Analysis of recombination junctions in extrachromosomal circular DNA obtained by in-gel competitive reassociation

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Abstract **Essentially all eukaryotic cells contain circular extrachromosomal DNA as a result of excision from the chromosomes. To obtain insight into the nature of recombination associated with the occurrence of such DNA species and its biological significance, we analyzed a library enriched in recombination junctions which was constructed by a novel DNA subtraction technique; in-gel competitive reassociation (IGCR). Furthermore, we also introduced inverse PCR to characterize chromosomal DNA fragments containing the recombination junctions. At least 45% of the clones in the library constructed by the IGCR procedure comprised DNA with recombination junctions. Nucleotide sequence analysis of the recombination junctions indicated that three of four extrachromosomal DNAs thus analyzed were produced through recombination between sequences with a 3–5 bp homology in the chromosomes. One extrachromosomal DNA was apparently generated through non-homologous recombination, possibly by end-to-end joining. These results have demonstrated the usefulness of IGCR in concentrating recombination junctions, which provide the most direct evidence for the mechanism of the recombinational events involved, from highly complex genomes.**

Key words: Recombination junction; Extrachromosomal DNA; IGCR; Subtraction

1. Introduction

Various types of extrachromosomal (EC) DNA or small polydisperse circular DNA are found in eukaryotic cells [1–3]. They are either self-reproducing DNA fragments, such as plasmids or defective viruses [1,4], or fragments excised from chromosomal DNA through homologous as well as non-homologous recombination [5–8]. Although most EC DNA molecules are the products of random excision events, the recombination can take place at specific sites, for example at the immunoglobulin and T cell receptor genes [9–11]. As a result of their abundance in cells, most likely the result of relatively high excision frequencies, EC DNA has provided a good model for studying the mechanism of recombination, mostly through analysis of the DNA structure at recombination junctions [5–8,12–14]. Previously, we reported a strong preference for recombination, leading to the generation of circular DNA, in an alphoid satellite sub-family, the *Sau*3A family [6,15,16]. Similar modes of recombination have been reported by others, including the L1 repetitive family, where only a perfect homology of 9 bp long was observed at the recombination sites [5].

One of the drawbacks in using EC DNA to study the molecular mechanism of recombination, however, is the lack of techniques to concentrate recombination junctions from restriction-digested EC DNA molecules. Since recombination junctions comprise only a small portion of EC DNA, the majority of the clones from EC DNA are derived from fragments carrying no recombination junctions. To eliminate cumbersome procedures such as Southern hybridization against chromosomal DNA to screen for clones carrying recombination junctions and also to systematically collect as many clones with the junctions, we applied an improved DNA subtraction technique, the in-gel competitive reassociation (IGCR) method, developed recently in this laboratory for this purpose [17,18]. The IGCR method was designed for cloning DNA fragments of altered size as observed in recombination junctions [19]. The higher reassociation efficiency in the gel enables us to clone even a single copy of an altered sequence present in a complex higher eukaryotic genome, as well as repetitive sequences such as *Alu* or L1 families among 10^6–10^7 different restriction fragments.

2. Materials and methods

2.1. Materials

Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (USA), ATP-dependent DNase was from Toyobo (Japan), and *Ex Taq* DNA polymerase and bacterial alkaline phosphatase were from Takara (Japan). Oligonucleotides were synthesized with a Milligen Cyclone Plus DNA Synthesizer and purified with Milligen Oligo-Pak columns. Primers used in PCR were GTTTTCCCAGTCACGAC (P4) and CAGGAAACAGCTATGAC (P5) for pBluescript, AGGGTTGATAAAGGGTCATT (a1), ACTGCATAATATCATGCTGA (a2), CATTATTGCTCCTGCTAAGA (b1) and GACTGTAAGCAAATCCTTAT (b2) for pIG11, TTCACTTGCTGCTTCTCATC (a1), CTGCTGACAAAGGATTTCTC (a2), GTGATTTGTATTGGGTGTAA (b1) and CTCAATCCAGCAGTGCTTCT (b2) for pIG21.

2.2. Purification of EC DNA from human cells

EC DNA from human cells (HeLa cells) was purified according to Kiyama et al. [6] with minor modifications. Briefly, crude EC DNA was prepared according to Hirt [20] from 6×10^9 cells. After treatment with 0.1 mg/ml proteinase K, DNA samples were extracted with phenol and chloroform/isoamylalcohol (24:1) and purified with ethanol. The covalently closed circular DNA fraction of the crude extracts was recovered as the mitochondrial DNA fraction through CsCl/ethidium bromide density gradient centrifugation (three times) at 55,000 rpm for 19 h at 22°C. The DNA samples were then desalted by ethanol precipitation. Purity of EC DNA was confirmed by Southern hybridization with the *Sau*3A family DNA as a probe [6]. Mitochondrial DNA was removed by ATP-dependent DNase treatment [21] as follows: EC DNA from 6×10^8 cells was treated with 4 U of *Pac*I to linearize mitochondrial DNA, followed by extraction with phenol and ethanol precipitation, and then incubated with 1.75 U of ATP-dependent DNase for 1 h at 37°C. The sample was extracted with phenol and purified with ethanol. Removal of mitochondrial DNA was confirmed by Southern hybridization.

2.3. IGCR procedure

IGCR was performed according to Yokota et al. [18]. EC DNA was sed as target DNA (from which common DNA species were

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subtracted) and chromosomal DNA was used as a reference. Target DNA was prepared from EC DNA after digestion with *Bfa*I followed by PCR amplification with adaptors attached to the ends. These adaptors were removed by *Bfa*I treatment after PCR. Chromosomal DNA was prepared from pellets at the Hirt extraction step by solubilization with 1 M guanidine isothiocyanate, 25 mM Tris-HCl (pH 7.5), 0.25% (v/v) 2-mercaptoethanol, which was followed by CsCl (0.4 g/ml) density gradient centrifugation and by dialysis against 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl. Reference DNA was prepared from purified chromosomal DNA by digesting 50 μg of the DNA with *Bfa*I followed by treatment with 9 U of bacterial alkaline phosphatase. IGCR was performed with 200 ng of target DNA mixed with 10 μg of reference DNA for the first cycle and 4.4 ng of target DNA and 10 μg of reference DNA for the second cycle. SV40 DNA (3.3 pg/μl) was mixed as an exogenous marker for enrichment. After 2 cycles of the procedure, the DNA sample was treated with *Not*I to cleave the site within the adaptors, cloned into the *Not*I site of pBluescript SK(−) (Stratagene) and used to transform *E. coli* DH5α. The library of clones was screened by Southern hybridization with each clone as a probe.

2.4. Screening of phage library

The chromosomal DNA fragments for clones pIG11, pIG30 and pIG46 were obtained from a phage library containing *Sau*3AI partial fragments of human chromosomal DNA [22]. A total of 8×10^5 plaques were screened with each clone as a probe, and 1–6 independent plaques were analyzed for each clone. The phage library was obtained from Japanese Cancer Research Resources Bank.

2.5. Inverse PCR

Chromosomal DNA fragments containing recombination junctions were amplified directly by inverse PCR [23–25]. Chromosomal DNA was first digested with *Apo*I or *Eco*RI and purified by phenol extraction. For circularization, 10^4 or 10 pg/μl of chromosomal DNA mixed with 10 pg of *Eco*RI-digested plasmid pBluescript/μg chromosomal DNA in 70 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 10 mM DTT, 0.2 mM ATP was incubated with 4 U/μl of T4 DNA ligase for 16 h at 16°C, followed by purification with phenol and ethanol. Ligation products were first examined by PCR with primers which amplify the region including the *Eco*RI site of pBluescript to confirm circularization. 3 ng of the ligation products was used for PCR amplification with 0.025 U/μl of *Ex Taq* DNA polymerase and primer sets for each clone (see Figs. 3 and 4) under the following conditions; 25 cycles of 1 min at 92°C for denaturation, 1 min at 50°C for annealing and 5 min at 74°C for extension, with 10 min extension at 72°C after the final cycle. PCR amplification with pairs of secondary primers was used to remove non-specific products, and *Ex Taq* DNA polymerase was used to amplify longer DNA fragments. PCR products were analyzed by electrophoresis through 1% agarose gels.

3. Results

3.1. Enrichment of recombination junctions by IGCR

The IGCR technique was used to construct a library enriched in recombination junctions present in EC DNA from human cells. EC DNA from HeLa cells were first purified by CsCl/ethidium bromide centrifugation as covalently closed circular DNA. Mitochondrial DNA in the purified EC DNA was then removed by treatment with a linear DNA-specific DNase (ATP-dependent DNase) after linearization of the circular DNA with *Pac*I. We used *Pac*I since this 8-base cutter restriction enzyme cleaves mitochondrial DNA specifically, while EC DNA is likely to be resistant to attack because of its relatively small size. The reaction products were then digested with the 4-base cutter *Bfa*I and amplified by PCR using adaptors attached to the ends. The PCR products, after *Bfa*I treatment to remove the adaptors, were used for IGCR as target DNA (from which clones were to be isolated) against *Bfa*I-digested chromosomal DNA as reference DNA (serving as a reference as well as competitor). The IGCR procedure was repeated twice, and 150-fold overall enrichment of the exogenous control marker (SV40) DNA was achieved (15-fold for the first and 10-fold for the second). Since the enrichment reached a plateau after the second cycle, we analyzed the clones in the library from the twice-cycled products.

A total of 22 clones were randomly picked up from the library and subjected to Southern hybridization analysis. 45% (10/22) of the clones exhibited hybridization patterns in which the size of the cloned fragments did not correspond to those of the chromosomal DNA. 41% (9/22) showed identical sizes with chromosomal DNA and 14% (3/22) were not analyzed because of their high repetitiveness. Thus, approximately half of the clones in the library carried possible recombination junctions. All of the 9 clones which exhibited identical bands between the chromosomal and EC DNA showed more than 10-fold higher signals for EC DNA than for chromosomal DNA on Southern hybridization (data not shown), suggesting that these fragments were originally enriched in the EC DNA fraction. On the other hand, none of the clones (0/16) randomly selected from the control EC DNA which had not been subjected to IGCR exhibited the apparent presence of recombination junctions (data not shown). 38% (6/16) showed high-repetitiveness.

Fig. 1 shows three clones (pIG11, pIG30 and pIG46) which exhibited differences in the *Bfa*I fragment size between EC and chromosomal DNAs (lanes 1 and 4 for pIG11, lanes 5 and 9 for pIG30, and lanes 10 and 13 for pIG46) or the PCR-amplified chromosomal DNA (lanes 2, 6 and 12, for the respective clone). *Bfa*I fragments were enriched approximately 100- (pIG11, lanes 3 and 4), 50- (pIG30, lanes 7–9) or 100-fold (pIG46, lanes 12 and 13) after IGCR.

3.2. Analysis of recombination junctions

To confirm the presence of the recombination junctions and to investigate the mode of recombination in the excision, we also screened clones carrying sequences present in these clones (pIG11, pIG30 and pIG46) in the phage genomic library constructed from *Sau*3AI partial digests of HeLa DNA. All of the phage clones thus screened contained a single region which matched the sequences of each clone isolated by IGCR (data not shown). Fig. 2A shows maps of typical phage clones (λIG11-1, λIG30-3 and λIG46-4). Also shown in the figure are the maps of EC DNA clones obtained by IGCR and possible structures of EC DNA from which these clones were derived. The structure of all EC DNA clones was divided into two regions, L and R, which were flanked by a *Bfa*I site used for cloning at the one end and a recombination junction for circularization at the other. Fig. 2B shows the nucleotide sequence of their recombination junctions. Comparison of the nucleotide sequences between EC and chromosomal DNA allowed localization of the exact site of recombination. In all cases, sequence homologies of 3–5 nucleotides (GTC for pIG11, TGGAT for pIG30, and TCTG for pIG46) were identified between the parental recombination sites. There were apparently no specific sequence motifs or secondary structures around the junctions (data not shown).

3.3. Characterization by inverse PCR of fragments containing recombination junctions

To facilitate screening for chromosomal DNA sequences responsible for excision in a phage library which generally involves many lengthy steps, we adopted the method of direct

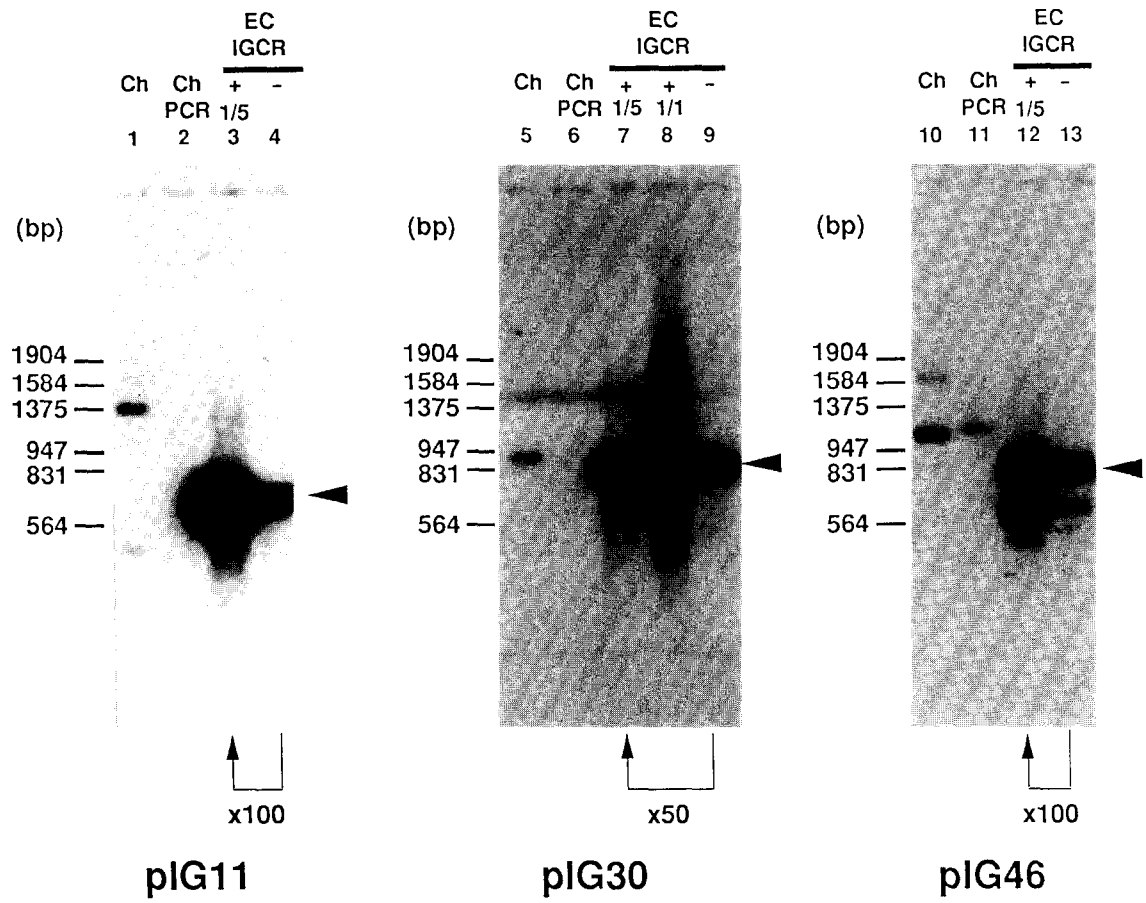
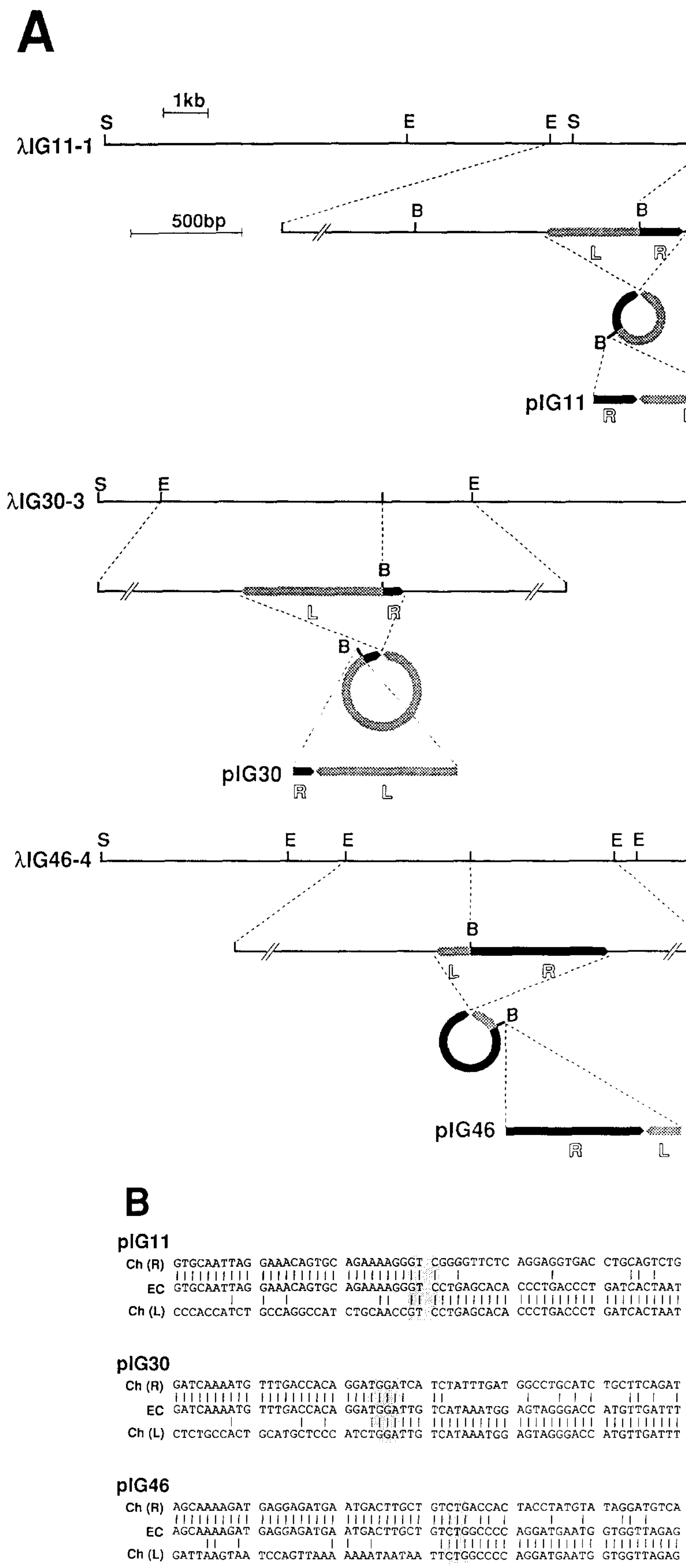


Fig. 1. Southern blot analysis of clones from the library constructed by IGCR. Three clones (pIG11, pIG30 and pIG46) from the library were used as probes for Southern hybridization. Chromosomal (indicated as Ch) DNA (1.5 μg) digested with *Bfa*I (lanes 1, 5 and 10) or amplified by PCR after adaptor ligation (lanes 2, 6 and 11), or the samples before or after IGCR (30 ng for lanes 3, 7 and 12, or 150 ng for lane 8) were electrophoresed through 1.5% agarose gels, transferred onto nylon membranes, and then probed with ^{32}P-labeled plasmid DNA (10^8 cpm/μg specific activity). Only DNA fragments between 0.3 and 1.3 kb were recovered and used for IGCR. The positions of the DNA fragments recovered as clones are shown by arrows. The degrees of enrichment by IGCR are shown at the bottom.

inverse PCR. For this, primers were designed to proceed outward on the restricted fragments and then the fragments were subjected to circularization before PCR. Only fragments circularized within the molecule containing both right and left recombination junctions (self-circularized products) were selectively amplified by PCR, provided that the following conditions were satisfied; (i) when the circularized products consist of only self-circularized products, and (ii) the restriction enzyme used for the digestion of chromosomal DNA neither cleaves the region between the two junctions nor produces the fragments which are too large in size for PCR. After finding optimal DNA concentrations for exclusive self-circularization (data not shown), *Eco*RI-digested plasmid pBluescript DNA with excess amounts of *Apo*I (Fig. 3A, lanes 3–5) or *Eco*RI (lanes 6–8) digests of chromosomal DNA were subjected to inverse PCR with primers P4 and P5, which face each other beyond the *Eco*RI site. The two enzymes (*Eco*RI and *Apo*I) recognize GAATTC and UAATTY (U, purine; Y, pyrimidine) sequences, respectively, sharing the AATT motif and, therefore, when *Eco*RI produces fragments too large for PCR, *Apo*I can be used instead. After PCR, only the sample where ligation was performed at 10 pg/μl showed the 246 bp band derived from the plasmid (lanes 5 and 8). Fig. 3B shows the amplification of the 4 kb *Eco*RI fragment containing the junction for pIG11 (lane 3) which was neither observed without ligation reaction nor ligation at higher DNA concentrations (lanes 1 and 2, respectively). Identity of this amplified fragment with the original fragment was confirmed by experiments using several restriction enzymes (data not shown).

Inverse PCR was also applied to the clone pIG21 (Fig. 4). This clone exhibited different patterns between EC and chromosomal DNAs (Fig. 4A, lanes 2 and 5), suggesting the presence of the recombination junction. Chromosomal DNA digested with *Apo*I and subjected to ligation at 10 or 10^4 pg/μl was PCR amplified with two pairs of specific primers (Fig. 4B). A 0.8 kb band appeared only under conditions where self-ligation prevailed (10 pg/μl, lane 3), suggesting that this 0.8 kb fragment included both junctions. No amplification of specific bands was observed when *Eco*RI-digested chromosomal DNA was used, probably because *Eco*RI digestion generated fragments which were too large to be amplified by PCR (data not shown). Nucleotide sequencing of the 0.8 kb fragment confirmed the presence of the junctions (Fig. 4C). As shown in Fig. 4B, one of the junctions was located close to an *Apo*I end. Unlike the three junctions described above, the recombination junction in pIG21 was probably formed through non-homologous recombination.


A
1kb
500bp
λIG11-1
S E E S E S
B B B
L R
pIG11
R L
λIG30-3
S E E S
B
L R
pIG30
R L
λIG46-4
S E E E E S
B
L R
pIG46
R L
B
pIG11
Ch (R) GTGCAATTAG GAAACAGTGC AGAAAAGGGT CGGGGTTCTC AGGAGGTGAC CTGCAGTCTG
EC GTGCAATTAG GAAACAGTGC AGAAAAGGGT CCTGAGCACA CCCTGACCCT GATCACTAAT
Ch (L) CCCACCATCT GCCAGGCCAT CTGCAACCGT CCTGAGCACA CCCTGACCCT GATCACTAAT
pIG30
Ch (R) GATCAAAATG TTTGACCACA GGATGGATCA TCTATTTGAT GGCCTGCATC TGCTTCAGAT
EC GATCAAAATG TTTGACCACA GGATGGATTG TCATAAATGG AGTAGGGACC ATGTTGATTT
Ch (L) CTCTGCCACT GCATGCTCCC ATCTGGATTG TCATAAATGG AGTAGGGACC ATGTTGATTT
pIG46
Ch (R) AGCAAAAGAT GAGGAGATGA ATGACTTGCT GTCTGACCAC TACCTATGTA TAGGATGTCA
EC AGCAAAAGAT GAGGAGATGA ATGACTTGCT GTCTGGCCCC AGGATGAATG GTGGTTAGAG
Ch (L) GATTAAGTAA TCCAGTTAAA AAAATAATAA TTCTGGCCCC AGGATGAATG GTGGTTAGAG

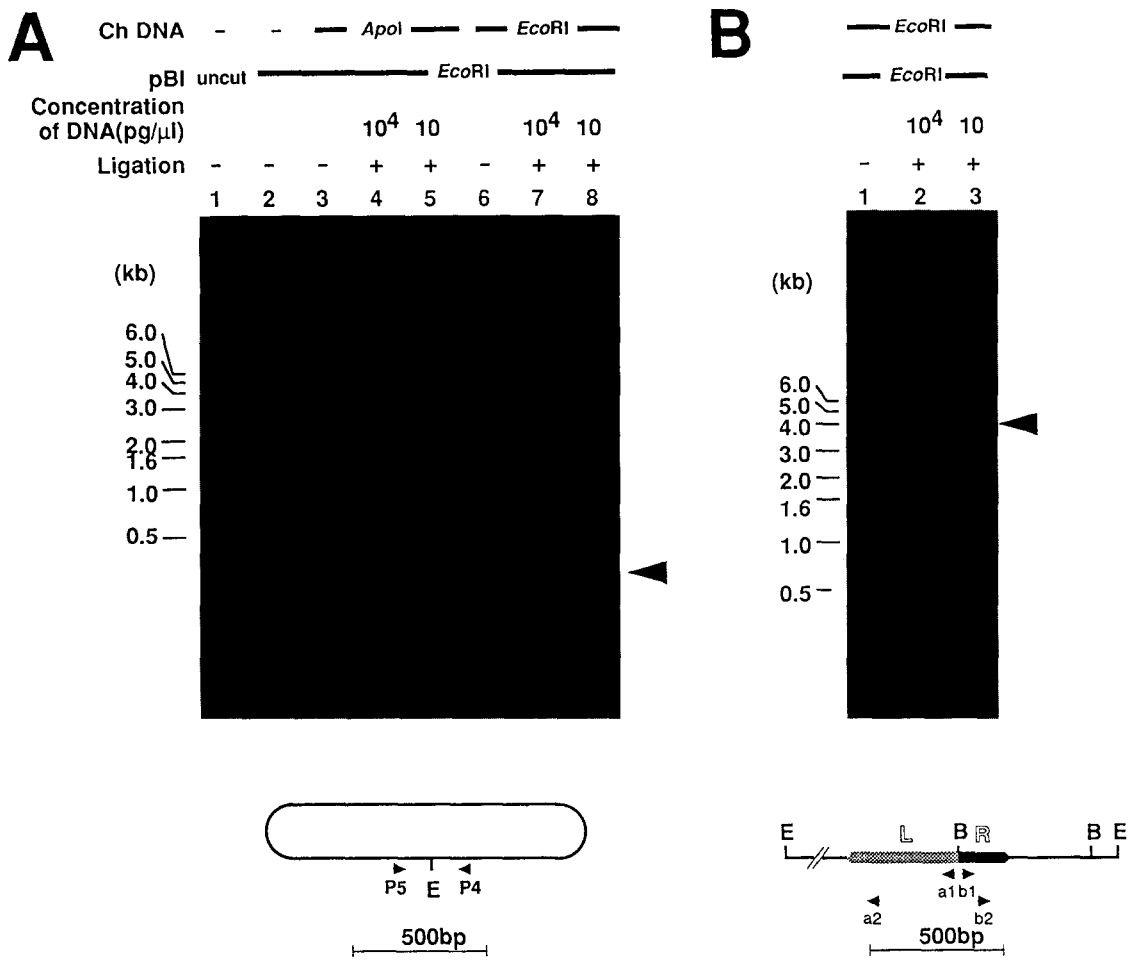


Fig. 3. Amplification of fragments containing recombination junctions by inverse PCR. Inverse PCR was applied to pBluescript vector (control: panel A), and the EC DNA clone pIG11 (panel B). (A) 3 ng of chromosomal (indicated as Ch) DNA digested with *Apo*I (lanes 3–5) or *Eco*RI (lanes 6–8) and mixed with 0.03 pg of *Eco*RI-digested pBluescript (pBl) was used for PCR amplification with primers specific to each clone or plasmid after ligation of the DNA sample at a DNA concentration of 10^4 pg/μl (lanes 4 and 7) or 10 pg/μl (lanes 5 and 8), or without ligation (lanes 3 and 6). Uncut (0.03 pg, lane 1) or *Eco*RI-digested (lane 2) pBluescript was used for PCR without mixing with chromosomal DNA as positive and negative controls, respectively. (B) The samples in lanes 6–8 in panel A were used for PCR with primers specific to the clone pIG11 (lanes 1–3, respectively). Maps of the control plasmid and the chromosomal sequence for pIG11, and the positions of the primers used for PCR are shown at the bottom of each panel. B, *Bfa*I; E, *Eco*RI.

4. Discussion

4.1. Recombination junctions in EC DNA

In this paper, we applied an improved differential cloning method for enrichment of recombination junctions present in EC DNA from cultured human (HeLa) cells. The method, termed in-gel competitive reassociation (IGCR), because dissociation and competitive reassociation of duplex DNA is performed in a gel, should provide a powerful tool for cloning of altered DNA sequences. Since the average size of EC DNA in HeLa cells is 1.9 kb [26], it was necessary to linearize the DNA using restriction enzymes with 4-base recognition sites, which would generate many DNA fragments without the junctions. van Loon et al. [27], on the other hand, used *Eco*RI for construction of an EC DNA library, where at least 9% (4/45) of the clones apparently carried recombination junctions. We used *Bfa*I, which recognizes CTAG, and the digested EC DNA was amplified first by PCR and then subjected to IGCR. As reported previously by other groups [26,28], Southern hybridization with several repetitive sequences (L1 and *Alu* families) as probes indicated that these fragments were not significantly enriched in the EC DNA fraction (data not shown). Our library was constructed after two cycles of IGCR with the digested EC DNA as target against 50- (for the first cycle) or 2,000- (for the

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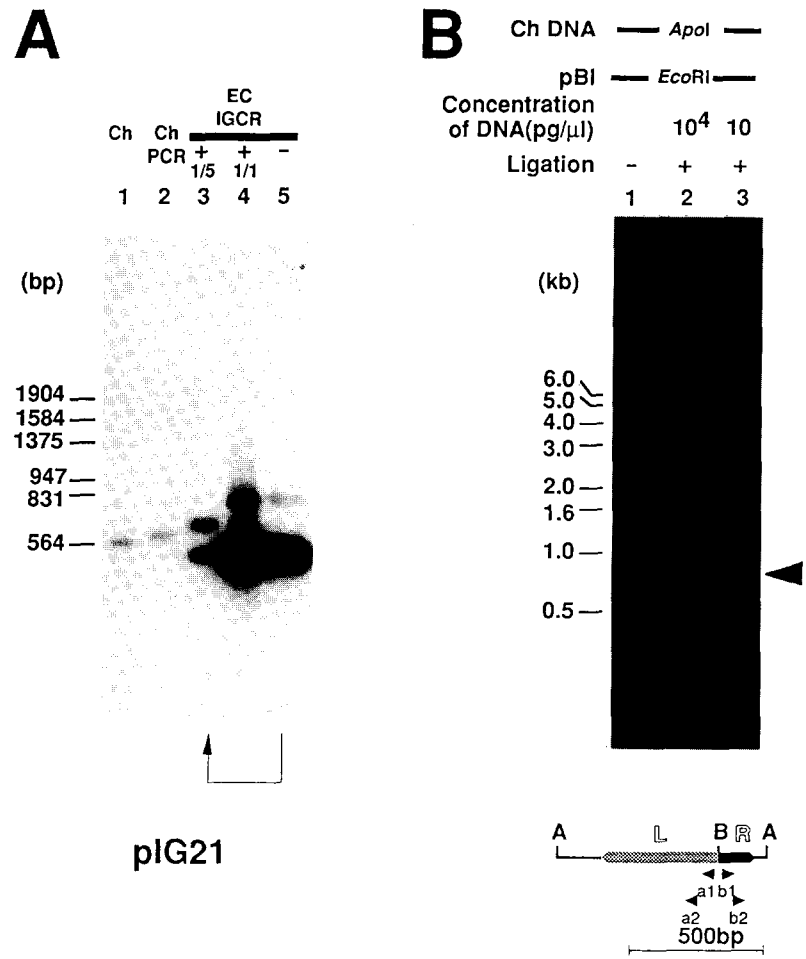
Fig. 2. Recombination junctions in EC DNA. (A) Maps of phage clones (λIG11-1, λIG30-3 and λIG46-4) which contain the whole regions of cloned fragments along with the clones (pIG11, pIG30 and pIG46) and their parental EC DNAs. The clone sequences were divided into two regions (L and R) flanked by a recombination junction at one end and a *Bfa*I site at the other. B, *Bfa*I; E, *Eco*RI; S, *Sal*I. (B) Nucleotide sequences of recombination junctions. The nucleotide sequences of EC DNA were aligned with their parental DNA sequences in chromosomal (Ch) DNA (L and R regions). Possible recombination junctions are shadowed.



C

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Ch (R)  ACTGCAAGAA CCTGTTAAAC GCAGGGAATT Y
        |||||||||| |||||||||| |||||     |
EC      ACTGCAAGAA CCTGTTAAAC GCAGGAGTCT CAGTGTTTCC CAGACTGCTT GACAAATGAA
        |||     ||       ||   |      |||||| |||||||||| |||||||||| ||||||||||
Ch (L)  ACTCACTGAG TGGATTTCAT TATGTAGTCT CAGTGTTTCC CAGACTGCTT GACAAATGAA
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Fig. 4. Amplification by inverse PCR of fragments containing the recombination junctions for clone pIG21 and the nucleotide sequences of the junctions. (A) Southern blot analysis of chromosomal (lanes 1 and 2) and EC (lanes 3–5) DNA using pIG21 as a probe. See Fig. 1, lanes 5–9, for details of samples. (B) Results of inverse PCR. Inverse PCR was performed as shown in Fig. 3 with the ligation products shown in lanes 6–8 in Fig. 3A (lanes 1–3, respectively) using specific primers to pIG21. The 0.8 kb PCR product was arrowed. Map of the *Apo*I chromosomal fragment containing both junctions and the positions of primers used for PCR are shown below. A, *Apo*I; B, *Bfa*I. The *Apo*I site at the right (R) recombination junction is underlined. (C) Nucleotide sequence of the recombination junction on pIG21. The recombination junction deduced by the comparative alignment is indicated by arrowhead.

second) fold excess amounts of similarly digested chromosomal DNA as reference. Analysis of the clones from the library revealed that 45% (10/22) of the clones exhibited Southern hybridization patterns which were different from those of chromosomal DNA. The rest of the clones exhibited either no apparent differences between EC and chromosomal DNA or highly repetitive patterns which could not offer conclusive evidence regarding the degree of enrichment. All of the clones showing bands identical in size between chromosomal and EC DNAs, however, were also derived from the EC DNA fragments which were originally enriched in the EC DNA fraction. These results were contrasted by the ones before enrichment, where less than 8% showed the presence of recombination junctions and, therefore, have demonstrated the usefulness of the IGCR procedure for enriching recombination junctions.

Three clones (pIG11, pIG30 and pIG46) further analyzed by nucleotide sequencing to locate the exact site of the junctions showed that the EC DNA from which these clones were derived were mini-circles of 580 to 800 bp in size and contained only one *Bfa*I site. As described above, these mini-circles could not possibly be cloned when restriction enzymes such as 6-base cutters were employed because of their small size.

4.2. Mechanism of recombination appeared in the excision of chromosomal DNA

The nucleotide sequences of the recombination junctions appeared in three clones indicated that the recombination had most likely occurred by recognizing three (pIG11), five (pIG30) or four (pIG46) nucleotides of homology over distances of 580 to 800 bp. Since theoretically 3 bp of homology (GTC for pIG11), for example, would appear every 81 bp in the human genomic DNA (41% G + C), recombination could have occurred at either site. We detected no specific motifs or secondary structures that might enhance the recombination frequency at least in these clones. Short stretches of sequence homology at the recombination junctions are commonly observed in EC DNA as well as chromosomal DNA [5,14]. Although these short sequences could be generated by topoisomerases as well as recombinases which are responsible for homologous recombination, the recombination junctions described here was likely to be produced by homology-directed end joining, which required only short stretches of homology [29]. On the other hand, the junction which appeared in the clone pIG21 (Fig. 4) was a product of non-homologous recombination. A class of EC DNA generated by non-homologous recombination, probably through end-to-end ligation, has been reported recently [27].

To facilitate the analysis of recombination junctions, we also adopted inverse PCR in addition to IGCR. As described above, the use of inverse PCR greatly increased the efficiency of cloning for the chromosomal fragments carrying the junctions. We believe that the approach presented here is quite useful, particularly for future studies on the mechanism of recombination, in obtaining as yet unidentified site-specific recombination products with possible biological significance from specific tissues and cells because they produce genomic fragments with unique size by digestion with appropriate restriction enzymes.

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