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Isolation and characterization of endonuclease J: a sequence-specific endonuclease cleaving immunoglobulin genes

Tohru Kataoka[1], Sigeru Kondo, Miyuki Nishi, Mieko Kodaira and Tasuku Honjo*

Department of Genetics, Osaka University Medical School, 4-3-57, Nakanoshima Kita-ku, Osaka, 530 Japan

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ABSTRACT

An endonuclease activity which cleaves close to the recombination sites of the immunoglobulin J_K segments was found in extracts of chicken bursa of Fabricius and characterized after partial purification. The enzyme preparation also cleaved a V_K segment at its 3' end. A similar activity was found in mouse liver, mouse myelomas and Hela cells. The enzyme designated as endonuclease J introduces double-stranded cleavages preferentially at sequences containing G clusters of pBR322 as well as the J_K segments. However, not all the G clusters were cleaved by endonuclease J, suggesting that the enzyme recognizes additional sequences. Deletion of the conserved nonamer (GGTTTTTGT) located immediately 5' to the J_{K4} segment drastically reduced the cleavage activity of its immediate downstream G cluster. Although biological function of endonuclease J is not clear at this stage, the possibilities of its involvement in the immunoglobulin gene recombination and general recombination were discussed.

INTRODUCTION

Two types of somatic recombination play essential roles for creation as well as expression of the immunoglobulin gene during differentiation of B-lymphocytes. One type assembles two or three germline DNA segments to create the variable region gene; variable (V) and joining (J) segments for the light (L) chain V gene and V, diversity (D) and J segments for the heavy (H) chain V gene (1-8). Since these recombination reactions take place within the coding regions, the reactions are relatively site-specific although they are not exactly precise. The flexibility of the joinig site, in turn, contributes to augmentation of the V region diversity. The other type of recombination unique to the H chain gene takes place between switch (S) regions located in the 5'-flanking region of the H chain constant (C_H) gene (7,9,10). This recombination termed as S-S recombination replaces the C_H gene linked to the expressed V_H gene, providing genetic bases for the class switch phenomenon. Since the S-S recombination takes place in the intervening sequence, this reaction is not necessarily site-specific but rather region-specific (11,12). The S-S recombination is accompanied by

deletion of intervening DNA segments (13).

Nucleotide sequence determination of the V, D and J segments has shown that two common blocks of nucleotide sequences are conserved at their immediate flanking regions, i.e. a consensus heptamer (CACAGTG) and a consensus nonamer (ACA_5C_2) at the 3' flanks of the germline V and D segments; a consensus nonamer (G_2T_5GT) and a consensus heptamer (CACTGTG) at the 5' flanks of the germline D and J segments (3-8). The heptamer and nonamer sequences are mutually separated by spacers of 12 bp and 23±1 bp which correspond to the lengths of one and two turns, respectively, of Watson-Crick B helix and let the heptamer and nonamer align on one side of the helix (6,7). The recombination occurs only between two germline segments with different spacers (the 12/23 bp spacer rule). The conserved sequences are complementary between two recombining DNA segments and considered to serve as recognition signals for the recombination at least in part.

Three models have been proposed for the mechanism of the V-J and V-D-J recombination; a looping out-excision model (3-5), an unequal sister chromatid exchange model (14) and an inversion model (15-18). Recently, Lewis et al. (19) reported that the V_K gene and the J_K segment in a retroviral vector introduced into lymphoid cells were joined via inversion. The results favor the inversion model although the other models are not excluded. Deletion of D segments is accompanied by V-D-J recombination (20).

To elucidate detailed mechanisms of the immunoglobulin gene rearrangement it is essential to establish an *in vitro* recombination system. Our previous attempt to construct the *in vitro* system for the S-S recombination using λ phage vectors encountered various difficulties such as a novel recombinational activity in *in vitro* packaging extracts (21). We have also constructed recombinant plasmids carrying a genetic marker sandwitched between the V_L and J_L (or D and J_H) segments. The genetic marker was selected so that recombinants that lost the marker allow host bacteria to grow in a selection media (22). When these plasmids were incubated with extracts of various lymphoid cells, they produced a number of recombinants which partially deleted the genetic marker, and a few which recombined close to the expected site for the immunoglobulin gene recombination. Since the actual V-J or D-J recombination is not exactly precise, it is not easy to distinguish recombinants produced by the recombinational machinery of the immunoglobulin gene from the *in vitro* artifact. We have, therefore, decided to disect the reaction and looked for the enzyme which digests the

immunoglobulin gene segment at specific positions.

We and others reported preliminary evidence for the existence of a novel specific endonuclease in eukaryotic cells which cleaves the immunoglobulin J_K segments (22, 23). Further purification and characterization of the enzyme termed endonuclease J revealed that the enzyme introduces double-stranded cleavages at clusters of G bases of not only immunoglobulin gene segments but also pBR322. However, not all the G clusters were cleaved. The digestion of the J_{K4} segment was greatly reduced by deletion of its nonamer sequence. Possible biological functions of endonuclease J are discussed.

EXPERIMENTAL PROCEDURES

Materials Restriction endonucleases were purchased from Takara Shuzo Co. (Kyoto), Bethesda Research Laboratories (Bethesda) and New England Biolab (Boston). E.coli DNA polymerase I and polynucleotide kinase were obtained from New England Biolab. γ-^{32}P-ATP (specific activity 5000 Ci/mmole) and α-^{32}-dCTP (specific activity 2000-3000 Ci/mmole) were purchased from New England Nuclear (Boston).

Construction of recombinant plasmids A recombinant plasmid pJ-1 was prepared by inserting the XbaI-HindIII (1.7 kb) fragment of the J_K segments (3,4) into a unique BalI cleavage site of pNO1523 (24) after filling the ends with the large fragment of E.coli DNA polymerase I as illustrated in Fig. 1. pNO1523 contains the ribosomal protein (S12) gene of E.coli which makes streptomycin resistant host bacteria sensitive to the drug. A germline form of the mouse V_{K41} segment (2.1 kb) was excised from pBR-V_{K41} (5) by TaqI digestion. The 2.1-kb V_{K41} fragment was inserted into a unique ClaI cleavage site of pJ-1 to construct a recombinant plasmid pV•J-1 in which the V_{k41} and J_k segments were oriented in the same direction of transcription. pV•J-1 was partially cleaved with MstII and digests with a single cut were isolated. A XhoI linker (CCTCGAGG) was inserted into a MstII cleavage site after filling the cleaved ends by the large fragment of E.coli DNA polymerase I. The plasmid that has a XhoI site near the J_{K4} was selected and called pV•J-1(XhoI). Preparation of plasmid DNAs was carried out as described (8).

Assay of endonuclease J activity To assay specific cleavage activities of the J_k segments, 0.5 µg of pV•J-1 plasmid DNA (closed circle) was incubated at 37°C for 30 min with varying amounts of the cellular extracts in a 25-µl reaction mixture containing 12mM HEPES, pH7.9, 60mM NaCl, 7mM $MgCl_2$, 0.3mM

DTT, 2mM ATP, 0.1mM EDTA and 12% glycerol. Assay mixtures using unpurified extracts contained 5mM ATP to inhibit nonspecific DNases. After the incubation, the reaction mixture was deproteinized by phenol-chloroform-isoamyl alcohol extraction. DNA was precipitated with ethanol, cut with 2 units of BstEII at 65°C for 1 hour and electrophoresed in a 1% agarose gel. The gel was cut horizontally into two pieces and blotted separately to nitrocellulose filters (25) to avoid contamination of full-length plasmid DNA into lower molecular weight region during Southern blotting. Subsequently, the filters were hybridized with the 568-bp BstEII-AvaI fragment (probe A in Fig. 2) labeled by nick-translation (specific activity $2\text{-}5\times10^8$ dpm/μg DNA) and exposed to X-ray film after washing and drying as described (26). The amount of ^{32}P-labeled probes used for hybridization of the upper half of the gel was 20 times lower than that used for the lower half.

Preparation and purification of endonuclease J Whole cell extracts were prepared from chicken bursa Fabricius cells obtained from 17-19 day chick embryo essentially as described by Manley et al. (27). Extracts from mouse bone marrow and liver cells were prepared similarly. Whole cell extracts of chicken bursa cells (1.4mg protein) were applied onto a 0.3-ml Affi-gel Blue (Biorad) column and eluted stepwise with 0.1, 0.25, 0.5, 0.75, 1.0 and 2.0 M NaCl (5 ml each) in a buffer containing 20mM HEPES, pH7.9, 5mM $MgCl_2$, 1mM DTT, 0.1mM EDTA and 10% glycerol. A 15-μl aliquot of each fraction was assayed for its activity cleaving V_k and J_k segments. The 0.5 and 0.75 M eluates which have the endonuclease J activity contained 3.0 μg per 15 μl protein. Mouse liver extracts (17.6 mg protein) were purified similarly by an Affi-gel Blue column and 0.5 M NaCl eluate (4 mg) was further fractionated by chromatography on a Mono S column (Pharmacia). Enzymic activity was eluted from a Mono S column with a linear concentration gradient of NaCl from 0 to 1 M in the buffer described above. The final preparation contained 0.05 μg protein/μl.

Construction of deletion mutants of pV•J-1 The 4.4-kb MstII fragment of pV•J-1 containing the β-lactamase gene (see Fig. 1) was partially digested with Bal-31, ligated with XhoI linkers and cleaved with EcoRI. The XhoI-EcoRI fragments thus produced were ligated with the 4.5-kb XhoI-EcoRI fragment (carrying the V_k segment) of pV•J-1(XhoI). Reconstructed plasmids that make host cells ampicillin-resistant and streptomycin-sensitive were isolated and assessed for their extents of deletion by nucleotide sequence determination around their unique XhoI cleavage sites. For the introduction

of bidirectional deletions, pV•J-1(XhoI) was cut with XhoI, treated with Bal-31 and then circularized by ligation.

Determination of cleavage sites of endonuclease J Three µg each of pV•J-1 DNA were incubated with 90 µl (4.5 µg) of the fraction 7 of liver endonuclease J (Fig. 5) and the 5' ends of cleaved products were labelled by polynucleotide kinase and γ-^{32}P-ATP after bacterial alkaline phosphatase treatment. In some experiments 90 µl (18 µg) of 0.75M Affi-gel Blue fraction of chicken bursa enzyme were used. When the labeled DNAs were cut with BstEII and electrophoresed in a 1.2% agarose gel and autoradiographed, in addition to several faint bands four major bands corresponding to the molecular sizes expected by the cleavage close to the J_{K1}, J_{K2}, J_{K3} and J_{K4} segments were detected. The major bands were isolated from the gel and then cleaved with appropriate restriction enzymes. To determine the cleavage sites at the opposite strand of the J_K segments and in pBR322, BstEII was replaced by BamHI. The digests were then electrophoresed in DNA sequencing gels (28) together with molecular size markers. A 3.0-kb BamHI band produced by cleavage in the pBR322 sequence was also isolated and cleaved with two separate restriction enzymes. The molecular markers used were Maxam-Gilbert cleavage products (27) of a 115 bp DdeI-AccI fragment containing the J_{K4} coding region and its 5'-flanking sequence labeled at its 3' AccI cleavage site. Maxam-Gilbert cleavage products of a V_H gene of Xenopus laevis and pBR322 laveled at position 886 (AvaII site) were also used. As control experiments all the procedures were carried out exactly as above without incubation with endonuclease J.

RESULTS AND DISCUSSION

Specific cleavage of the J_K segment by extracts of bursa Fabricius Our strategy to detect a specific double-stranded cleavage of J_K segments is summarized in Fig. 2a. A plasmid pV•J-1 which contains the mouse germline V_{K41} gene and mouse J_K segments (Fig. 1) is incubated with whole cell extracts of B-lymphocytes, deproteinized and cut with BstEII. If a specific double-stranded cleavage is introduced at the immediate 5'-flank of each J_K segment of the plasmid, five bands of the expected lengths corresponding to the cleavage at the five J_K segments should emerge by Southern blot hybridization using the J_{K4}-J_{K5} segment (fragments A in Fig. 2) as probe.

In fact, when we incubated pV•J-1 DNA with cell extracts of bursa Fabricius an avian organ harboring developing B-lymphocytes, five discrete fragments hybridizing to probe A were produced as shown in Fig. 3 (lane

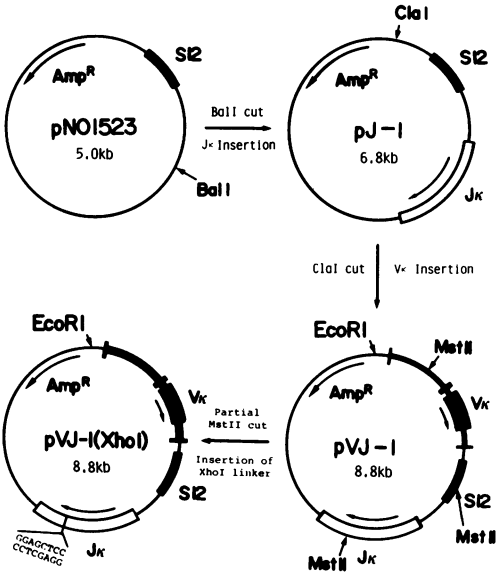


Figure 1. Construction scheme of pJ-1, pV•J-1 and PV•J-1(XhoI). Three steps to construct pV•J-1(XhoI) from pNO1523 were schematically shown. Inserted sequences were shown by wider lines. Directions of transcription of V and J sequences were indicated by arrows.

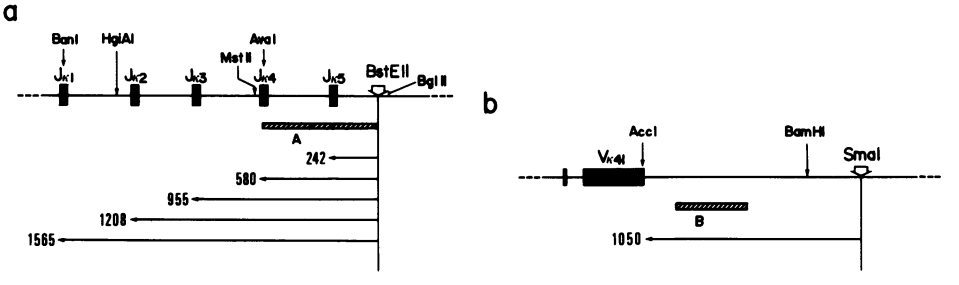


Figure 2. Assay strategies of site-specific cleavage products. Organization and restriction sites of the J_K and V_K segments (closed rectangles) are shown. Horizontal arrows with numbers (bp) indicate expected lengths of fragments produced by combination of site-specific cleavages at the 5' end of each J_K segment or 3' end of the V_K segment and the second digestion with restriction enzymes indicated. a) Assay of the J_K-specific cleavage of pV•J-1. BstEII was used as the second cut enzyme. Fragment A, a 568 bp-long BstEII-AvaI fragment of pJ-1 was used as probe for Southern hybridization experiment to detect cleaved products of the J_K segments. b) Assay of the V_K-specific cleavage of pV•J-1. SmaI was used as the second cut enzyme. Fragment B, a 346-bp HindIII-BamHI fragment of pBR322, was used as probe for detection of a cleaved product of the V_K segment.

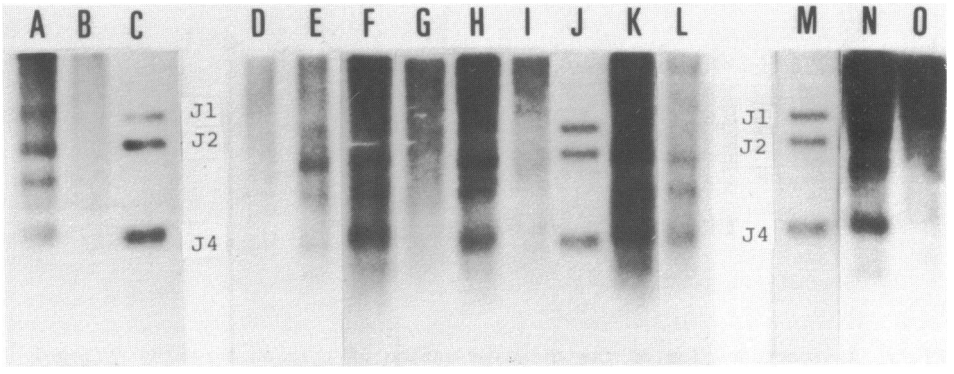


Figure 3. Characterization of endonuclease J. Endonuclease J preparation purified by Affi-gel Blue column chromatography (2.0 µg/10µl of 0.75M fraction of Fig. 4) was used except that whole cell extracts of chicken bura (35µg) were employed for lane A. Lanes C, J and M contain molecular markers i.e. a mixture of 0.3ng each of the digestion products of pV•J-1 with BanI+BstEII, HgiAI+BstEII and AvaI+BstEII which produce 1549-, 1265-, and 568-bp fragments, respectively. Standard assay conditions described in Experimental Procedure were used except for that specified in each lane. Special conditions in each lane are: A, none; B, without enzyme; D, with pretreatment of the enzyme with proteinase K (100µg/ml) for 10 min at 37°C; E, with the same proteinase K treatment as D after the reaction; F, none; G, without $MgCl_2$; H, 30mM NaCl; I, 200mM NaCl; K, without ATP; L, pJ-1 as substrate ; N, pV•J-1 cleaved by BstEII as substrate; O, DNaseI (10ng) was used in stead of endonuclease J. J_1, J_2 and J_4 indicate expected lengths of cleaved J_k segments.

A). The sizes of the hybridized bands were 1500, 1200, 950, 600 and 250 bp, in agreement with to those expected from the cleavage at the J_{K1}, J_{K2}, J_{K3}, J_{K4} and J_{K5} segments, respectively. Indeed the sizes of these bands were similar to those of molecular markers produced by combined digestion of pV•J-1 with BstEII and a restiction enzyme cleaving near each J_k coding sequence (BanI for J_{k1}, HgiAI for J_{k2} and AvaI for J_{k4}). The results indicate that the specific cleavage took place close to the immediate 5' flank of each J_K segment. The band corresponding to the J_{k5} segment is faint probably because small DNA fragments are inefficient to be transferred to nitrocellulose filters.

Appearance of these bands was dependent on digestion with BstEII (data not shown), but the restriction enzyme alone did not produce such bands (lane B). The presence of magnesium and a high concentration of ATP were required at this stage of purification.

Partial purification of the J_k-cleaving activity Since the whole cell extracts of the chicken bursa cells had considerable non-specific nuclease

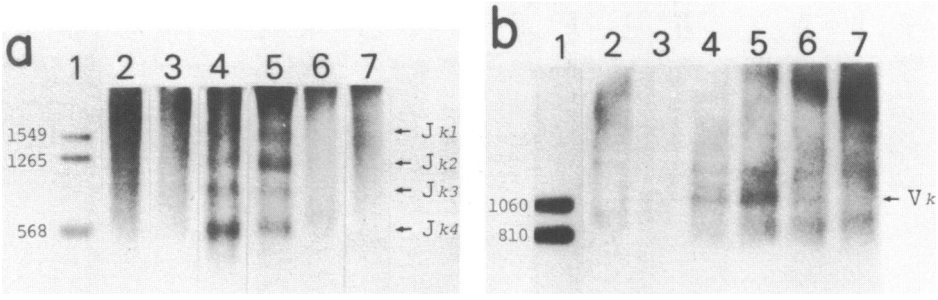


Figure 4. Fractionation of the cleavage enzyme of chicken bursa by Affi-gel Blue column chromatography. Whole cell extracts of chicken bursa cells were fractionated by an Affi-gel Blue column as described in Experimental Procedure. Aliquots (10µl) of fractions eluted with 0.1, 0.25, 0.5, 0.75, 1.0 and 2.0 M NaCl were assayed for cleavage activities in lanes 2, 3, 4, 5, 6 and 7, respectively. a) The J_K segment cleavage activity. Size markers (lane 1) are the same as used in Fig. 3. Arrows indicate expected lengths of cleaved J_K segments. b) The V_K segment cleavage activity. Size markers (lane 1) were prepared by mixture of digestion products of pV•J-1 with SmaI+AccI, and BamHl+AccI which produce 1060- and 810-bp fragments, respectively.

activities, we fractionated them by Affi-gel Blue column chromatography to purify the specific cleavage activity. Six fractions were obtained by stepwise elution with increasing salt concentrations. As shown in Fig. 4a, the J_k segment-cleaving activity was eluted with 0.5M and 0.75M NaCl. The cleaving activity in the 0.75M fraction was purified about 100-fold from the crude extracts.

We have tested a specific cleavage at the 3'-flanking sequence of the V_{K41} segment according to the strategy shown in Fig. 2b. pV•J-1 was incubated with each fraction of the Affi-gel Blue chromatography, deproteinized and cut with SmaI. The specific cleavage close to the V_K gene was shown by Southern-blot hybridization using the HindIII-BamHI fragment of pBR322 (fragment B in Fig. 2b) as probe. As shwon in Fig. 4b, incubation with the 0.5M and 0.75M eluates yielded a discrete band which is similar in size to the SmaI-AccI fragment of pV•J-1 (see Fig. 2b), indicating that the cleavage was introduced close to the immediate 3' flank of the V_{K41} segment.

The J_K-cleaving activity was found in extracts of other B cells such as mouse myelomas and bone marrow cells of chicken, mouse and rat. Unexpectedly, a similar activity was found in liver extracts partially purified by an Affi-gel Blue column although crude extracts did not yield the discrete bands probably because of overwhelming other DNases as shown in

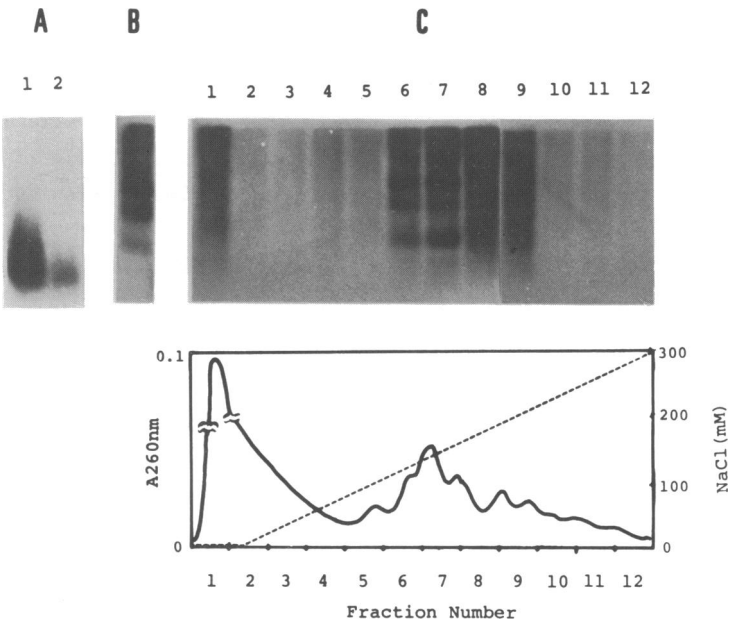


Figure 5. Purification of endonuclease J from mouse liver extracts. Enzyme preparations used are: (A) 1, 1μg crude liver extracts; 2, 10μg crude liver extracts: (B) 10μl of 0.5M NaCl eluate of Affi-gel Blue column chromatography: (C) 10μl aliquot of each fraction of Mono S chromatography. The number of lane corresponds to that of the enzyme fraction shown below. Solid line, A_{260}. Broken line, NaCl concentration.

Fig. 5A. The enzymic activity of mouse liver was further purified by Mono S column chromatography. The final preparation was purified about 500 folds from crude extracts. The J_K-cleaving activities in chicken bursa and mouse liver were indistinguishable from each other with respect to their enzymological properties and the specificity for the cleavage site as shown below. HeLa cells also contained the similar activity.

Characterization of the endonuclease activity Since the J_K-cleaving activity was destroyed by the treatment with proteinase K (lanes D and F in Fig. 3) or heat treatment at 90° for 5 min (data not shown), there is no doubt about the enzymic nature of the cleavage activity. RNase A treatment had no effect on the J_K-cleaving activity. We have tentatively referred to this enzyme as endonuclease J. The enzymic properties of chicken bursa endonuclease J were studied using the 0.75M fraction of the Affi-gel Blue chromatography (Fig. 4). The cleavage activity increased in proportion to the amount of enzyme. A 10-μl aliquot of the 0.75M fraction which

corresponds to the extracts of about 10^6 bursa cells cleaved approximately 0.3ng of pV·J-1 in 30 minutes as estimated from the intensity of the hybridized band. The partially purified exnzyme required magnesium ion and low salt concentration (Fig. 3 lanes G-I) but did not require ATP (lane K) or other ribo- or deoxyribo-nucleotides for its activity. The role of ATP on the cleavage using crude extracts seems to be ascribed to inhibition of nonspecific DNase activities.

Since pJ-1 which has only the J_K segment was cleaved with the comparable efficiency by endonuclease J (lane L in Fig. 3), pairing of the J_K and V_K segments is not prerequisite for the cleavage activity. The linearized pV·J-1 plasmid yielded bands of the same sizes as the closed circular plasmid (lane N), excluding the possibility that the cleavage activity is due to non-specific DNase recognizing pallindromic sequences in closed circular DNA molecules as reported by other investigators (9,30). This possibility is considered because the 5'-terminal sequences of the J_K-coding regions and their immediate 5'-flanks usually contain relatively long pallindromic sequences, a part of which comprises the conserved heptamer sequence. DNase I was incapable of yielding discrete bands (lane O).

Deletion of the conserved heptamer and its immediate flanking sequence abolished the the J_K-cleaving activity If endonuclease J is involved in the V-J recombination, it is likely to recognize the conserved heptamer and nonamer sequences flanking the V and J segments. To address this question we have constructed series of mutants carrying unidirectional (toward downstream) and bidirectional deletions surrounding the MstII site located 6 bp upstream of the nonamer sequence of the J_{K4} segment. DNAs of these deletion mutants were tested for the cleavage by partially purified endonuclease J of chicken bursa. Structure of the deletion mutants and experimental results using them as substrate are shown in Fig. 6.

The intensity of the band due to the cleavage at the J_{K4} segment was decreased considerably when the nonamer sequence was interrupted or totally deleted (Δ1-Δ3). When the deletion of mutants passed 27 bp 5' to the heptamer sequence (lanes Δ4-Δ10), the band of the J_{K4} segment virtually disappeared whereas the intensities of the other three bands corresponding to the cleavages at the J_{K1}, J_{K2} and J_{K3} segments remained the same as those of the intact plasmid. Estimation of the densities of the bands by spectrophotometric scanning of the autoradiogram indicates that the intensities of the J_{K4} segment bands of pV·J-1(XhoI), clone Δ2 and clonesΔ4-Δ11 correspond 28%, 10% and less than 1%, respectively, of the sum of the

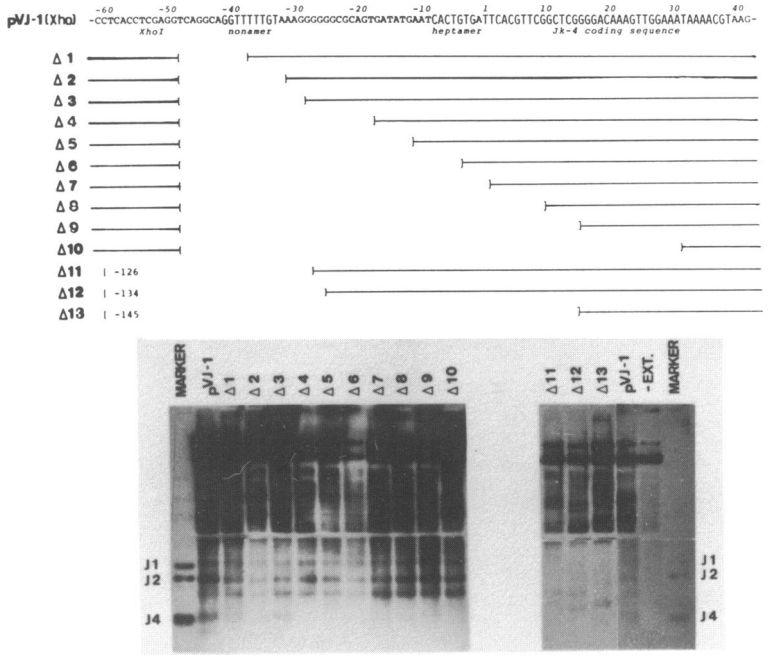
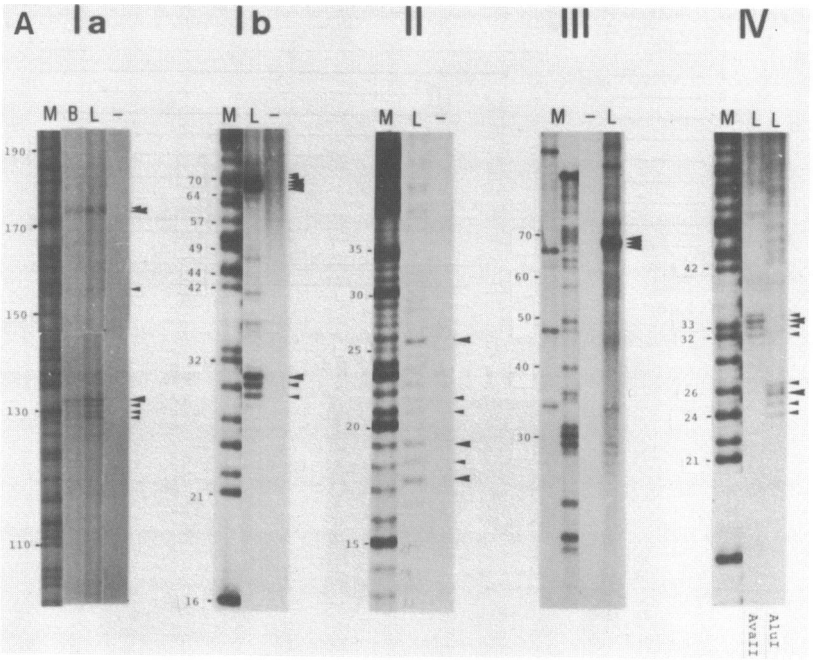


Figure 6. Cleavage of plasmid DNAs containing deletions in the J_{k4}-flanking sequence by endonuclease J. The top line represents the nucleotide sequence around the J_{k4} segment of pV•J-1(XhoI). Clones △1-△10 have unidirectional deletions, and clones △11-△13 bear bidirectional deletions. The 5' and 3' ends of deleted segments are indicated by brackets and numbers of deleted nucleotides are shown when the 5' deletions exceed the XhoI cleavage site. Autoradiograms of the Southern blots of J_K cleavage products using deletion plasmids are shown at the bottom. Plasmid DNA used in each lane is indicated. Marker lane contains the same fragments as described in the legend of Fig. 3. A 10-μl aliquot of the 0.75M fraction of the Affi-gel Blue chromatography was used for each lane except for the lane (-Ext) which does not contain any enzyme.

densities of all the five bands of each digest. Similar results were obtained using plasmids carrying bidirectional deletions (clones△11-△13) and the sizes of the bands became smaller in accord with the extent of deletion. These results clearly suggest that endonuclease J recognizes nucleotide sequence(s) between the nonamer and the heptamer sequences and that the nonamer sequence is effective but not indispensable for the cleavage. Endonuclease J of mouse liver showed the same profile of the cleaving activity with series of the deletion mutants.

Hochtl and Zachau (18) found that a DNA segment of unknown function, which is flanked by the heptamer but not by the nonamer-like sequence, recombined precisely with the J_K segment, suggesting that the heptamer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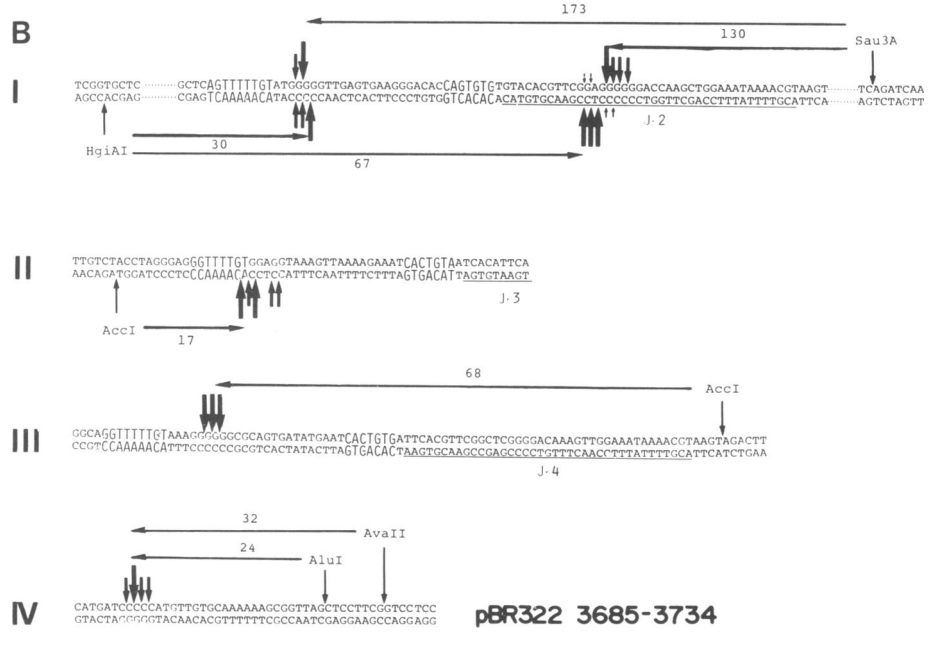
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sequence is more important for V-J recombination. It is conceivable that the nonamer sequence may not be essential for the cleavage step but indispensable for the pairing of the J_K segment with the V_K segment. In any case, the nonamer sequence seems to be less important and less conserved than the heptamer sequence although experiments using base substitution mutations are required to further investigate the nucleotide sequences recognized by endonuclease J.

Endonuclease J cleaves sequences containing clustered G We have determined the cleavage sites of endonuclease J by post labeling of the cleaved termini and subsequent digestion with restriction endonucleases. Double-stranded cleavage sites of endonuclease J were estimated by lengths of terminally labeled fragments produced by the second cleavage at known restriction sites. As shown in Fig. 7, endonuclease J cleaved the J_{K2} segment at 130 bp and 173 bp 5' to the Sau3A site which correspond to 14 bp 3' and 27 bp 5' to the 5' end of the J_{K2} coding sequence, respectively. Similar experiments with the anti-coding strand revealed essentially the same cleavage sites surrounding the J_{K2} segment. A few base discrepancy between two strands may be due to the usage of different size markers. The enzyme cleaved 28-30 bp 5' to the J_{K3} coding sequence and 25-27 bp 5' to the J_{K4} coding sequence. All the cleavage sites of the J_{K2}, J_{K3} and J_{K4} are similar in the location relative to the respective coding region, namely close to the 3' end of the nonamer sequence except for one within the coding region of the J_{K2} segment.

The sequences surrounding the cutting sites of the J_{K2} and J_{K4} segments are comprised of $\frac{GGGGG}{CCCCC}$ or $\frac{GGGGGG}{CCCCCC}$. GTGGAGG is the sequence surrounding the cutting site of the J_{K3} segment. The sequence immediately 5' to the

Figure 7. (A) Determination of double-stranded cleavage sites of endonuclease J. pV•J-1 DNA was cleaved with endonuclease J and termini of the digests were labeled. Detailed strategy for cleavage site determination is described in Experimental Procedure. The digests were cut with BstEII (Ia and III) or BamHI (Ib, II and Iv). Isolated DNA fragments and restriction enzymes used for the second cleavage are as follows; Ia, J_{K2} and Sau3A; Ib, J_{K2} and HgiAI; II, J_{K3} and AccI; III, J_{K4} and AccI; IV, pBR322 and AvaII or AluI as indicated. DNA fragments used for size markers (M) are; Ia, a V_H segment of Xenopus laevis; Ib, pBR322; II, pBR322; III, J_{K4}; IV, pBR322. Enzymes used are; B, endonuclease J of chicken bursa Fabricius. L, endonuclease J of mouse liver. -, control without endonuclease J. (B) Schematic representation of the putative cleavage sites of the J_k segments. The nucleotide sequences around the J_{K2} (I), J_{K3} (II) and J_{K4} (III) segments are taken from literature (3, 4) on which the putative cleavage sites of endonuclease J and restriction sites used are shown by arrows. The heptamer and nonamers are shown by larger letters and coding sequences are underlined. Cleavage sites in pBR322 sequence (IV) of positions 3686-3734 was taken from literature (34).

J_{K1} coding region which comprises a G-rich sequence, GTGGTGG is also cleaved (data not shown). The possibility that the $\binom{G}{C}_n$ sequence alone can serve as a recognition signal for endonuclease J was tested with use of pBR322 as substrate. The enzyme digested pBR322 at positions 3692-3696 $\binom{CCCCC}{GGGGG}$ (Fig. 7). Other positions digested are 1876-1899 ($C_5N_{14}C_5$), 2195-2211 ($GGCG_3TGTCG_4CG$) and 2546-2556 ($CCGC_4TG$). These results suggest that endonuclease J recognizes and digests $\binom{G}{C}_n$. However, endonuclease J cleaves pBR322 much more poorly than J_K segments except for positions 1876-1899. In addition, not all the G clusters of pBR322 were cleaved. For example, $(G)_6$ sequence at positions 2797-2803 is hardly cleaved. Similarly, clone $\Delta 9$ containing $\frac{GGGGG}{CCCCC}$ surrounding the artifitial deletion site was not cleaved with the enzyme (Fig. 6). These results indicate that endonuclease J recognizes other structure in addition to $\binom{G}{C}_n$. It is noteworthy that all the cleaved G clusters shown in Fig. 7 are flanked by T clusters.

Disappearance of J_{K4} cleaving activity in clones $\Delta 4$-$\Delta 10$ is explained by the absence of the G cluster specifically digested by endonuclase J. Reduction of the cleaving activity in clones $\Delta 1$-$\Delta 3$ also suggests that the endonuclease J activity is affected by other sequences than the G cluster.

Our endonuclease J preparation also contained a nicking activity replatively specific to G clusters although we do not know whether or not the nicking activity is ascribed to endonuclease J. The G clusters close to D_{Q52}, J_{H2} and J_{H4}, position 3448 of pBR322 were all nicked. The J_K-cleaving activity observed by Desiderio and Baltimore (23) shares many properties with endonuclase J. The cleavage sites around the J_{K2} segment are identical. However, they were unable to detect the cleavage close to the J_{K3} segment in contract to our results. It is not known whether or not the J_{K3} segment is inert for V-J recombination although the J_{K3} segment is incapable of producing a light chain because of a mutation in the splicing donor site.

Biological roles of endonuclease J The absence of the tissue specificity and the loose specificity of the cleavage site are unfavorable to the hypothesis that endonuclease J alone can give specific cleavages for the immunoglobulin gene rearrangement. What is the biological function of endonuclase J? Since the total activity of endonuclease J is far less than other non-specific DNases, especially those found in liver, endonuclase J would be ineffective as a degradative enzyme. It is possible to consider that endonuclase J is involved in general recombination, the precise mechanism of which is not known. Recent model (31) postulates that double-

stranded breakage is involved for DNA recombination.

It is also possible that endonuclase J can cut the immunoglobulin gene more specifically in collaboration with other regulatory proteins which may constitute subunits of putative recombinase. Regulatory proteins may help endonuclease J be more specific by alteration of the chromatin structure of the immunoglobulin gene. It is well known that the holoenzymes of RNA and DNA polymerases require many other proteins to recognize the correct initiation site. Although exonuclease V encoded by rec B and rec C genes (32) is shown genetically to recognize the G rich-sequence χ $\binom{GCTGGTGG}{CGACCACC}$ (33), purified exonuclease V cannot recognize χ sequence in vitro. Since chromatin structure correlates with DNase hypersensitive sites, the site specificity of DNA cleavage would certainly be controlled by chromatin structure at least in part.

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1 Present address; Cold Spring Harbor Laboratory, P.O.Box 100, Cold Spring Harbor, New York 11724, U.S.A.

*, To whom correspondence should be addressed.

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