions were probed with BPV or mouse *Alu*-like probes. The positions of the LL, HL, and HH DNA are indicated.

TABLE 4. Random-choice replication of autonomous BPV plasmids

Cell line and expt no.[a]	% Hybridization of DNA from[b]:					
	BPV			Chromosome		
	HH	HL	LL	HH	HL	LL
ID13-3						
I	27	47	26	0	75	25
II	27	49	24	0	68	32
III	22	58	20	0	87	13
ID13-70						
I	17	51	32	0	82	18
II	23	55	22	0	89	11

[a] I, II, and III are three separate labeling experiments performed at different times.
[b] Percentages were determined as described in Table 1, footnote *a*.

the cell population does not alter the density labeling pattern responsible for the conclusion that normal replication of extrachromosomal BPV occurs by a random-choice mechanism. Consistent with the finding that jackpot cells are not responsible for the presence of HH and LL BPV DNA in density gradients after one cell cycle is recent evidence (20) suggesting that the unusually high copy number of BPV in jackpot cells results from abnormal partitioning of BPV rather than "runaway" lytic-type replication.

We are still left with a discrepancy between the results obtained in our laboratory and those reported at a papillomavirus symposium (4), in which once-per-cell-cycle replication of BPV was observed in a continuous labeling experiment with nonsynchronously growing ID13 cells. To ensure that our results were not simply due to differences in experimental design, continuous labeling experiments were performed with the cell lines ID13, clone B, and clone D. Again, the results were consistent with a random-choice mechanism for the replication of BPV plasmids and not with once-per-cell-cycle replication. HH BPV DNA appeared more rapidly than did HH chromosomal DNA, while LL BPV DNA persisted longer than LL chromosomal DNA (8). In our experience, results consistent with once-per-cell-cycle replication of BPV have been obtained only for BPV plasmids that have integrated into the host chromosome (21).

BPV previously has been found to exist in different intracellular states in cells infected with the wild-type virus; these include supercoiled extrachromosomal monomers, multimeric plasmids, and integrated multimers (2, 25, 28). Considerable heterogeneity among different BPV-transformed cell lines has been observed, and Bostock and Allshire (3) have shown that the method used to introduce BPV-based cloning vectors into the cell can influence whether the vector DNA remains autonomous or integrates into the genome. It has been suggested that once a cell line is established, the copy number and state of the BPV molecules remain stable (16). However, few reports have examined stability on a cell-to-cell basis rather than in the population as a whole. Moar et al. (17) found with isotopic in situ hybridization to bovine cells infected with BPV-2 that 0.02 to 0.1% of the cells contained large amounts of viral DNA while the majority of the cells were indistinguishable from background. The results reported here confirm and extend that observation with more sensitive FISH methods. We show that there is in fact a continuum of BPV copy number, as reflected by the variability of the FISH signal. We also show that BPV molecules can integrate into the host chromosomes at apparently random sites throughout the genome.

The observation that a large percentage of the subclones of ID13 contained no detectable BPV sequences while others contained large amounts of BPV DNA was surprising and is indicative of the instability of the BPV DNA in cells grown in culture. Lusky and Botchan (14) found that subclones of cell lines derived from transformation of C127 cells with a low-copy-number BPV-based vector all showed equivalent copy numbers per cell. The apparently conflicting results may result from the difference between the wild-type BPV contained in ID13 and the mutant vector used in the studies of Lusky and Botchan. The demonstration of the heterogeneity of the FISH signal in three separately isolated clonally derived cell lines and the observation that even the newly isolated ID13 subclones described here give rise to cells without detectable BPV (data not shown) support the idea that the replication and segregation of BPV molecules in cultured cells are not perfectly controlled processes.

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