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Illegitimate recombination in a bovine papillomavirus shuttle vector: a high level of site specificity

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Recombination in a bovine papillomavirus shuttle vector carrying direct repeats of Moloney murine leukemia virus LTR sequence was examined. Differently from similar vectors carrying direct repeats of SV40 polyA addition signal or neomycin resistance gene, the vector exhibited no homologous recombination between the repeats. Instead, illegitimate recombination took place. There were two major types of recombination products from the restriction cleavage pattern. The plasmids in independent cellular clones belonging to the same recombination type shared the identical crossover point. Thus, in this plasmid, illegitimate recombination occurred at preferential sites involving exactly the same sequences.

Illegitimate recombination is defined operationally as the joining DNA sequences with little or no homology. It results in the formation of deletions, duplications, fusions of separate DNA molecules such as chromosome translocation, and other rearrangements. These rearrangements often cause genetic disease and cancer in humans. Study of illegitimate recombination is interesting from another perspective. It is a nuisance factor in any experiment designed to replace a mammalian chromosomal gene with another by homologous recombination. The ratio of targeted to random events is more than 1,000-fold less in mammalian cells than yeast cells, and it cannot be accounted for by the genome size (1). The basis for the rarity of homologous recombination in mammalian cells remains unknown. The study on the illegitimate recombination is difficult on account of the fact that any one event cannot be experimentally reproducible. We report here that in a bovine papillomavirus (BPV) shuttle vector illegitimate recombination occurred in a site specific manner. This may provide a new tool for studying the mechanism of illegitimate recombination.

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MATERIALS

Structure of pLTRDR used as recombination substrate is shown in Fig. 1. The plasmid was propagated in a recombination defective Escherichia coli strain DH 1 (2).

Mouse C127 cells (3) which support extrachromosomal replcation of BPV were maintained Dulbecco's MEM supplemented with 7% heat-inactivated fetal calf serum. The cells transfected with pLTRDR at a dose of 2 μg per plate by calcium phosphate method (4). Three days after transfection G418 (GIBCO,Inc.) was added to the cultures at a concentration of 200 μg/ml. Well separated G418-resistant colonies which appeared 10-14 days later were isolated by the cylinder method.

Extrachromosomal DNA was extracted according to the Hirt's method (5). Southern blot analysis, DNA cloning, sequencing and other recombinant DNA techniques were performed as described (6).

RESULTS

We previously reported that, in BPV shuttle vectors carrying direct repeats of polyA addition signal (7) or neomycin resistance gene (8) homologous recombination took place at a high frequency. The plasmid used in this report had a structure similar to the previous ones except that it contained direct repeats of 845 bp sequence derived from the junction of two long terminal repeats (LTR) of Moloney murine leukemia virus (9). The repeats were separated by total 2.4 kb consisting of neo gene and SV40 polyA addition signal (Fig. 1).

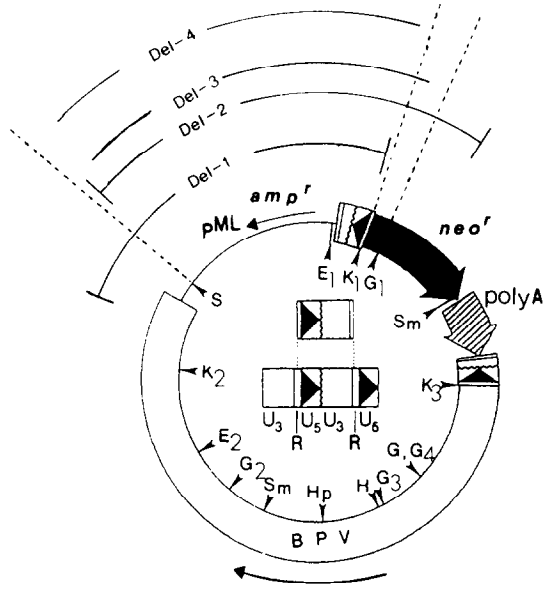

Figure 1. Restriction map of pLTRDR and those of deletion products. Neo gene (neo^r) combined to SV40 polyA addition signal (polyA) was flanked by direct repeats of 845 bp 2-LTR junction fragment derived from circular provirus of Kirsten murine sarcoma virus [shown in the center of the map]. E1 and E2: EcoR1 sites. G1, G2, G3 and G4: BglII sites. H: HindIII site. Hp: HpaI site. K1, K2, and K3: KpnI sites. S:SalI site. Sm: SmaI sites. Del-1, Del-2, Del-3 and Del-4: deleted sequence in the recombination products.

Among 20 G418-resistant clones obtained by transfection with pLTRDR, 13 clones had extrachromosomal copies, and 7 had integrated copies only (Table 1). The undigested DNA in the Hirt's supernatant of clones with extrachromosomal copies was analyzed by Southern blot using the whole pLTRDR plasmid as probe. Except for clone #13 which contained the intact pLTRDR plasmid and clone #41 which contained plasmids with a minute deletion, they had plasmids with similar sized deletion (representative blots are shown in Fig. 2A).

Eight independent clones were chosen for further analysis. Extrachromosomal DNA was digested with HindIII which cuts pLTRDR at a single site. All the clones, #12, #15, #16, #21, #23, #24, #25, #27, #42 and #43 produced 10.5 kb fragment (Fig. 2B). As the deletion size in these clones was uniformly 3.5 kb which nearly equals the distance between the two repeats plus the size of the repeats, the homologous recombination between the repeats appeared to have produced the deletion. But, it actually was not the case as shown below.

EcoRI, KpnI and BglII cleave pLTRDR into 9.1 and 4.9 kb fragments, 7.0, 4.1 and 2.9 kb fragments, and 6.6, 4.2, 2.5, and 0.7 kb fragments, respectively (see diagrams in Figs. 2B-2E and lane M in each figure). EcoRI digestion of DNA from all these clones produced only one band whose size was ca.10.5 kb. Either one of the EcoRI sites (E1, see below) is missing (Fig. 2C). KpnI digestion of DNA from clones #12, #15, #16 and #21 produced 7.0 kb and 2.9 kb bands, while the same digestion of DNA from clones #23, #24, #25 and #27 produced 7.0 kb and 3.6 kb fragments (Fig. 2D). In the former clones, the 3.5 kb deletion must reside in the 4.1 kb KpnI fragment. In the latter clones, a stretch of 3.4 kb covering the junction of 4.1kb-2.9kb KpnI fragments (site K1) must be deleted so that the 4.1 kb and 2.9 kb KpnI fragments were fused to produce the 3.6 kb fragment. BglII digestion of DNA from the former clones, #12, #15, #16, and #21, produced 4.2 kb, 2.5 kb, (0.7 kb) and 3.1 kb fragments (Fig. 2E). The 3.1 kb fragment must be derived from the 6.6 kb BglII fragment by 3.5 kb deletion. BglII digestion of DNA from the latter clones, #23, #24, #25 and #27, produced 7.4 kb, 2.5 kb and 0.7 kb fragments as major products. The 7.4 kb fragment must be produced by fusion of 6.6 kb and 4.2 kb BglII fragments caused by the 3.4 kb deletion involving the junction of 6.6 kb-4.2 kb BglII fragments (site G1). The structures of the two major types of recombination products deduced from the above cleavage analysis are shown in Fig.1 (Del-1 for clones #12, #15, #16 and #21 and Del-2 for clones #23, #24, #25 and #27). In addition to these major recombination products, clones #23, #25 and #27 contained minor products, which was represented by 4.2 kb and ca. 3.5 kb BglII bands. As presence of 4.2 kb band indicates retention of site G1, this plasmid population must have ca. 3.1 kb deletion within the 6.6 kb BglII fragment. This deletion type (#25 and #27, but not #23, see below) will be called Del-3. The faint 2.9 kb

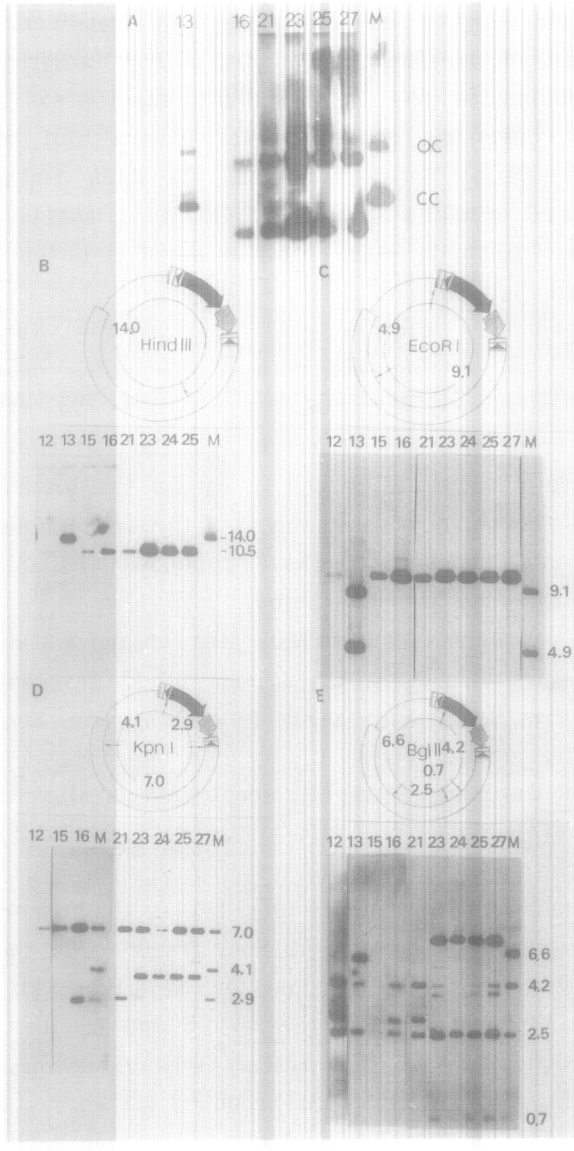

Figure 2. Southern blot analyses of Hirt's DNA. One fifth of Hirt's DNA prepared from a 100-mm plate culture, without digestion (A) or after digestion with 20 units of HindIII (B), EcoRI (C), KpnI (D), or BglII (E) for 2 hours, was eletrophoresed through 0.7 % agarose gel and blotted onto Nylon membrane with 0.4 N NaOH. The membranes were incubated for hybridization overnight at 65°C with ^{32}P-labelled pLTRDR DNA in 5xSSPE, 0.5 % BLOTTO, 1 % SDS, 0.5 mg/ml of denatured sheared salmon sperm DNA. They were washed once with 2xSSC contaning 0.1 % SDS, once with 0.5xSSC containing 0.1 % SDS and then with 0.1xSSC containing 0.1 % SDS each for 15 minutes at room temperature. The clone numbers are shown on the top of the slots. M: pLTRDR DNA (100 pg).

band found in KpnI digest of clone #23 indicates that the minor population in clone #23 must retain site K1 in addition to site G1. This type will be called Del-4 (Fig.1). The deletion was precisely determined for Del-1 and Del-2 by nucleotide sequencing (see below) but not for Del-3 and Del-4. The site of deletion junction relative to SalI site was determined by SalI

Table 1. Various types of pLTRDR-transformants

Extrachromosomal copies		
absent	7	
present	13	
Clones with parental copy only		1
Clones with parental and rearranged copies		0
Clones with rearranged copies		12
Eight clones were examined in detail		
Type of rearrangement	cell clone	
Del-1	#12,#15,#16,#21	
Del-2	#23,#24,#25,#27	
Del-3	#25,#27	
Del-4	#23	

digestion (data not shown). Frequencies of the deletion types are summarized in Table 1.

The deletion pattern obviously does not fit the deletion caused by homologous recombination between the two LTR repeats. It indicates recombination between non-homologous sequences. In order to know whether the sequences involved in recombination was the same in four clones each of the two major recombination types, the sequence involved in the recombination was determined. For this purpose, the sequence around the deletion site was amplified by means of polymerase chain reaction (PCR) (10). One-tenth of Hirt's DNA prepared from 100-mm dish was digested with 10 units of HindIII followed by 30 cycles of PCR amplification DNA denaturation at 95°C for 1.5 min, primer annealing at 52°C for 2 min, and primer extension 72°C for 4 min, in 100 μl of 50 mM Tris-HCl (pH 9.0 at 25°C), 1.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 1 μM of each primer, 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP, 0.3 mM dTTP and 2.5 units of AmpliTaq (Perkin Elmer Cetus Corp.). PCR products were then converted blunt-ended by T4 DNA polymerase, kinased by T4 polynucleotide kinase and ligated with pUC119 or pUC118 (11) at HincII site. They were sequenced by dideoxy sequencing method (6) using Sequenase Version 2.0 (USB, Corp., Ohio).

0.5 kb or 0.6 kb sequence containing the both sides of the deletion was amplified from clones #12, #15, #16 and #21 or from clones #23, #24, #25 and #26, respectively (Fig. 3B). The sequence around the recombination junction was determined for all these eight clones (Fig. 3C). The recombination products in clones #12, #15, #16 and #21 shared the identical sequence at the crossover point, and so did the major recombination products in clones #23, #24, #25 and #27, i.e., both for Del-1 and Del-2 type deletions, the sequence at the crossover point was identical in the respective four clones. In neither case was there sequence homology at

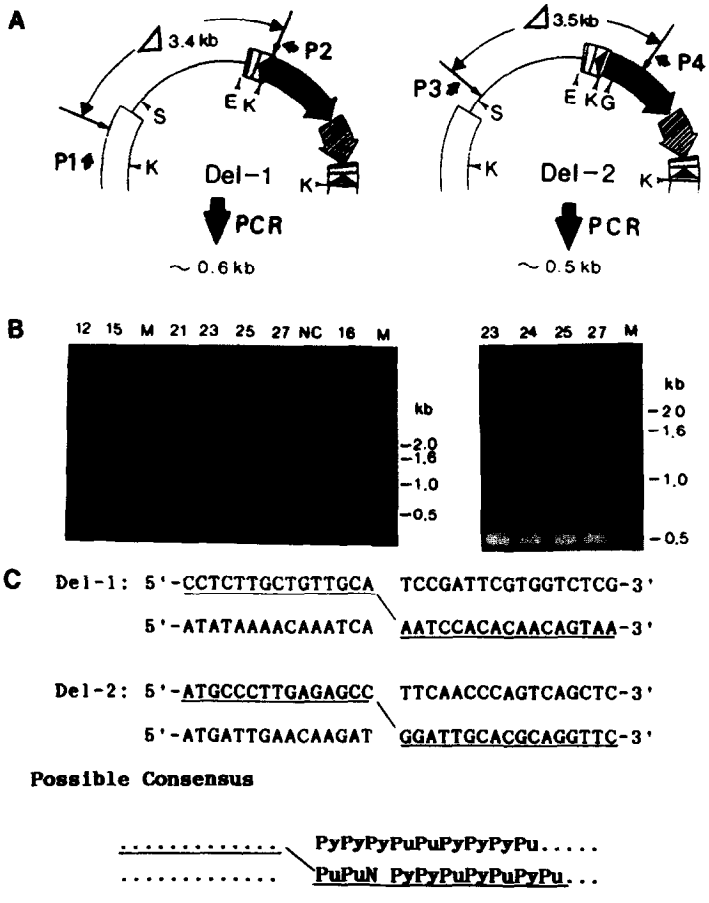

Figure 3. Determination of sequence involved in the deletion. **(A)** Locations of primers for PCR amplification. p1 and p2 are for amplification of sequences surrounding Del-1, and p3 and p4 for those surrounding Del-2. p1: 5'-CGGTACCGGTGGACTTGGCATCAAG-3' which is complementary to BPV (3444-3458). p2: 5'-GCCCGAGTCTTGCGGCAGCGTG-3' complementary to 5' part of neo. p3: 5'-GAGTCGCATAAGGGAGAG-3' complementary to pML (16) sequence. p4: 5'-GTCGGGCATGCGCGCGCCTT-3' compelemtary to middle part of neo which contains BssHII restriction site. **(B)** PCR amplified products. One-tenth of Hirt's DNA prepared from 100-mm plate culture was subjected to 30-cycle PCR amplification. One-tenth of the reaction products was applied onto 2.0 % agarose gel and electrophoresed. Clone numbers are shown on the top of the slots. M: molecular size marker. NC:10 ng of C127 cell genomic DNA. With primers p1 and p2, the 0.6kb fragment was amplified in clones #12,#15,.#16 and #21 only. **(C)** Sequence surrounding deletion site. All of the 0.5 or 0.6 kb PCR products from the eight clones were sequenced. Only the sequences surrounding the deletion are shown. The 5' side sequence of the upper row is joined to the 3' side sequence of the lower row, the 3' side sequence of the upper row continuing to the 5' side sequence of the lower row being deleted (i.e., the underlined sequences were present on the recovered plasmid and the sequences without undeline were deleted). Possible consensus shows the alternating pattern of purines and pyrimidines common to Del-1 and Del-2 on the 3' side of the strand exchange site. Pu : purine, Py : pyrimidine, N : purine or pyrimidine.

the recombination junctions. The crossover occurred without any extra-bases added between the junctional ends.

DISCUSSION

Our experiments showed that, in the BPV vector with directly repeated 845 bp derived from the junction of two LTRs, the homologous recombination between the LTR repeats was not detected. Instead, illegitimate recombination involving specific sequences took place. As the size of the repeat 845 bp was far above the lower limit of the size required for homologous recombination, 200 bp (12, 13), the homologous recombination might have well occurred between the repeats. The 506bp homology used to restitute the neo gene by homologous recombination, in the previous report (8), was even shorter than the 845 bp. The LTR sequence thus may have contained a sequence non-permissible for the homologous recombination. This may be related to the wide observation that spontaneous deletion of integrated retroviruses leaving a solo LTR (caused by homologous recombination between LTRs at the both sides of the genome) in mammalian cells is rare.

It was surprising that the illegitimate recombination took place involving exactly the same sequences in the independent clones. Among the four clones with Del-1 type deletion, clone #21 and the other three clones were obtained in two independent transfection trials. It was improbable that the transfecting DNA preparation was contaminated by the rearranged DNA molecules because the rearranged DNA had no prokaryotic replication origin. Between Del-1 and Del-2 type deletions was there no common sequence involved. One possible common feature is the alternating pattern of purines and pyrimidines in nine to ten nucleotide sequences on the 3' side of the recombination site on the remaining and deleted DNA fragments (Fig. 3C). The chance of the occurrence of this pattern in a random case is 2^{-18}. The topoisomerase I mediated illegitimate recombination has been suggested (14, 15), but the consensus sequence for the strong break site, 5'-(A or T)-(G or C)-(A or T)-T-3' was present only in the deleted sequence in Del-2 and not in any where else.

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