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Expression of a Human U1 RNA Gene Introduced into Mouse Cells via Bovine Papillomavirus DNA Vectors

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We introduced a gene for human U1 small nuclear RNA, HU1-1, into mouse C127 cells via bovine papillomavirus (BPV) vectors. After transfection, up to 15% of the total U1 RNA in transformed cells was encoded by the introduced human genes. High levels of expression of the human gene were observed when the recombinant viral DNAs were maintained either as plasmids or after integration into high-molecular-weight DNA. As few as 400 and 35 base pairs of 5′ and 3′ flanking region sequences, respectively, were sufficient for transcription of human U1 RNA, and no increase in the level of expression was observed with HU1-1 DNA containing several kilobases of flanking region sequences. Several of the transformed cell lines contained the recombinant BPV DNA apparently integrated into the host genome. Integration or rearrangement or both of the U1-BPV DNA was promoted when the HU1-1 gene was positioned at the *Bam*HI site downstream of the BPV transforming region. At least two variants of the U1-BPV DNAs were able to cause morphological transformation of cells despite the fact that these DNAs lacked a BPV transcriptional enhancer element.

In humans, U1 RNA is encoded by a large multigene family comprising about 30 (20) or 100 to 125 (6, 22) gene copies per haploid genome. The sequence of this RNA is conserved among higher eucaryotes (2, 3), in keeping with its apparent role in the splicing of pre-mRNA (14, 16, 17, 24, 27, 32, 41). Human U1 RNA genes are homologous in sequence for several kilobases (kb) in both the 5′ and 3′ directions (12, 20, 22; L. Bernstein, T. Manser, and A. Weiner, submitted for publication). This homology between the human U1 RNA gene flanking sequences suggests that extensive regions might participate in the mechanism or control of U1 RNA synthesis. However, we have recently shown that 231 and 35 base pairs (bp) of 5′ and 3′ flanking region sequences, respectively, are sufficient for faithful transcription of a cloned human U1 RNA gene, HU1-1, in *Xenopus laevis* oocytes (25, 36).

To study the transcription of the human U1 RNA gene in mammalian cells, we incorporated it into bovine papillomavirus (BPV) DNA vectors which we then transfected into mouse cells; the multicopy plasmid form of BPV DNA has been shown to support transcription of several other exogenous genes (7, 8, 15, 28, 35, 42). We were able to monitor expression of the human HU1-1 gene in mouse cells because human and mouse U1 RNAs were distinguishable by both their electrophoretic mobilities and RNase T_1 fingerprints (19).

We report here that the human U1 RNA gene is efficiently expressed in U1-BPV transformed cells both when present extrachromosomally (as stably maintained multicopy plasmids) and when integrated into the mouse genome. We found, however, that the presence of a transcriptionally active, 0.6-kb HU1-1 DNA fragment in certain positions of the BPV vector DNA frequently resulted in rearrangements or integration or both of the recombinant BPV DNAs in transformed cells. The inclusion of several kb of flanking region sequences of the human gene diminishes the occurrence of these rearrangements. Some of the HU1-BPV chimeric DNAs are able to cause morphological transformation of mouse C127 cells even in the absence of an enhancer element (4, 21) normally located at the end of the early gene region. The significance of these findings is discussed with respect to the structure of the promoter region of the U1 RNA gene and the regulatory elements of the BPV genome.

MATERIALS AND METHODS

Construction of U1-BPV hybrid DNA molecules. The composite vectors $pBPV_{69T}$ (35) and pdBPV-1 (34) were kindly provided by P. Howley, National Institutes of Health, Bethesda, Md. The plasmids pHU1-1D and pHU1-1HB, carrying the human U1 RNA gene HU1-1, are described in detail elsewhere (20). The 0.6-kb fragment of pHU1-1D (U1D) was inserted into two derivatives of BPV DNA; the physical maps of the hybrid U1-BPV DNA molecules are shown in Fig. 1 and 4.

In one series of experiments, the human DNA was linked to the 69% transforming fragment of BPV-1 DNA (BPV_{69T}) following the scheme used by Sarver et al. (35). The 0.6-kb *Bam*HI fragment of pHU1-1D was converted to a *Hin*dIII-*Bam*HI fragment by the addition of *Hin*dIII linkers after cleavage with *Rsa*I; this fragment was then ligated to the *Hin*dIII-*Bam*HI-fragment of $pBPV_{69T}$ via the *Hin*dIII sites. The $U1D\text{-}BPV_{69T}$ DNA was inserted into *Bam*HI-cleaved pBR322 DNA and cloned by transformation of *Escherichia coli* HB101. Similarly, the 5-kb *Hin*dIII-*Bam*HI fragment of pHU1-1-HB (U1HB) was linked via the *Bam*HI site to the BPV_{69T} fragment before ligation with *Hin*dIII-cleaved pBR322 DNA.

In the other series of experiments, the U1D fragment was inserted in both orientations into the two *Bam*HI sites and the *Hin*dIII site of the pdBPV-1 vector. When the map of pdBPV-1 is drawn as in Sarver et al. (34), the two *Bam*HI sites are located at the 5 and 7 o'clock positions; we refer to these sites as B5 and B7, respectively (see Fig. 4). The recombinant plasmids were designated pU1D-BPV-B5, pU1D-BPV-B7, and pU1D-BPV-H. The orientation of the HU1-1 gene is indicated as either "s" or "o" to denote

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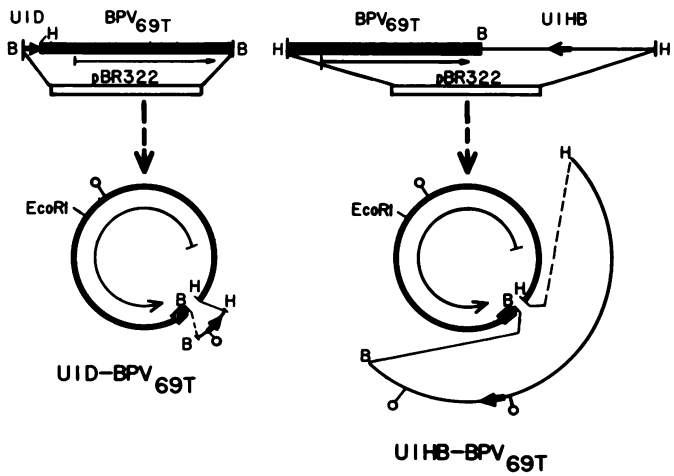

FIG. 1. Structure of U1D-BPV$_{69T}$ and U1HB-BPV$_{69T}$ recombinant DNAs. The human U1 DNA fragments, U1D and U1HB, were ligated to the 69% transforming fragment of BPV-1 DNA (BPV$_{69T}$) and to pBR322 DNA to generate pU1D-BPV$_{69T}$ and pU1HB-BPV$_{69T}$. Before use in transfection of C127 cells, the recombinant DNAs were treated with *Bam*HI or *Hin*dIII to release pBR322 DNA sequences. The plasmids that would be generated by intracellular ligation (dotted lines) of the linear transfecting DNAs are shown in the lower portion of the figure. The position and direction of transcription of the U1 RNA coding region are indicated by the heavy arrow. BPV transcripts are represented by the thin arrow, and the hatched box indicates the BPV enhancer element. B, *Bam*HI; H, *Hin*dIII; ♀, *Bgl*II.

whether transcription of the U1 RNA gene would be in the same or opposite direction as that of BPV early genes. *Hin*dIII linkers were added to the *Bam*HI-generated termini of the U1D and HB fragments to allow insertion into *Hin*dIII-digested pdBPV-1 DNA.

Plasmid DNAs used for the transfection experiments were propagated in *E. coli* HB101 or the *dam* GM33 (23) or GM2163 strains (obtained from G. Marinus). The lack of adenine methylation in plasmid DNAs isolated from GM33 or GM2163 cells was verified by cleavage with *Bcl*I restriction endonuclease. Plasmid DNAs were isolated from *E. coli* by the Triton X-100 (13) or alkaline lysis (1) method, and supercoiled DNAs were twice purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients.

Transfection and establishment of transformed cell lines. Mouse C127 cells (18), generously provided by P. Howley, were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Transfections with linearized U1-BPV$_{69T}$ or circular pU1-BPV DNAs were carried out by the protocols of Wigler et al. (40), using the calcium phosphate precipitation method (11) with a 20 to 25% glycerol shock (10). Before transfection, pU1-BPV$_{69T}$ recombinant DNAs were cleaved with *Hin*dIII or *Bam*HI to separate pBR322 DNA from U1-BPV$_{69T}$ DNA; pdBPV-1 recombinant DNAs were used without prior cleavage. About 12 to 18 days after transfection, the foci of morphologically transformed cells were counted and individual foci were picked and expanded into cell lines. Pure lines of transformed cells were obtained by growth in soft agar or by isolation of colonies derived from endpoint dilution.

The plasmid and chromosomal DNAs of transformed cells were separated by equilibrium centrifugation in cesium chloride-ethidium bromide gradients. Plasmid DNAs were recovered from the portion of the gradient where supercoiled DNAs band, as estimated by comparison with a control gradient containing a visible band of plasmid DNA; linear fragments of chromosomal DNAs were also collected. Approximately 20 ng of episomal DNA or 10 μg of total chromosomal DNA was used for transfection of C127 cells.

Analyses of cellular RNA and DNA. The labeling of cells with $^{32}P_i$ (29) and subsequent isolation and analysis of total U1 RNAs and U1 RNA containing ribonucleoprotein particles (U1 snRNPs) (17) were carried out as previously described (19). The preparation of total cellular DNA (33) and the analyses by genomic blot hybridization (37, 38) have also been described elsewhere in detail (20). Total cellular DNA was sheared by passage 10 times through a 22 gauge needle.

^{32}P-labeled DNAs to be used as hybridization probes were synthesized by nick translation (31) in the case of BPV$_{69T}$ and pdBPV-1. DNA probes specific for the U1 RNA coding and 5′ flanking regions were prepared by primer extension with U1D-M13mp7 phage DNAs, or by 5′ end labeling with polynucleotide kinase, respectively (20).

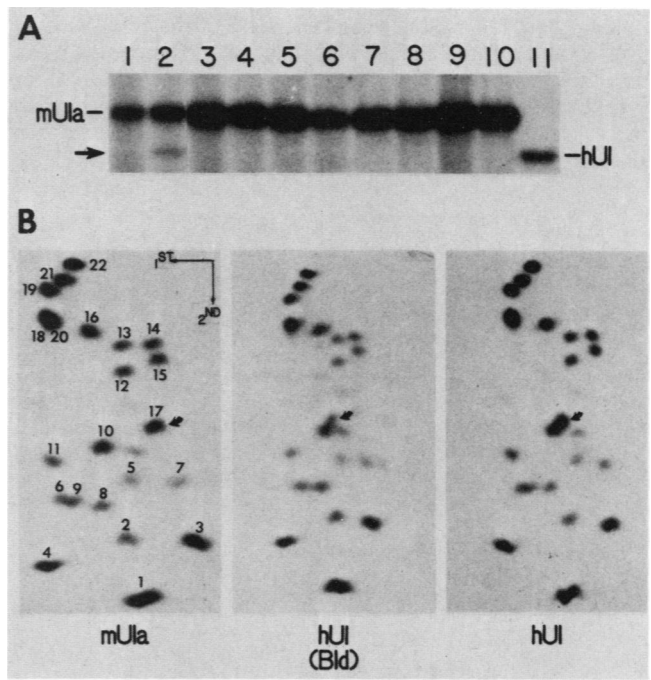

FIG. 2. Identification of human U1 RNA in mouse cells transformed with U1D-BPV$_{69T}$ DNA. (A) Total U1 RNAs isolated from ^{32}P-labeled cells by precipitation with anti-RNP antiserum were analyzed by electrophoresis in a nondenaturing 15% (19:1) polyacrylamide gel (19). The U1 RNAs were from mouse C127 fibroblasts (lane 1), human fibroblasts (lane 11), and nine independent isolates of C127 cells transformed by U1D-BPV$_{69T}$ DNA (lanes 2 through 10). The positions of mouse U1a (mU1a) and human U1 (hU1) RNAs are indicated. The arrow indicates U1 RNA with the gel mobility of human U1 RNA which is present in B1d cells (lane 2). (B) RNase T_1 fingerprints of gel-purified, uniformly labeled U1 RNAs from mouse C127 fibroblasts (mU1a), mouse fibroblasts transformed with U1D-BPV$_{69T}$ DNA [hU1 (B1d)], or human fibroblasts (hU1), as shown in A. The mU1a and hU1 RNAs were from lanes 1 and 11, respectively; hU1 (B1d) RNA was from the minor band in lane 2. The oligonucleotides are numbered by the system of Reddy et al. (30). The 3′-terminal oligonucleotides that distinguish human U1 from mouse U1a RNAs are indicated by arrows. The first dimension of the fingerprint was electrophoresis on cellulose acetate at pH 3.5 (right to left), and the second dimension was homochromatography on polyethyleneimine thin-layer plates (top to bottom). Autoradiograms are shown.

RESULTS

Transformation of C127 cells with linear U1D-BPV DNA. Since we had shown previously that the 0.6-kb U1D DNA fragment contained all of the DNA sequences necessary for human U1 RNA synthesis in *X. laevis* oocytes (25, 36), we first tested whether the U1D sequences also sufficed for expression in mouse cells. Mouse C127 cells were transfected with linear U1D-BPV$_{69T}$ DNA (Fig. 1), and nine individual foci of morphologically transformed cells were expanded into cell lines. Expression of human U1 RNA was assayed by analysis of the U1 RNAs present in anti-RNP antibody-precipitable U1 snRNPs (17). One of the cell lines, B1d, produced detectable amounts of human U1 RNA (Fig. 2A, lane 2), although the other cell lines did not. The U1 RNAs accumulated in B1d cells were further characterized by RNase T_1 fingerprinting (Fig. 2B). These fingerprints confirmed the assignments of mouse U1a (mU1a) and human U1 (hU1) RNAs based upon their relative mobilities in the 15% nondenaturing gel.

Since only one cell line synthesized significant amounts of human U1 RNA, we examined the state of the U1D-BPV$_{69T}$ DNA in all of the established cell lines. Total genomic DNAs were digested with *Bgl*II and analyzed by Southern blot hybridization, using probes specific for the 5′ flanking region or U1 RNA coding region of the HU1-1 gene (data not shown). In all but two cases, at least some coding and flanking region U1D sequences were detectable; however, rearrangements of the input DNA had occurred in all cell lines. Since sequences required for U1 RNA gene expression were located within 200 bp from one end of the linear transfecting U1D-BPV$_{69T}$ DNA (36; Fig. 1), such sequences could easily have been lost due to exonucleolytic degradation of the input DNA.

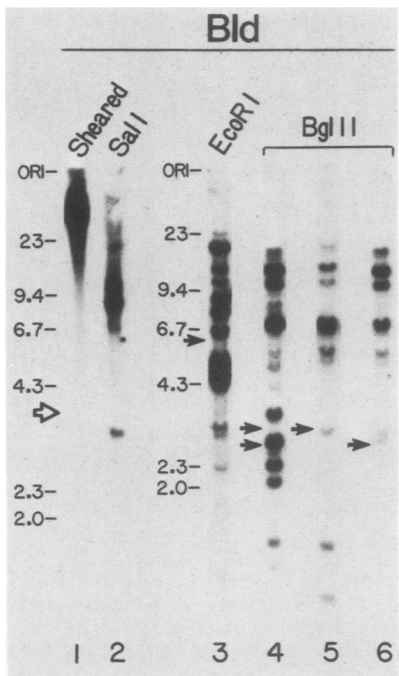

FIG. 3. Southern blot analyses of genomic DNA from B1d cells. Total cellular DNA, sheared (lane 1) or digested with *Sal*I (lane 2), *Eco*RI (lane 3), or *Bgl*II (lanes 4 to 6) restriction enzyme, was fractionated on 0.8% agarose gels and transferred to nitrocellulose filters. The filters were hybridized to ^{32}P-labeled probes of BPV$_{69T}$ (lanes 1 to 4), 5′ flanking region (lane 5), or U1 RNA coding region (lane 6) sequences. Molecular weight markers of λ DNA digested with *Hin*dIII are indicated in kb pairs. The open arrow (lane 1) denotes the expected position of full-length U1D-BPV$_{69T}$ plasmid DNA (form I) (Fig. 1). The closed arrows (lanes 3 to 6) indicate the pattern of hybridizing U1D-BPV$_{69T}$ fragments expected in the absence of rearrangements of the input transfecting DNA. Autoradiograms are shown.

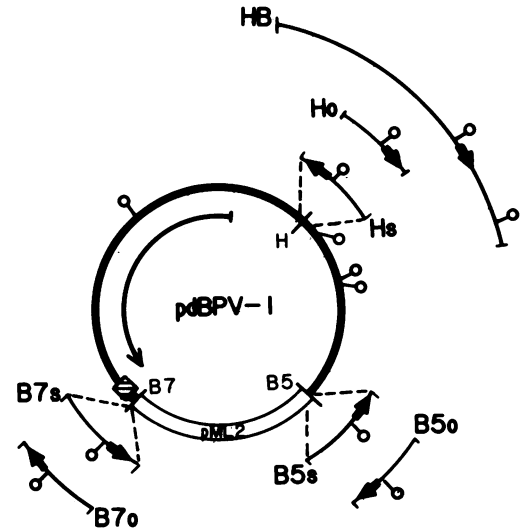

FIG. 4. Structure of pU1D-BPV and pU1HB-BPV recombinant DNAs. The 0.6-kb U1D fragment was inserted into pdBPV-1 DNA (34) at the *Hin*dIII site (H), the *Bam*HI site at 5 o'clock (B5), or the *Bam*HI site at 7 o'clock (B7). The 5-kb U1HB fragment was inserted at the *Hin*dIII site. The BPV-1 DNA (heavy line), pML2 DNA (open box), and U1D or U1HB DNAs (thin lines) are indicated. The position and direction of transcription of the U1 RNA coding sequences are shown by the heavy arrow. Transcription of the BPV genome is represented by the thin arrow, and the BPV enhancer element is indicated by the hatched box. ⚲, *Bgl*II sites.

Unexpectedly, all of the U1D-BPV$_{69T}$ DNA sequences in B1d cells were associated with high-molecular-weight DNA. U1D-BPV$_{69T}$ sequences migrated with the sheared chromosomal DNA (Fig. 3, lane 1). However, multiple fragments of U1D-BPV$_{69T}$ DNA were released by treatment with *Sal*I (lane 2), an enzyme that does not cleave within the U1D-BPV$_{69T}$ DNA. Presumably, these fragments resulted from cleavage at *Sal*I sites in genomic (or carrier) DNA near the integrated U1D-BPV$_{69T}$ sequences. Because of the large size of these complexes, we believe that they reflect genomic integration, but we cannot rule out the possibility that they represent highly catenated, rearranged structures (39).

The complicated patterns of hybridization observed after digestion with *Sal*I or with other enzymes that cut U1D-BPV$_{69T}$ DNA only once or twice (e.g., *Eco*RI [Fig. 3, lane 3] and *Bgl*II [Fig. 3, lanes 4 through 6]) indicated that this DNA was integrated at multiple sites or in multiple rearranged forms or both. It is unlikely that this complexity results from the B1d cells being a mixture of cell types since the same complex patterns of hybridization were observed from B1d cells harvested after several months of continued passage. Likewise, comparable results were obtained from DNAs isolated from three sublines of B1d cells derived by selection for growth in soft agar (data not shown).

Unfortunately, it was not possible to determine which of the multiple human genes in these cells were being transcribed. Furthermore, since the input DNA appeared to be

TABLE 1. Characteristics of cells transformed by circular pU1-BPV DNA

Input DNA	No. of cell lines tested	State of recombinant DNA[a]		
		Extrachromosomal		Integrated
		No. intact	No. rearranged[b]	
pU1D-BPV-B7o	13	0	9	5[c]
pU1D-BPV-B7s	11	0	10	1
pU1D-BPV-B5o	14	8	6	0
pU1D-BPV-B5s	16	11	4	1
pU1D-BPV-Ho	9	5	3	2[c]
pU1D-BPV-Hs	11	3	2	6
pU1HB-BPV	10	9	0	1

[a] Total cellular DNAs (sheared or digested with restriction enzymes) were analyzed by Southern blots to determine whether the transforming DNAs were extrachromosomal or integrated, and intact or rearranged.
[b] Rearrangements involved deletions of U1 DNA sequences.
[c] One of the cell lines contained both integrated and extrachromosomal U1D-BPV sequences.

integrated into mouse DNA, we could not rule out the possibility that endogenous mouse sequences, rather than the exogenously introduced human DNA sequences, were acting as promoters for human U1 RNA synthesis in B1d cells.

Transformation of C127 cells with circular U1-BPV recombinant DNAs. In an effort to prevent the loss of U1D DNA sequences due to rearrangements of linear transfecting DNAs, we introduced the U1 RNA gene into the circular vector pdBPV-1 (Fig. 4). Transfection of cells with these circular DNAs, however, still resulted in numerous DNA rearrangements or integrations. The frequencies of such events were strongly influenced by the location of the human gene within the BPV genome.

When the HU1-1 gene was inserted into the circular BPV vector at the "7 o'clock" *Bam*HI site (pU1D-BPV-B7o), multiple rearrangements of the transfecting DNA were observed. Southern blot analyses of total genomic DNA isolated from 14 cell lines showed the presence of plasmid BPV DNA sequences in nine cases (Table 1), but the plasmid DNAs differed in size from the original transfecting plasmid DNA (Fig. 5A). All of these rearranged DNAs contained little or none of the U1D DNA sequences present in the input pU1D-BPV-B7o DNA (Fig. 5A and B, lanes 1, 4, and 5; other data not shown). Complete and unrearranged U1D-BPV-B7o DNA sequences were found only in those cell lines that contained BPV DNA sequences associated with high-molecular-weight DNA (Fig. 5A and B, lanes 2, 3, and 6). From the patterns of DNA fragments generated by several different restriction endonucleases, it was evident that most of these high-molecular-weight U1D-BPV-B7 DNA sequences were present in head-to-tail tandem arrangements.

Similar results were obtained with recombinant DNA containing the U1D sequences in the same *Bam*HI site, but in the other orientation (pU1D-BPV-B7s) (Fig. 5C). Thus, pU1d-BPV-B7 DNAs apparently could not be maintained extrachromosomally unless most or all of the U1D sequences were deleted (Table 1).

Extensive deletions and rearrangements also occurred in cells that were transfected with pU1D-BPV DNAs lacking methylation of adenine residues (data not shown). This result is in contrast to a previous report (21) that the frequency of rearrangements decreased when the transfect-

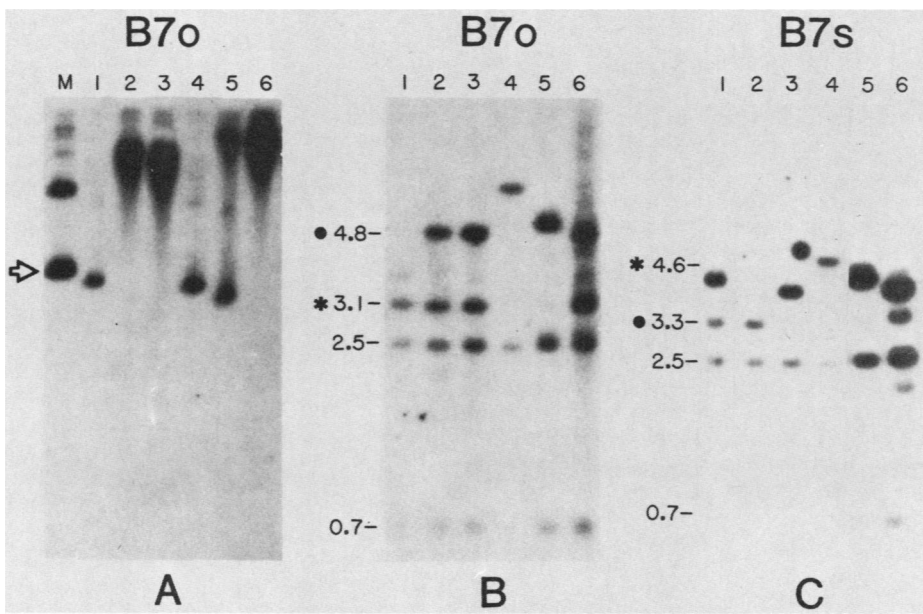

FIG. 5. Southern blot analyses of DNAs isolated from cell lines transformed with pU1D-BPV-B7o and pU1D-BPV-B7s recombinant plasmids. Total cellular DNA, sheared or digested with *Bgl*II (ca. 5 μg per lane), was fractionated on 0.8% agarose gels. After transfer to nitrocellulose filters, the DNAs were probed with ^{32}P-labeled pdBPV-1 DNA. (A) Hybridization pattern of sheared DNA from six cell lines (lanes 1 to 6) transformed with pU1D-BPV-B7o DNA. The open arrow indicates the position of form I of marker pU1D-BPV-B7o DNA (lane M). (B) Hybridization pattern of *Bgl*II-digested DNA from the same six cell lines shown in A. The pattern and sizes (in kb pairs) of *Bgl*II restriction fragments derived from the input pU1D-BPV-B7o DNA are shown on the left. ★ and ● denote fragments containing U1 coding and 5′ flanking region sequences, respectively (Fig. 4). (C) Hybridization pattern of *Bgl*II-digested DNA from six cell lines transformed with pU1D-BPV-B7s DNA; size markers correspond to the *Bgl*II fragments of this input plasmid. Although not represented well in the photographs, the 0.7-kb fragment was present in the original autoradiograms for all DNAs.

ing plasmid DNA (a thymidine kinase-BPV recombinant) was propagated in a *dam* strain.

In contrast to the results obtained with the U1 gene at the 7 o'clock *Bam*HI site, U1D-BPV DNAs were stably maintained extrachromosomally when the U1D DNA fragment was inserted into the other *Bam*HI site, at 5 o'clock (pU1D-BPV-B5s and pU1D-BPV-B5o), or into the *Hin*dIII site (pU1D-BPV-Hs and pU1D-BPV-Ho). Most of these transformants contained multiple copies of extrachromosomal U1D-BPV DNAs which were indistinguishable from the transfecting plasmid DNAs (Fig. 6A to C). Chromosomal integration of the recombinant DNAs was detected in some cell lines, particularly when the U1D sequences were inserted into the *Hin*dIII site. The data are summarized in Table 1.

To determine whether the human U1 RNA gene was active when maintained in an extrachromosomal state, we tested eight cell lines containing unrearranged pU1D-BPV-B5 and pU1D-BPV-H DNAs for their ability to produce human U1 RNA. Independent of the orientation of the human gene, human U1 RNA was expressed in all of these cases and represented between 5 and 10% of the total U1

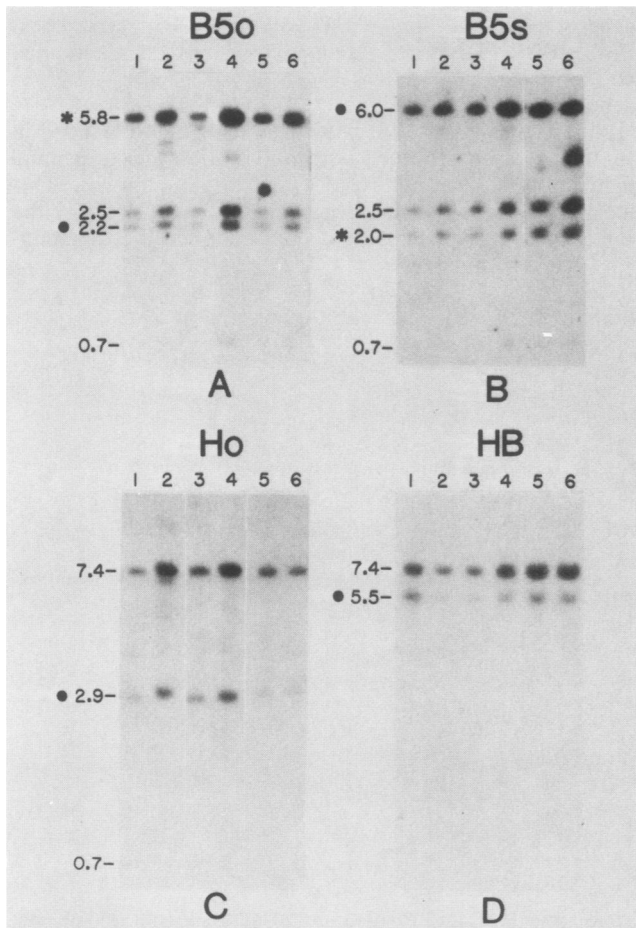

FIG. 6. Hybridization of the pdBPV-1 probe to total DNAs isolated from six cell lines transformed with (A) pU1D-BPV-B5o, (B) pU1D-BPV-B5s, (C) pU1D-BPV-Ho, or (D) pU1HB-BPV DNAs. All cellular DNAs were digested with *Bgl*II. The size markers represent the *Bgl*II digestion pattern of the transfecting plasmid DNAs as in Fig. 5. In panels C and D, the *Bgl*II DNA fragments containing the U1 coding region were 0.2 kb and did not hybridize to the pdBPV-1 probe, respectively.

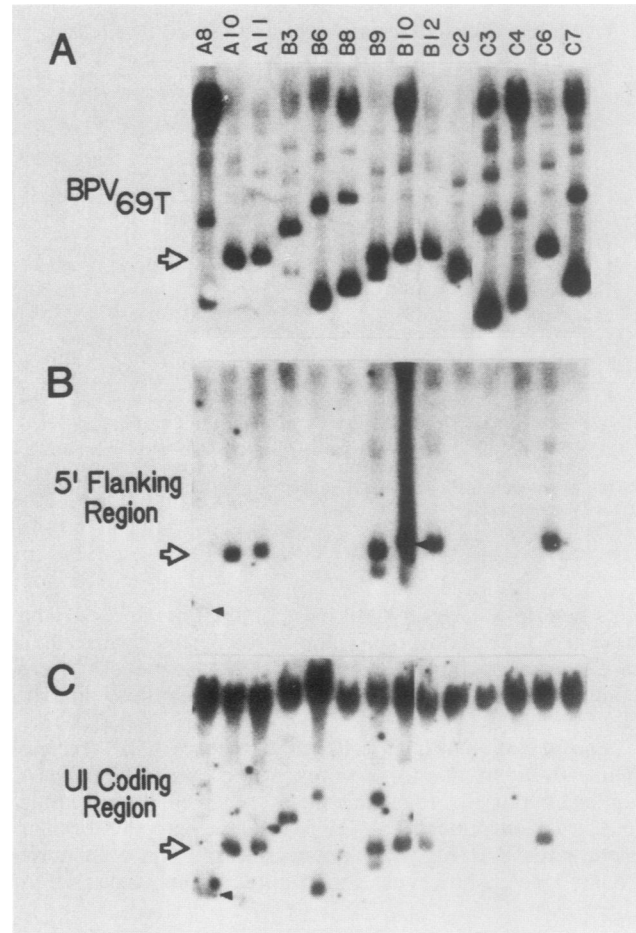

FIG 7. Hybridization of (A) BPV_{69T}, (B) U1 RNA gene 5′ flanking region, and (C) U1 RNA coding region probes to sheared, total DNAs from cells transformed with U1HB-BPV_{69T} DNA. The cellular DNAs were fractionated on an 0.8% agarose gel, transferred bidirectionally to nitrocellulose filters, and hybridized to the appropriate probes. One filter was used for sequential hybridization to the BPV_{69T} (A) and the U1 coding region (C) probes. The open arrow indicates the position of the expected recircularized full-length U1HB-BPV_{69T} plasmid DNA (form I). Arrowheads indicate the position of faint or nondistinct bands of hybridization. The B10 sample in panel B is smeared due to an imperfection of the gel.

RNA (data not shown). Thus, 400 and 35 bp of 5′ and 3′ flanking region sequences, respectively, were sufficient for expression of the human U1 gene in mammalian cells.

The human U1 RNA gene was also expressed in several cell lines that contained only integrated copies of HU1-1 sequences. However, as was the case with the B1d cell line described earlier, we could not rule out the possibility that this expression was due to transcription directed from promoter elements in the mouse genome.

Transfection of C127 cells with U1HB-BPV DNA. Possible effects of additional flanking region sequences upon expression and stability of the human U1 RNA gene were tested by transfection with DNA containing 3.0 and 2.0 kb of human U1 gene 5′ and 3′ flanking region sequences, respectively (U1HB DNA). Comparison of the level of human U1 RNA accumulating in cells transfected with either pU1HB-BPV-H DNA or pU1D-BPV-H DNA showed that the additional human flanking region sequences did not significantly alter the level of accumulation of human U1 RNA. However, the frequency of DNA rearrangements was lower in pU1HB-

TABLE 2. Characteristics of human U1 RNA gene sequences in cells transformed by U1HB-BPV$_{69T}$ linear DNA[a]

Region	Type(s) of sequence in the following cell line[b]:													
	A8	A10[c]	A11[c]	B3	B6	B8	B9	B10[c]	B12[c]	C2	C3	C4	C6[c]	C7
5′ Flanking	*p*	P	P/I	*p*/I	—[d]	—	P	P/I	P	—	—	I	P	—
U1 coding	P	P	P/I	P/I	P	—	P	P/I	P	—	—	—	P	—

[a] All cell lines have plasmid DNAs that contain BPV$_{69T}$ sequences (Fig. 7A). 5′ Flanking and U1 coding region sequences were detected by hybridization to total cellular DNAs that were sheared (Fig. 7B and C) or digested with *Bgl*II (data not shown).

[b] P (or *p*) and I denote plasmid or apparently integrated sequences, respectively. *p* indicates a faint hybridization signal of the 5′ flanking region probe relative to that of the U1 coding region probe (Fig. 7); in the case of A8 cells, restriction enzyme mapping of the plasmid DNA showed that the majority of the 5′ flanking region sequences were deleted.

[c] Cell line containing full-length plasmid DNAs (see the text).

[d] —, absence of detectable 5′ flanking or U1 coding region sequences.

BPV-H-transformed cells than in cells transformed by pU1D-BPV DNAs (Fig. 6D and Table 1).

The presence of additional human flanking region sequences also appears to reduce rearrangements of linear transfecting U1-BPV$_{69T}$ DNA. More than one third of the cells transformed by U1HB-BPV$_{69T}$ DNA (Fig. 1) carried full-length plasmid copies of recircularized input DNA (Fig. 7; Table 2). This was in contrast to the results obtained with U1D-BPV$_{69T}$ DNA, in which no cells were found to contain unrearranged input DNA.

Three cell lines transformed by U1HB-BPV$_{69T}$ DNA (A10, B10, B12) were assayed for the copy number and expression of the human U1 RNA gene. About 100 copies of unrearranged plasmid DNA were present per cell in each of these lines; in addition, one of them (B10) also carried fewer than 10 copies of apparently integrated U1-BPV DNA. In all cases, human U1 RNA was present, representing 10 to 15% of the total cellular U1 RNA; this level was not changed after more than 50 cell divisions (Table 2).

Sequences in U1HB DNA substitute for a BPV enhancer region. Continued passage of B10 cells led to the generation of smaller than full-length plasmid DNAs. Three sublines of B10 cells (B10-3, B10-6, and B10-9) were established by cloning through soft agar. Like the parental B10 cells, all three subclones carried integrated U1HB-BPV$_{69T}$ DNA sequences. Two sublines, B10-3 and B10-9, maintained U1HB-BPV$_{69T}$ extrachromosomal DNAs (Fig. 8A); these plasmid DNAs lacked sequences located in the middle, rather than at the ends, of the original U1HB-BPV$_{69T}$ DNA (Fig. 8B). The deletion endpoints were mapped by using several different restriction enzyme digestions. In both DNAs, the entire 3-kb 5′ flanking region of the human U1 RNA gene was intact. In B10-3 DNA, one deletion endpoint was within the first 25 bp of the U1 RNA coding region, whereas in B10-9 DNA it was ca. 600 bp downstream of the 3′ end of the U1 RNA coding region. As expected from the extent of these deletions, human U1 RNA accumulation was observed only in B10-9 cells (data not shown). The other ends of the deletions were located within the BPV$_{69T}$ sequences, 300 to 400 bp from the original *Bam*HI site; the endpoints of the B10-3 and B10-9 deletions were mapped by *Pst*I and *Bcl*I digestions (data not shown). The results (Fig. 8B) show that the *Bam*HI site (position 4450 in BPV) and *Pst*I sites at positions 4382 and 4172 were deleted in the plasmid DNAs. In wild-type BPV-1 DNA, an enhancer element reported to be required for BPV-mediated morphological transformation of cells is located between positions 4391 and 4450 (4, 21); this BPV-derived element was therefore deleted from the B10-3 and B10-9 plasmid DNAs.

To determine whether the morphological transformation function in B10-3 and B10-9 cells was provided by the extrachromosomal or integrated copies of U1-BPV DNA (Fig. 8A), we tested these DNAs separately for their ability to retransform C127 cells. Each of the extrachromosomal DNA preparations produced several foci of transformed cells, whereas the chromosomal DNAs did not. All of the cell lines established from these foci contained exclusively plasmid U1-BPV DNAs. These extrachromosomal DNAs were indistinguishable (by restriction enzyme mapping) from the input transfecting plasmid DNA (Fig. 8C; other data not shown).

Cell lines transformed by cellular plasmid DNAs (e.g., E-3) had the characteristic morphology associated with BPV-transformed C127 cells (Fig. 8D). We conclude, therefore, that these BPV-derived plasmid DNAs do not require the viral enhancer element located between positions 4391 and 4450 to establish and maintain morphological transformation.

DISCUSSION

We have demonstrated here that a human U1 RNA gene can be maintained in an active state in mouse cells when introduced via BPV vectors. After selection of stably transformed cells, the HU1-BPV DNA sequences often contained deletions or were integrated into the chromosomal DNA of the host. The frequencies of these rearrangements were strongly dependent on the point of insertion of the human U1 DNA sequences within the BPV vector. Both extrachromosomal and integrated copies of HU1-BPV recombinant DNAs were transcriptionally active.

The human U1 RNA that accumulated in HU1-BPV-transfected cells was indistinguishable from normal human U1 RNA isolated from tissue culture cells. For example, the RNase T_1 oligonucleotides containing the 5′ and 3′ ends were those of normal human U1 RNA, and all posttranscriptional modifications were observed. The human U1 RNA was found associated with proteins in anti-RNP precipitable snRNPs. The accumulation of human U1 RNA did not appear to affect the mouse cells adversely since we could detect no consistent changes in the generation time of producing and nonproducing cells.

Cells harboring U1-BPV DNAs with as little as 400 bp of 5′ flanking region sequences of the U1 RNA gene were capable of synthesizing and accumulating human U1 RNA. This implies that the highly conserved 5′ flanking region sequences upstream from position −400 are not absolutely required for transcription in mammalian cells. This observation is in agreement with our previous results obtained by injection of human U1 RNA genes into *X. laevis* oocyte nuclei (25).

These results do not address the question of efficiency of U1 RNA transcription or accumulation. We note that the human genes may not have been as actively transcribed as the mouse genes. For example, the copy number of human U1 RNA genes was often comparable to the number of mouse U1 RNA genes (19), but in no case did we observe

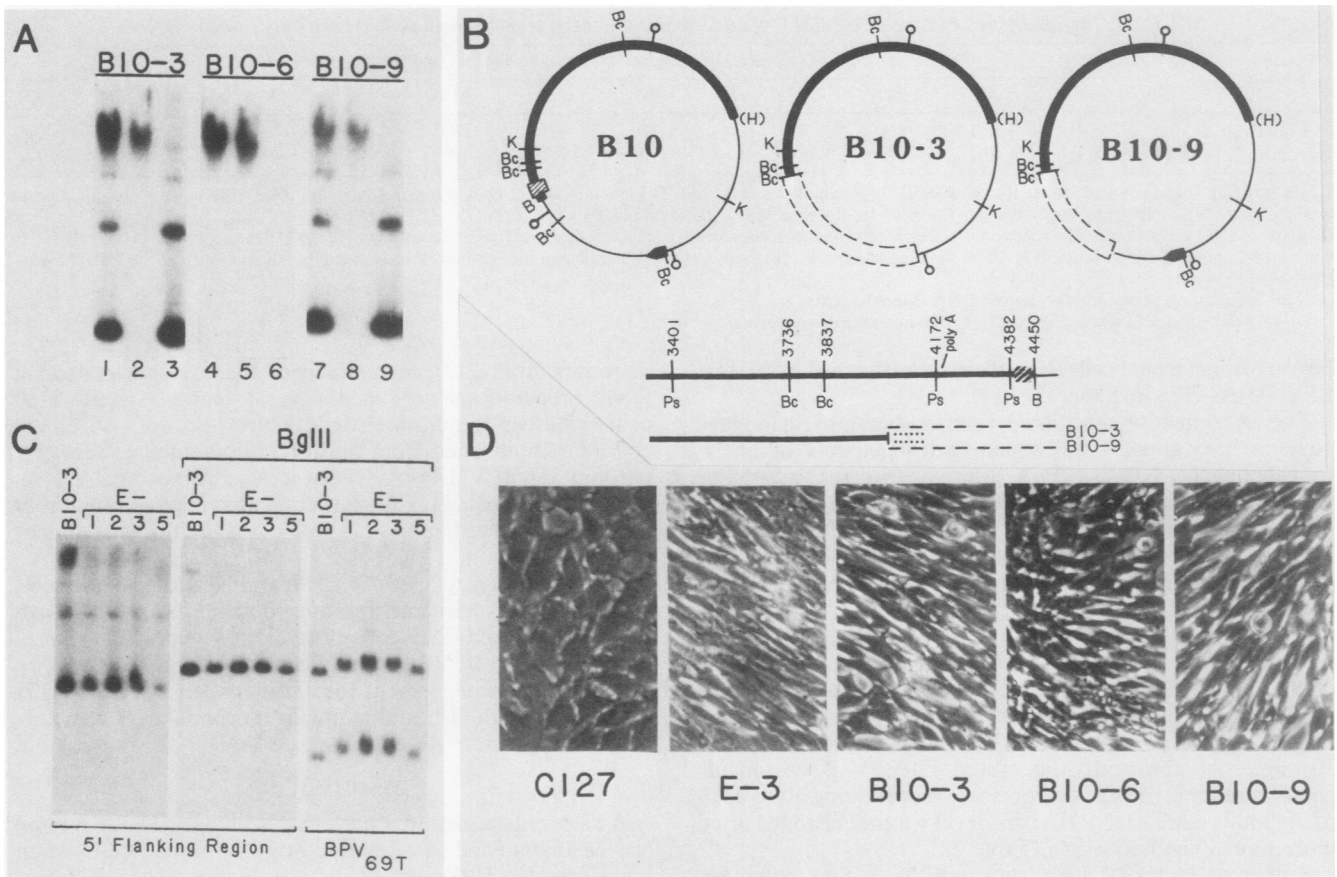

FIG. 8. U1HB-BPV$_{69T}$ plasmid DNAs lacking a BPV enhancer element retain the capacity to transform C127 cells. (A) Chromosomal and plasmid DNAs from B10-3, B10-6, and B10-9 cells were prepared by cesium chloride-ethidium bromide gradient centrifugation. Each panel shows the analyses of total, unfractionated DNA (lanes 1, 4, 7) or gradient-purified chromosomal (lanes 2, 5, 8) and plasmid (lanes 3, 6, 9) DNAs. All DNAs were electrophoresed in 0.8% agarose gels, transferred to nitrocellulose filters, and probed with ^{32}P-labeled BPV$_{69T}$ DNA. (B) Structure of the recombinant plasmid DNAs isolated from B10, B10-3, and B10-9 cells. BPV and human DNA sequences are shown as in Fig. 1. The deleted regions in B10-3 and B10-9 DNAs are indicated by the box outlined in broken lines. The lower linear map enlarges the BPV$_{69T}$ region near the enhancer element (hatched box) and indicates that the deletion endpoints for B10-3 and B10-9 DNAs were mapped within the stippled area. The polyadenylation site (poly A) is at position 4179. Restriction sites are abbreviated as follows: B, *Bam*HI; Bc, *Bcl*I; , *Bgl*II; (H), former *Hin*dIII site; K, *Kpn*I; Ps, *Pst*I. (C) Analysis of total cellular DNAs isolated from four (E-1, -2, -3, -5) lines of cells transformed by B10-3 plasmid DNA. Lightly sheared or *Bgl*II-digested DNAs were separated in 0.8% gels and probed with 5′ flanking region or BPV$_{69T}$ sequences. The control lane, B10-3, contained total DNA from the original B10-3 cells. The faint extra bands in the E-2 sample were due to partial digestion by *Bgl*II. (D) The spindle-shaped morphology of established transformed cell lines (E-3, B10-3, B10-6, and B10-9) was in contrast to the flat appearance of uninfected C127 cells.

more than 15% of the total U1 RNA as human U1 RNA. This percentage did not increase when the U1-BPV DNAs contained more extensive flanking region sequences. It is still possible, however, that sequences located more than 2.6 kb upstream could facilitate the efficient expression of the U1 RNA genes in mammalian cells. Alternatively, a difference in efficiency between expression of endogenous and exogenous U1 RNA genes in mouse cells might result from the locations of U1 DNA templates in active or relatively inactive chromatin structures, or from the action of species-specific transcription factors. In addition, the human U1 RNA may be relatively unstable in mouse cells and more accessible to nuclease activity than the endogenous mouse U1 RNA.

In both normal and BPV-transformed C127 cells, only one form of mouse U1 RNA (mU1a) was observed. This is in agreement with our recent observations that certain mouse cell lines express mU1a but not mU1b RNA (E. Lund, B. Kahan, and J. E. Dahlberg, submitted for publication). Neither BPV transformation nor the expression of the human U1 RNA genes appears to influence the type of mouse U1 RNA genes that are expressed in these cells. Since expression of the human U1 RNA genes never accounts for more than about 15% of the total U1 RNA, we are unable to determine whether the level of mouse U1 RNA is modulated in response to the accumulation of human U1 RNAs.

Unexpectedly, we found that the chimeric DNAs from B10-3 and B10-9 cells, which contain the U1 gene but lack a BPV enhancer element, replicated as plasmids and established and maintained morphological transformation. These plasmids differ from the one used to define the BPV enhancer element in that they lack both pML2 and the 31% nontransforming segment (BPV late genes) sequences, and they contain 5′ flanking regions of the human U1 RNA gene. It is presently unclear whether the lack of some sequences or the presence of others accounts for the ability of these plasmids to replicate and transform cells. In any case, the region reported to be a BPV transcriptional enhancer is not

essential in these plasmids. We note that the U1 RNA gene 5′ flanking region sequences can act as an enhancer element for the chloramphenicol acetyltransferase gene (E. Schenborn, unpublished data), and these sequences may substitute for the viral enhancer in B10-3 and B10-9 plasmids. An alternative explanation is that the total absence of pML2 sequences from the B10-3 and B10-9 DNAs may account for their ability to transform cells.

Th B10-3 and B10-9 plasmid DNAs also lacked the polyadenylation recognition signal located at position 4179. Nevertheless, the fact that transformation occurs in cells transfected with these plasmids implies that early viral mRNAs are made. Therefore, these early viral transcripts either may not require polyadenylation or they may utilize an alternative polyadenylation site (5) provided by human sequences or a previously cryptic site in BPV.

The presence of the human U1 RNA gene at various positions in the BPV genome had a very strong effect on the ability of the U1-BPV DNA to exist extrachromosomally. When the human U1 RNA gene was inserted into the *Bam*HI site adjacent to the BPV enhancer (the 7 o'clock position), all of the transformed cells contained recombinant DNAs that either had deletions in extrachromosomal DNAs or appeared to be integrated into the host genome. In fact, we were unable to obtain cell lines carrying unrearranged extrachromosomal U1-BPV DNA with the human gene present at this position, in either orientation. When the human gene was inserted into the *Hin*dIII site or when it was separated from the BPV enhancer region by the bacterial vector pML2 DNA sequences (i.e., the human gene was inserted at 5 o'clock), the plasmid U1-BPV DNAs were significantly more stable, although deletions were still observed. The most stable chimeric DNA was one in which 5 kb of human DNA was inserted into the *Hin*dIII site of pdBPV-1 DNA.

The reason for the position effect on the stability of U1-BPV recombinant DNAs is unclear. We note that the closer the U1 DNA is to the 3′ end of the BPV early gene sequences, the more unstable is the construct when transfected into mouse cells. Although there is no direct evidence that the enhancer region of BPV located there is influenced by the presence of the human U1 RNA gene, it is tempting to speculate that such might be the case. For example, transcription from the strong promoter of the U1 gene might interfere with the action of the BPV element on transcription of genes required for BPV transforming activity or for plasmid replication. Alternatively, the juxtaposition of BPV and U1 gene enhancer elements may be deleterious. Genomic integration and rearrangement of input DNA was also reported in cells transformed with BPV recombinant DNA containing multiple enhancer elements from mammalian retroviruses (26).

The integration of multiple copies of circular U1-BPV DNAs into high-molecular-weight (presumably genomic) DNA often occurred in a head-to-tail arrangement. There was relatively little rearrangement or deletion of these integrated sequences, indicating that integration may have occurred early after transfection in those cells (9). In a number of cases, the pattern of restriction enzyme cleavage sites surrounding the integrated DNA indicated that the U1-BPV sequences were associated with several regions in the host genome. It is unclear whether the multiple sites of integration are related to each other. One of the reasons for the high level of integration of human U1 genes into the mouse genome might be that the input gene and the endogenous U1 genes are both actively transcribed. Perhaps the relatively open chromatin structures of such genes make them good targets for homologous recombination. Support for this hypothesis comes from a recent report showing that BPV recombinant DNAs carrying a gene for an expressed histocompatibility antigen became integrated into the mouse genome with a very high frequency (7).

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