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HOMOLOGOUS RECOMBINATION IN BOVINE PAPILLOMAVIRUS SHUTTLEVECTER; EFFECT OF RELATIVE ORIENTATION OF SUBSTRATE SEQUENCES

Ayako Naito*, Yoshihiro Kitamura, and Hiroshi Yoshikura

Department of Bacteriology, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113 Japan

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Relative orientation of recombination substrates, neo gene, strongly influenced homologous recombination events in a bovine papillomavirus shuttle vector. Between direct repeats, recombination occurred at a high frequency while between inverted repeats, it was rare. Double strand break near the mutation site increased the recombination frequency between inverted repeats but not between direct repeats. Formation of long heteroduplex as a recombination intermediate may explain this apparently paradoxical phenomenon.

In most mammalian recombination studies, substrates were those which could be maintained only in integrated copies; less frequently used were SV40 and polyoma virus-based vectors which replicate extrachromosomally during the lytic cycle (1). BPV-based vectors which can be stably maintained as episomes, however, have been exploited to a limited extent. Interest in recombination in BPV is two-fold; one is possible difference in the recombinations between episomal and integrated DNAs, and another is that episomal DNA can be readily recovered and its structural analysis is easier. We previously reported that, in BPV vectors carrying direct or inverted repeats of SV40-derived poly A addition signal, homologous recombination occurred at a high frequency between direct repeats but undetectably between inverted repeats (2). In the present study , we used BPV vectors which had repeats with a selectable marker so as to make the quantitative estimation possible.

MATERIALS AND METHODS

Recombination substrate. Recombination substrates were pIK43 carrying inverted repeats of neo (3) and its derivative pDK8 carrying direct repeats of neo (Fig. 1A). One of the two neo segments designated as neo(+-) had 283 bp deletion between the two NaeI sites (Δ d in Fig. 1), which was filled by XhoI linker, and the other designated as neo(-+) had 248 bp deletion between the two NarI sites (Δ b in Fig. 1). Both pIK43

* To whom correspondence should be addressed.

Abbreviations: BPV (bovine papilloma virus), neo (neomycin resistance gene), E.coli (Escherichia coli), PCR (polymerase chain reaction).

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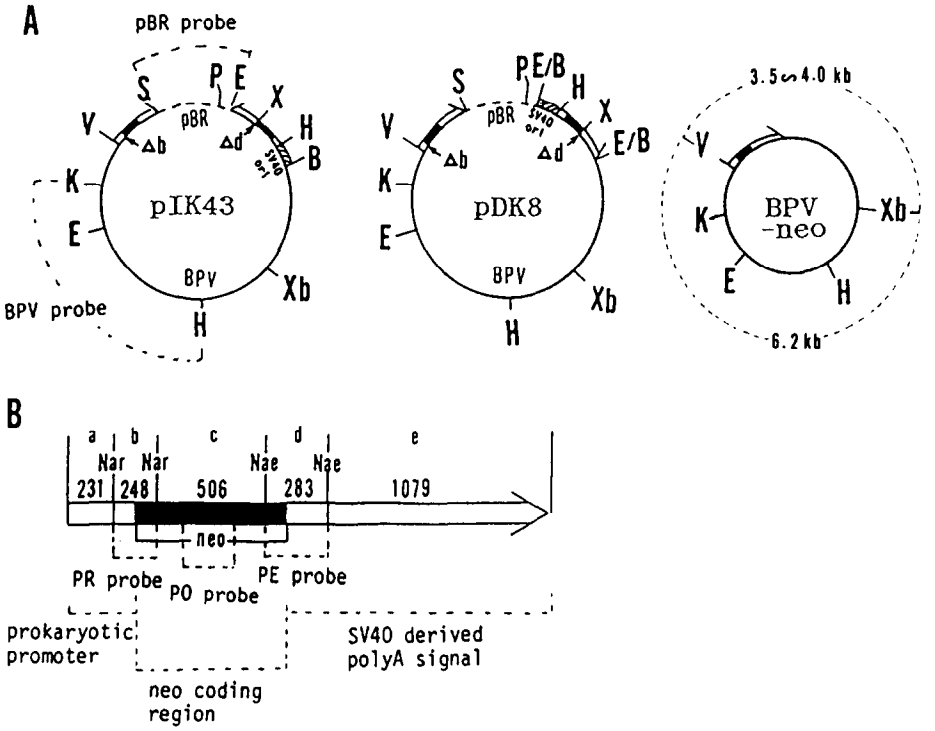

Fig. 1. Substrate plasmids, pIK43 and pDK8, and a recombination product of pDK8, BPV-neo. Probes used in Southern blot analysis are indicated in the maps of pIK43 in Fig. A and Fig. B. Abbreviations for restriction sites are as follows. E:EcoRI. X:XhoI. H:HindIII. B:BamHI. Xb:XbaI. K:KpnI. V:EcoRV. S: SalI. P: PvuI. Δ b and Δ d are deletions at NarI-NarI and NaeI- NaeI site, respectively.

and pDK8 had two mutated neo genes, and recombination between the two neo genes reconstitute intact neo. They had pBR322-derived 2323 bp for replication in E. coli, and BPV-derived 7953 bp for replication in mouse C127 cells. Though maintained stably in E. coli DH1 (4), either plasmid as the original structure failed to replicate in the mouse cells (see below).

Analysis of recombination products. Transfection of C127 cells was done under the standard calcium phosphate precipitation method (5). The transfected cells were treated with G418 (200-400μg/ml) for 10-14 days for selection of the transformants. The clones were isolated and expanded for analysis of DNA. DNA extraction and Southern blot analysis were done according to the standard procedures (6). The location of the probes are shown in Fig. 1. The extrachromosomal DNA was prepared according to the Hirt's method (7), and purified as follows. Extrachromosomal DNA obtained from 10^7 cells was mixed with 10μg of M13mp8 RF DNA as a closed circular (cc) DNA marker and 20μg C127 cell DNA as a linear DNA marker. The CC DNA was separted by cesium chloride equilibrium centrifugation [in 3.7ml TE sarcosyl (0.4% sarcosyl, 10mM Tris-Hcl pH 7.5, 1mM EDTA), 224μl ethidium bromide (5mg/ml) and 3.745g cesium chloride]. One tenth of the purified CC DNA were used for the PCR analysis (8). 100 μl mixture consisting of 50mM KCl, 10mM Tris-HCl pH8, 2.5mM $MgCl_2$, 200μM dNTP, 1μM each primer, 2.5 U Taq DNA polymerase (AmpliTaq, Perkin Elmer Cetus) and substrate DNA was subjected to 30 cycles of 94° C for 1 min, 62° C for 2.5 min, and 72° C for 2.5 min using DNA Thermal Cycler (Perkin Elmer Cetus).

RESULTS

Recombination frequency : Neither pIK43 nor pDK8 has intact neo genes. Only the cells harboring recombination products with a restored neo gene can form G418 resistant colonies. Results are summarized in Table 1. Production of G418 resistant colonies, i.e., recombination frequency, was far higher for pDK8 than for pIK43 (compare uncut pIK43 and pDK8). Digestion of pIK43 at XhoI or EcoRV site within or near the deletion marker resulted in 6 to 9-fold increase in the recombination frequency. Digestion at BamHI or SalI at the intact side of neo (Fig. 1A) rather decreased the frequency. In pDK8, digestion at XhoI site which stimulated recombination in pIK43 had no or even an adverse effect. Here, transfection efficiency can be roughly estimated from that of pSV2neo.

Recombination products of pIK43: The transfected pIK43 was found in the integrated form; no extrachromosomal copies were found. Presence of many aberrant bands made detailed analysis of all the products difficult. Only the major bands produced by the diagnostic EcoRI-HindIII-KpnI triple digests are considered. As shown in Fig. 2, aside from the parental type bands 1 and 7, prominent was band 4 for XhoI-cut and BamHI-cut plasmids, and bands 2 and 3 were major bands for EcoRV-cut plasmid (these bands are compatible with the model in Fig. 5C; see discussion). Though only three clones were analyzed for each form of transfecting DNA, but as the cells contained several tens of transfected DNA copies, this pattern reflects the recombination events in near hundred copies.

Recombination products of pDK8: In the extrachromosomal fraction, 10 kb circular DNA was detected in all the clones but no parental 15 kb DNA (Fig. 3A). The whole cell DNA was digested with PvuI which cleaves the plasmid at a single site in pBR portion, and probed with pBR-specific probe (pBR) or neo-specific probe (PO) (Fig. 3C). Presence of massive high molecular weight bands indicated chromosomal integration of variously rearranged copies. As

TABLE 1. Transformation frequency of pDK8 and pIK43

DNA	Enzymatic digestion	Input dose 4 μg	8 μg	10 μg
pIK43	uncut		6, 4	21, 33, 29, 36
	XhoI		21, 38	111, 99, 97, 124
	EcoRV		50, 41	48, 66, 62, 70
	BamHI			14, 0, 0, 0
	SalI			15, 1, 0, 1
pDK8	uncut	48, 65	83, 91	
	XhoI	31, 47	79, 89	
pSV2neo	uncut	47, 81	122, 138	

Figures indicate number of G418 resistant colonies per culture. pSV2neo has an intact copy of neo (20).

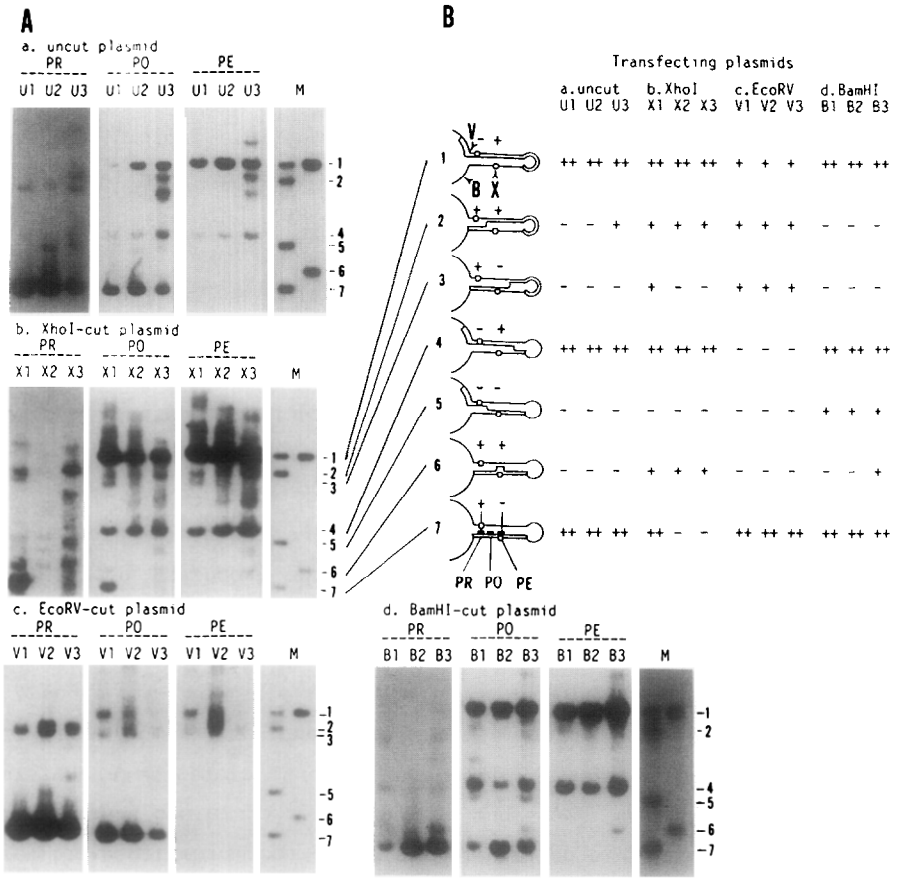

Fig. 2. Southern blot analysis of the EcoRI-HindIII-KpnI triple digests of whole cell DNA of pIK43 transformants. The transfecting DNA was either uncut (panel A-a), XhoI-digested (panel A-b), EcoRV-digested (panel A-c) or BamHI-digested (panel A-d). U1, U2, U3, X1, X2 etc. on the top of the slots indicate the transformant numbers and PE, PO and PR indicate probes used for Southern analysis. The specific probe for PR was 248 bp NarI-NarI (region b), that for PO was 255 bp AluI-BanII (the middle part of neo), and that for PE was 283 bp NaeI-NaeI (region d) sequence (Fig. 1B). All the probes were excised from the gel and ^{32}P-labelled by the random primer method (21). From the probes and the size of the bands, the recombinant structures could be deduced. Bands 1-7 correspond the size of the expected recombinant structures depicted in panel B. For example, band 6 is 2.3 kb band hybridizable with probes PE, PO and PR, and band 4 is 5.4 kb band hybridizable with probes PE and PO but not with PR. The site of deletion is indicated by □. The incidence of each structure roughly estimated from the intensity of the bands was represented by - (absent), + (present) or ++ (abundant) (panel B). Locations of probes are shown in Fig. 1.

meaningful conclusion was difficulty obtained from such heterogenously rearranged integrated DNAs, only the extrachromosomal 10 kb recombination products were analysed. The structure of these copies, however, must reflect the whole recombination events in the cells, and will provide us with the necessary information.

The 10 kb circular DNA was hybridizable with the BPV-specific probe or with probe PR (NarI-NarI) or PE (NaeI-NaeI) (Fig. 3A) , but not with pBR-specific probe (data not shown but see lanes 9 and 10 in panels a and b in Fig. 3C).

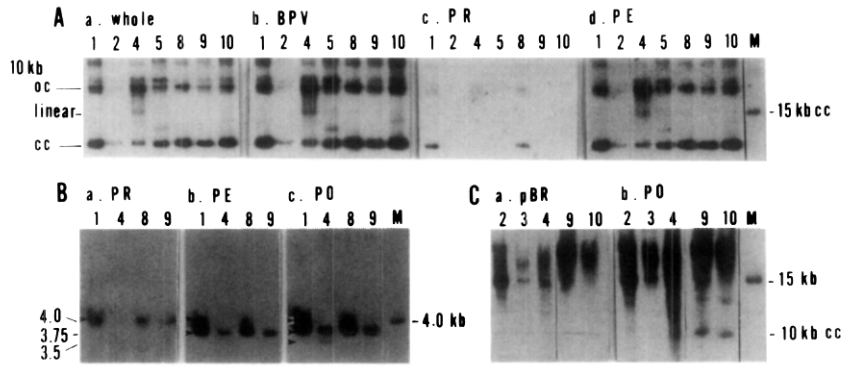

Fig. 3. Southern blot analysis of pDK8-transfected G418-resistant clones. Panel A: Extrachromosomal DNA was prepared according to Hirt's method (7). An aliquot of uncut DNA was electrophoresed and analyzed by Southern blot (6). The same filter was hybridized with the whole pDK8 probe labeled after EcoRI digestion(a), BPV probe (b), PR probe (c), or PE probe (d). The hybridization was performed in the order of c, d, b, and a, with removal of probes(22) in between. CC, linear and OC indicate electrophoretic markers obtained by a 10 kb plasmid. Panel B: XbaI-EcoRV double digests of extrachromosomal DNA probed with PR(a), PE(b) or PO(c). Panel C: PvuI digests of the whole cell DNA probed with pBR(a) or PO probe(b). Electrophoretic markers are indicated on the right.

Thus the 10 kb band must be derived from pDK8 by losing one of the two neo genes and pBR sequence in between. Molecules of this type will be called BPV-neo (Fig. 1A). Among the clones, clones 1 and 8 contained BPV-neo molecules hybridizable with both probes PE and PR, suggesting a possibility that the BPV-neo molecules in these two clones had a restored neo gene, neo(++). In order to test this, highly purified extrachromosomal DNA (see Material and Methods) was analysed by Southern blot and PCR. For Southern analysis, the purified DNA was double-digested with XbaI and EcoRV which cleaves BPV-neo into 3.5-4.0 kb and 6.2 kb fragments(Fig. 1A). As it is the 3.5-4.0 kb fragment which contains neo gene, the size will vary depending upon the recombination, 4 kb for the restored neo(++)gene, 3.75 kb for parental neo(+-) or neo(-+), and 3.5 kb for neo gene with the both deletions, neo(--) (Fig. 1B). As shown in Fig. 3B, clones 1 and 8 had 4 kb band hybridizable with PR, PE and PO probes, indicating that BPV-neo molecules in these two clones contained restored neo gene. This observation was confirmed by PCR (8) (Fig. 4). Two other clones, clone 4 and 9 devoid of 4 kb XbaI-EcoRV fragment were analyzed by PCR but no neo(++) sequence was amplified (Fig. 4). Southern blot(Fig. 3B) and PCR(Fig. 4) indicated that clone 1 had BPV-neo molecules with neo(++) and neo(-+) (and possibly neo(--) which was detected only in PCR), clone 4 those with neo(-+) and neo(--), clone 8 those with neo(++) and neo(-+), and clone 9 those with neo(-+) (and possibly neo(+-) detected only in PCR).

DISCUSSION

The previous results(2) showed that homologous recombination between direct repeats occurred at a high frequency while that between inverted re-

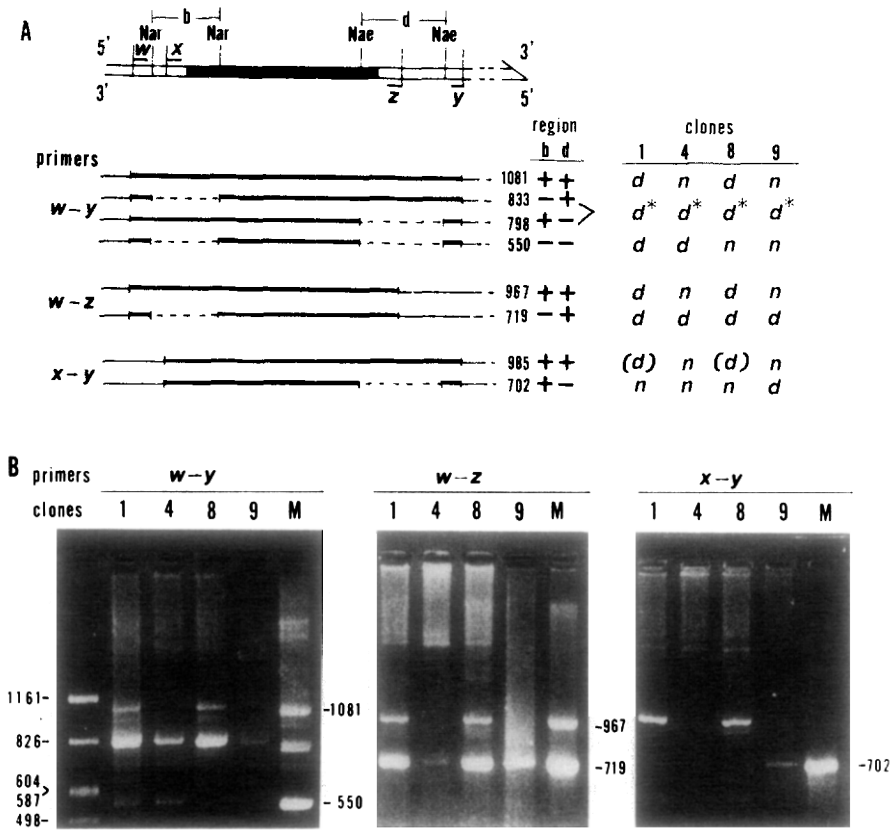

Fig. 4. Polymerase chain reaction (PCR) to detect recombination products in extrachromosomal BPV-neo type molecules. DNA extracted according to Hirt's method was purified by cesium chloride equilibrium centrifugation. DNA was extracted from the fraction of closed circular DNA, and used for templates in the PCR reaction. Four oligomers used as primers are:
w:5' (214) GCGAACCGGAATTGCCAGCTGG (235) 3', x:5' (310) GCAGGGGATCAAGATCTGATCA (331) 3',
y:5' (1295) CCCCGCGCTGGAGGATCATCC (1275) 3', z:5' (1181) GGTCGGTCATTTCGAACCCCA (1161) 3'
Sequences w and x are on the same strand, and y and z on the complementary strand. Combination of primers x and y, (x - y), will detect neo(++) and neo(+-), that of w and z, (w - z), will detect neo(++) and neo(-+) , and that of w and y, (w - y), will detect all the four possible recombinants. The dashed part of the line indicates deletion, and the figures on the right on each map indicate the size of the expected amplified neo segments. The gels of PCR are shown in panel B, and the data are summarized on the right side of panel A d: detected. n: undetected. *: 833 - 798 bp (either or both). Data on 985 bp amplified by x - y are in parenthesis, because the control 985 bp band was not included in the marker sample.

peats was rare. The present study using the G418 selectable marker as repeats, confirmed it. In addition, it was shown that a double strand break near the deletion marker increased the recombination frequency between the inverted repeats but not that between the direct repeats. Thus, the modes of the homologous recombination between direct and inverted repeats were different. The presence of this difference itself indicates that most, if not all, homologous recombination was an intramolecular event. If the recombination occurred between two molecules, its frequency would not have been influenced by the relative orientation of the repeats.

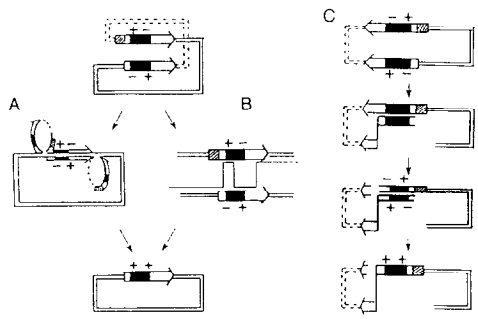

Fig. 5. Recombination giving rise to BPV-neo(++) molecules in pDK8 by heteroduplex intermediates (strand slip model is shown here, but other similar models are possible) (A) or by triple cross-over (B), and a model of double strand break followed by heteroduplex formation giving rise to neo(++) in pIK43 (C).

The recombination products between the inverted repeats, band 4 for XhoI-cut and BamHI-cut plasmids and bands 2 and 3 for EcoRV-cut plasmids, had a structure which is explained by postulating a double strand break followed by a formation of heteroduplex between the nearby and the other homologous sequence (Fig. 5C and Fig. 2). In pDK8 with the direct repeats, two out of seven clones harbored BPV-neo molecules with restored neo gene. Formally, formation of this molecule requires the triple independent cross-over at homologous regions a(231 bp), c(506 bp) and e(1079 bp) (Fig. 5B, see also Fig. 1B). In addition, the size of region a, 231 bp, is the lower limit of recombination to occur (9, 10). Thus, this will be an infinitesimally rare event and is inappropriate for explaining the observation. A better model may be formation of heteroduplex covering about 2000 bp neo gene (Fig. 5A). The strand slip (11, 12) or similar mechanism (13, 14, 15, 16) may produce the heteroduplex, and repair of the mismatch (17, 18, 19) will produce all the possible neo structures including neo(++). Formation of a long heteroduplex is a common feature in the hypothetical pathways shown in Figs. 5A and C for the recombination between the direct and the inverted repeats, respectively. This may explain the apparently different recombination modes between the direct and inverted repeats. Note that, in the model, the double strand break is a prerequisit for recombination between inverted repeats but not for that between direct repeats.

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