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Characterization of immunoglobulin enhancer deletions in murine plasmacytomas

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We have analyzed enhancer deletions found in murine plasmacytomas by DNA cloning. This analysis revealed that the deletions occurred between the J_H region and the switch region, removing the Ig heavy chain enhancer. The loss of the enhancer did not significantly affect the level of heavy chain expression as determined by RNA blots. Nucleotide sequence analysis revealed that there are no characteristic or homologous sequences around the recombination site. Extra nucleotides were found at the recombination sites, in a manner analogous to Ig and T-cell receptor V-D-J joining. The germline J_H and switch sequences involved in the deletion were analyzed by the *in vitro* DNA cleavage system with an endonucleolytic activity purified from mouse fetal liver nuclear extracts. It was found that the germline J_H DNA was strongly cleaved at the deletion recombination site.

Key words: enhancer deletions/mRNA expression/Ig heavy chain genes/*in vitro* cleavage of DNA

Introduction

DNA elements responsible for the enhanced expression of eucaryotic genes were first discovered near the origin of DNA replication in SV40 (Banerji *et al.*, 1981; Moreau *et al.*, 1981). Enhancers are usually associated with DNase I-hypersensitive sites and can act on a nearby promoter in either orientation (Gluzman and Shenk, 1983; Khoury and Gruss, 1983). In 1983, four groups independently reported an Ig heavy chain enhancer in the intron separating the J_H region and the C_μ exons (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Mercola *et al.*, 1983; Neuberger, 1983). This element conveniently lies 5′ to the switch recombination site, and is therefore unaffected by Ig class switching. Similar enhancer elements have also been identified in the κ light chain genes (Bergman *et al.*, 1984; Picard and Schaffner, 1984; Queen and Baltimore, 1983).

Recently, four groups have reported enhancer deletions in Ig heavy chain genes. For example, Wabl and Burrows (1984) found that a subclone of the pre-B cell line 18-81 (Alt *et al.*, 1982) lacks the Ig enhancer on the functional heavy chain allele. Klein *et al.*, (1984) reported another example of the enhancer deletion in an IgD hybridoma. In both cases the deletion did not affect the ability of the Ig genes to be expressed at the same level as in the parental cell line. A similar deletion was identified by Eckhardt and Birshtein (1985) when they isolated class switch variants of MPC11 in culture.

We have analyzed > 100 myeloma DNA samples for aberrant DNA rearrangements with various J_H region probes. We found that some of the myeloma samples such as M70A, M41, M467, M195 and M11 exhibited only one hybridizable allele when probed with the fragment containing the enhancer element (3′-J_H probe). This can be explained either by a chromosomal loss or by a local DNA deletion in the area where the enhancer resides. We found that 11 myelomas were of this type among the 110 myelomas tested. We have now characterized two examples of the enhancer deletion by DNA cloning and nucleotide sequencing. Enhancer deletions were further analyzed by the *in vitro* cleavage assay with a mouse fetal liver extract (Hope *et al.*, 1985) using germline J_H and switch region DNAs as subtrates.

Results

Enhancer deletion in the functional allele of a IgA myeloma

To study the 3′-J_H deletion found in myeloma M467, we cloned the DNA region containing the deletion into phage vector λ gtWES (Leder *et al.*, 1977). The cloned DNA was analyzed by restriction enzyme mapping and DNA sequencing. As shown in Figure 1A, two positive bands, 5.2 kb and 3.5 kb, were detected with the J_{H1}-J_{H4} (J) probe, however, only the 5.2-kb band hybridized to the 3′-J_H (E) probe. Because the 3.5-kb fragment does not hybridize with the 3′-J_H probe, it must contain a deletion in the 3′-J_H region. As shown in Figure 1B, nucleotide sequence analysis and restriction mapping revealed that this allele contains three types of DNA rearrangements, a functional V-D-J joining, an isotype switching to Cα, and an ~ 3-kb deletion between J_{H3} and the Cα switch region (Figure 1B). It should be mentioned that the V-D-J joining is in phase and the V-region sequence is derived from one of the members of the $V_{H\text{-}558}$ gene family (Figure 1C; Cohen *et al.*, 1982). This correlates with the previous demonstration that the M467 myeloma secretes IgA(κ) antibody specific for flagellin (Potter, 1970, 1972). The other J_H allele (5.2 kb) was also cloned from M467 DNA and analyzed by nucleotide sequencing. It was found that this allele is a D-J intermediate which is a common feature of non-functional heavy chain alleles (Sakano *et al.*, 1981).

Steady-state analysis of Ig mRNA expression in the plasmacytoma with the enhancer deletion

As mentioned earlier, two groups (Klein *et al.*, 1984; Wabl and Burrows, 1984) have recently reported that the deletion of the enhancer does not appear to affect Ig expression. We also found that the level of expression of α-mRNA is not significantly affected by the loss of the enhancer. The level of α-mRNA in M467 is almost identical to three other IgA(κ)-producing tumors M167, M460D and M603. As shown in Figure 2, little difference was found when the amount of α message in M467 was compared with that in M167 (lanes 1 and 2). As negative controls, we also isolated cytoplasmic mRNA from a pre-B cell line 38B9 (Alt *et al.*, 1984) and a fibroblast cell line L929 (Sanford *et al.*, 1948), neither of which should have mRNA hybridizable to the probes used in these experiments. As expected, no hybridizable RNA was detected in these two cell lines with both probes (lanes 3 and 4, Figure 2). As an internal control, we looked at κ mRNA expression and found that the level of the κ message is similar in all tumors analyzed (lanes 5 and 6, Figure 2).

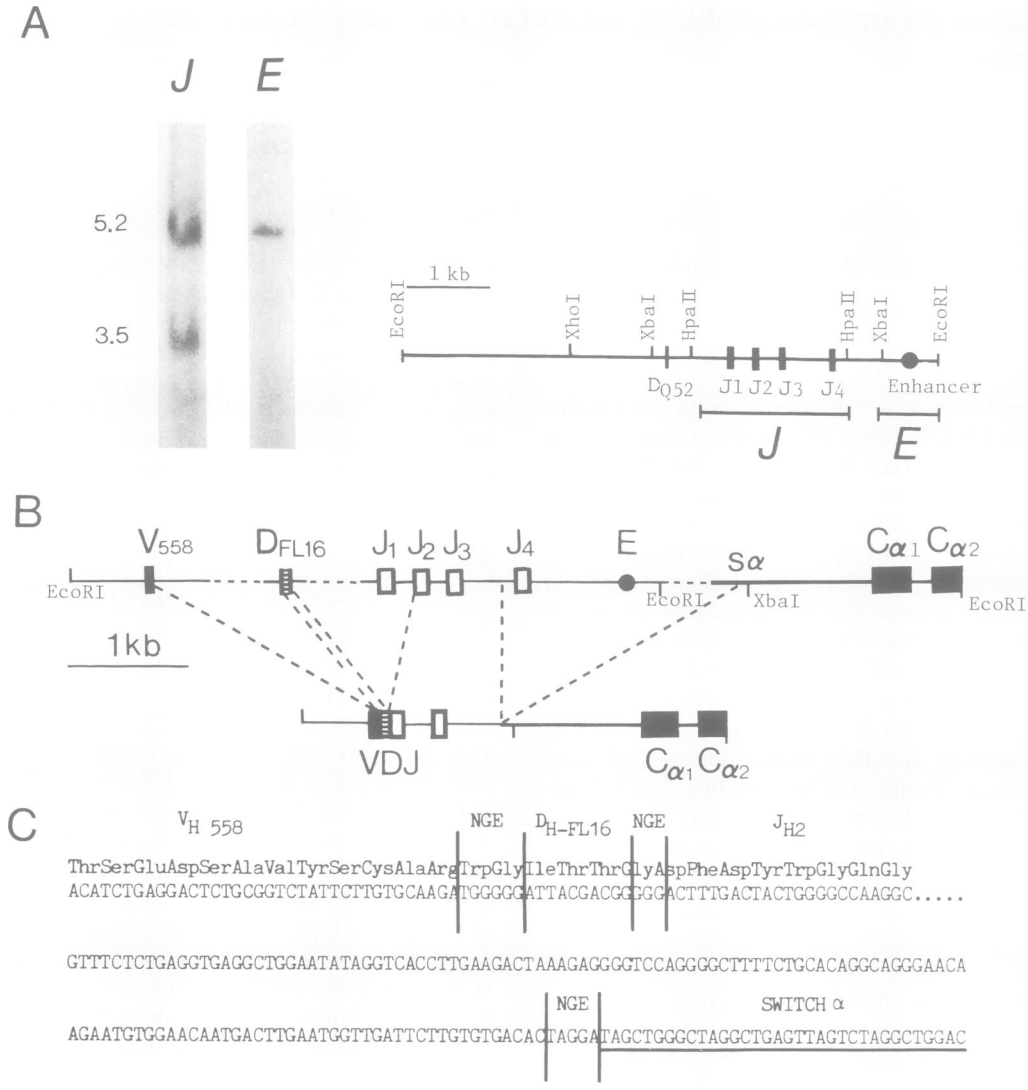

Fig. 1(A). Southern blot analysis of MOPC467 myeloma DNA. Duplicate filters were hybridized either to J_{H1}-J_{H4} (J) or to a 3′-J_H probe (E) containing the enhancer. (**B**) The J_{H1}-J_{H4} (J) probe detected an additional band (3.5 kb) in the *Eco*RI digest of MOPC467 DNA which had not been previously detected with the enhancer (E) probe. (**C**) Nucleotide sequences around the V-D-J joining and the enhancer deletion sites in the 3.5-kb MOPC467 clone. The determined nucleotide sequences were compared with published germline sequences for the $V_{H\text{-}558}$ (Cohen *et al.*, 1982) and C_α genes (Tucker *et al.*, 1981). Recombination sites are indicated by vertical lines. Note that extra nucleotides (non-germline elements) are present both at V-D-J joining and enhancer recombination sites.

Enhancer deletion is not restricted to the functional J_H allele

Some myelomas were found to contain the enhancer deletion in the non-functional J_H allele. For example, myeloma M195 showed a D_{Q52}-J_H fragment whose size (~ 15 kb in an *EcoRI* digest) could not be explained by a simple D-J recombination. As shown in Figure 3, this aberrant allele was cloned and characterized by restriction enzyme mapping and DNA sequencing. In addition to the D-J recombination, the non-functional allele of M195 was found to contain two more recombination events, a 1.5-kb deletion spanning from J_{H4} to the switch region of Cμ and a 15:12 *c-myc* translocation (Figure 3).

Nucleotide sequences around the deletion recombination sites

To study the molecular mechanisms of the enhancer recombination, two pairs of germline sequences containing the deletion site were compared (Figure 4). Although both deletions, one identified in M467 and the other in M195, begin around J_{H4} and end in a switch region, no characteristic sequences are evident around the recombination sites. Furthermore, there is no sequence homology between the 5′ and 3′ ends of the deletion. It is interesting to note that both in M467 and M195 extra nucleotides are inserted at the site of the deletion; TAGGA in M467 and A in M195 (Figure 4). These extra nucleotides appear to have been inserted enzymatically during the recombination process as in the V-D-J joining both in Ig heavy- and T-cell receptor β-chain genes (Alt *et al.*, 1982; Hagiya *et al.*, 1985, 1986; Siu *et al.*, 1984.

In vitro cleavage of germline sequences corresponding to the deletion recombination sites

Recently, we have identified an endonucleolytic activity which cleaves Ig DNA segments around the recombination sites (Hope *et al.*, 1985). The activity was identified in mouse fetal liver and chick embryo bursa cells. The activity cleaves DNA at dinucleotide pairs, $\frac{TG}{AC}$, in many cases at $\frac{TGG}{ACC}$. To test the possibility that the same endonucleolytic activity is responsible for the enhancer deletion occurring in the J_H region, we cleaved the germline J_H DNA *in vitro* with the mouse fetal liver

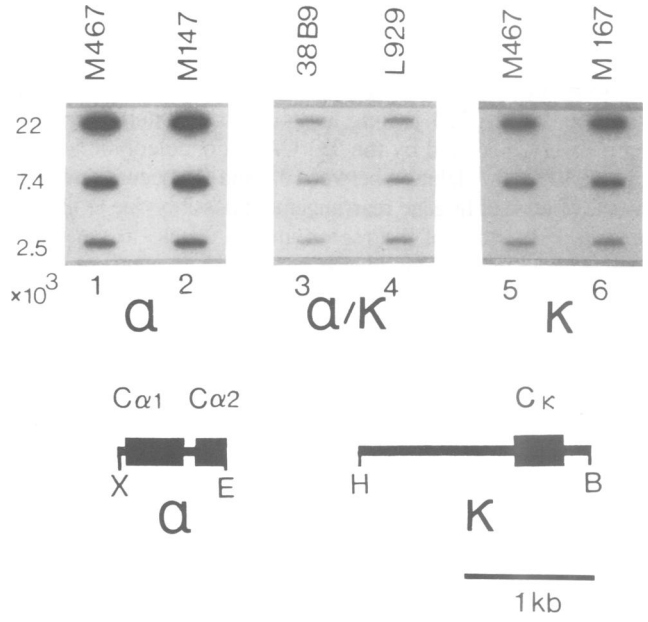

Fig. 2. Analysis of the steady-state level of mRNA in MOPC467. Cytoplasmic mRNA was obtained from 1 × 10^7 cells (White and Bancroft, 1982) and slot-blotted, after serial dilution, onto nitrocellulose filters. Samples 1 and 2 were hybridized with the C_α probe, samples 5 and 6 were probed with C_κ and samples 3 and 4 were hybridized to both probes. Probes used (α and κ) for this analysis are depicted at the bottom of this figure.

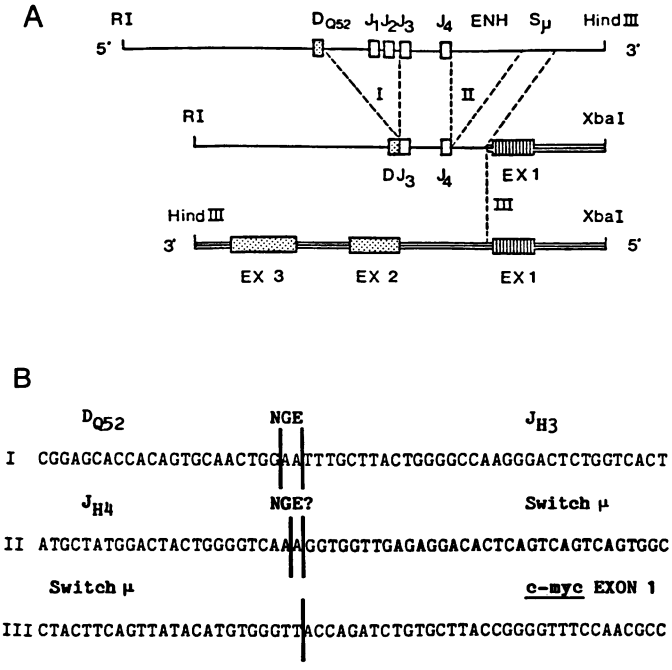

Fig. 3. Enhancer deletion identified in the non-productive J_H allele of plasmacytoma MOPC195. (**A**) Three recombination events, **I**, **II**, **III**, are involved in the formation of this non-productive allele. They are (**I**) a D_{Q52}-J_{H3} joining, (**II**) an Ig enhancer deletion and (**III**) a c-*myc* oncogene translocation. (**B**) Recombination sites, indicated by vertical lines, were determined by comparing the rearranged sequences with their germline sequences (Sakano *et al.*, 1981; Stanton *et al.*, 1983).

nuclear extract (Hope *et al.*, 1985). As shown in Figure 5, a strong cleavage was detected in the trinucleotide CCA at the deletion site. Since the cleavage occurred one base downstream to the recombination site, an exonucleolytic activity could have

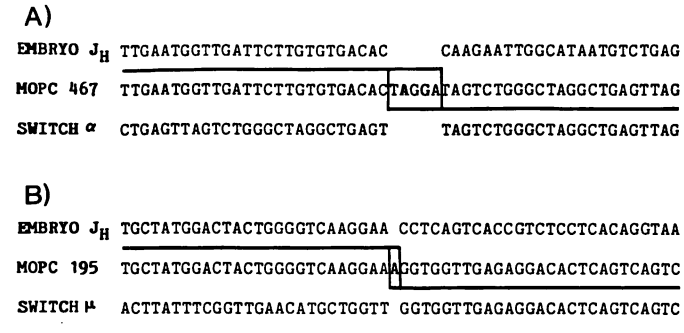

Fig. 4. Two sets of nucleotide sequences around the enhancer deletion sites identified in plasmacytomas MOPC467 (**A**) and MOPC195 (**B**). The embryonic (J_H and switch) and rearranged sequences are compared. Recombination sites are indicated by vertical lines. Non-germline elements (NGE) at the recombination sites are in boxes.

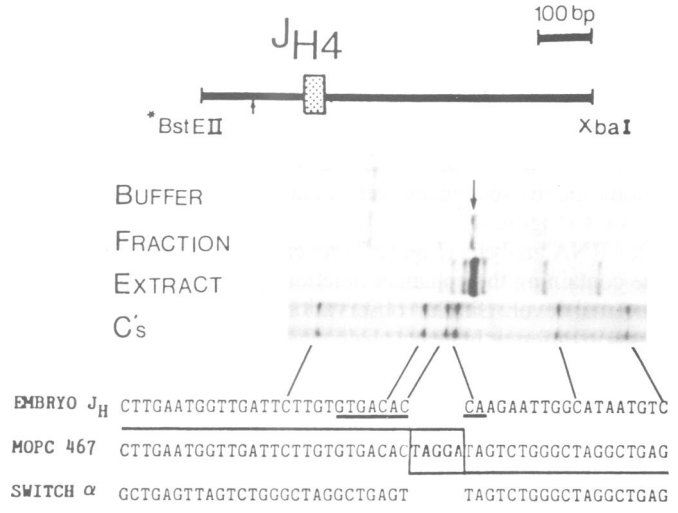

Fig. 5. *In vitro* cleavage of J_H DNA with the TG/CA cleaving activity of mouse fetal liver. The ^{32}P end-labelled fragment (*Bst*EII-*Xba*I) was reacted with the crude nuclear extract of mouse fetal liver (Extract), partially purified nuclease fraction (Fraction) or with reaction buffer without the extract (Buffer). The reaction products were precipitated with ethanol and separated in a 6% sequencing gel with the C-reacted sample (Maxam and Gilbert, 1980) as a size marker. Note that a strong cleavage (indicated by an arrow) is found one nucleotide 3′ from the enhancer deletion site identified in MOPC467. The inverted heptamer GTGACAC is underlined, and the non-germline element TAGGA is in a box.

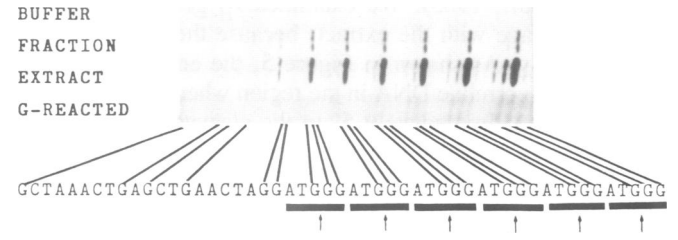

Fig. 6. *In vitro* cleavage of mouse Sα switch region. The *Xba*I-*Pst*I (Gerondakis *et al.*, 1984) fragment isolated from the Sα region was reacted with the TG/CA nuclease, and the cleavage products were separated in a DNA sequencing gel alongside a G reacted size marker. Repetitive sequences (ATGGG) found in the S_α region are underlined. *In vitro* cleavage sites for the TG/CA nuclease are indicated by arrows.

removed one nucleotide after the cleavage prior to the joining. Such exonucleolytic trimming is known to be a common feature in V-(D)-J recombination (Lewis *et al.*, 1985).

The recombination breakpoint in the switch α region has been identified in a 20-bp sequence (CTGGGCTAGGCTGAGT↓TAGT) which is tandemly repeated six times over a 120-bp stretch. The germline switch α region was also analyzed for the *in vitro* cleavage. Unlike the J_H breakpoint, we did not find the dinucleotide $\frac{TG}{AC}$ at the Sα deletion site and no *in vitro* cleavages were detected on the corresponding germline Sα sequences (data not shown). However, very strong cleavages were observed in the ATGGG repeats located ~50 bp from the deletion site (Figure 6). The implication of this observation will be discussed below.

Discussion

In this report, we characterized two examples of enhancer deletions identified in murine plasmacytomas by DNA cloning and nucleotide sequencing. Although enhancer deletions have been reported independently by other groups, this is the first attempt to charactertize the deletion at the nucleotide level. One of the examples is a functional α heavy chain gene isolated from the anti-flagellin IgA myeloma MOPC467, and the other is the non-functional J_H allele of MOPC195 which contains a *myc* oncogene translocation (Figures 1 and 3). Although both deletions begin within the J_H region and end in a switch region, no characteristic or homologous sequences were evident around the recombination sites (Figure 4).

Our RNA analysis (Figure 2) revealed that the M467 α chain gene containing the enhancer deletion is expressed at a relatively normal level. Similar observations were reported by other groups in different Ig heavy chain genes (Klein *et al.*, 1984; Wabl and Burrows, 1984; Zaller and Eckhardt, 1985). An intriguing question is why the enhancer deletion does not affect the level of transcription. It is possible that the Ig heavy chain enhancer is required for the transcriptional activation of the heavy chain gene, although it might not be necessary to maintain its transcriptional activity (Mason *et al.*, 1985; Zaller and Eckhardt, 1985). Alternatively, there might be other DNA elements, located in the vicinity of the Ig genes, which could replace the function of the deleted enhancer.

Recently, we have identified and isolated an endonucleolytic activity which cleaves Ig joining (J) sequences in the vicinity of the recombination sites (Hope *et al.*, 1985). This activity can be isolated from nuclear extracts of cells in mouse fetal liver or chicken embryo bursa. These tissues are sites where B-cell progenitors first appear during embryonic development. The activity is known to cleave Ig recombination sequences at the dinucleotide $\frac{TG}{AC}$ (Hope *et al.*, 1985). We examined J_H germline DNA for *in vitro* cleavage with the extract, because the 5′ deletion site is close to J_{H4}. As shown in Figure 5, the endonuclease does indeed cleave germline DNA in the region where the M467 deletion took place. Immediately 5′ to the cleavage site we found a heptamer GTGACAC which is identical, although inverted, to the consensus sequence CACTGTG used for V-(D)-J recombination. This might indicate that the cleavage activity which is normally used for the V-(D)-J joining was responsible for the cleavage at the 5′ deletion site. The switch region sequence was also examined for the cleavage, since the breakpoint of the enhancer deletion occurred in the Sα switch region. *In vitro* cleavage analysis revealed that the endonuclease cleaved Sα DNA ~ 50 bp from the recombination site, probably recognizing the TG dinucleotide in the ATGGG repeat unit (Figure 6). We speculate that cleavage occurred at one of these repeats and exonucleolytic trimming produced the observed recombinant. It is interesting to note that other switch regions including Sμ (Sakano *et al.*, 1980; Nikaido *et al.*, 1981) and Sγ (Kataoka *et al.*, 1981; Szurek *et al.*, 1985) also contain TG or TGG in their repeat units. We postulate that the enhancer deletion is an aberrant event related to the V-(D)-J joining and/or to the switch recombination possibly mediated by the TG/CA endonucleolytic activity. It appears that the deletion between J_H and S_H occurs during the process of normal Ig gene rearrangement possibly due to the open chromatin structure of the region and its access to the recombination machinery. This idea is supported by a recent finding by Eckhardt and Birshtein (1985) that switch recombination in cell culture (MPC11) sometimes leads to deletion of the Ig enhancer.

The *myc* oncogene translocation is another aberrant recombination event generally found at or in the vicinity of switch recombination sites (for reviews, see Perry, 1983; Robertson, 1983). The recombination usually occurs between an Ig switch region and c-*myc* exon 1. DNA sequence analysis of the recombination sites showed that there is no evident sequence homology between the two DNA regions, although some c-*myc* breakpoints were reported to lie around switch-like sequences (Dunnick *et al.*, 1983; Gerondakis *et al.*, 1984). Recently, we noticed that the dinucleotides TG/CA or trinucleotides TGG/CCA can be found at recombination sites in the c-*myc* sequence. Our preliminary experiments have shown that the endonucleolytic activity in fact cuts germline c-*myc* DNA precisely at the breakpoint identified in myeloma M195. These observations might imply that the TG/CA cleaving activity is also involved in the process of *myc* oncogene translocation.

Materials and methods

General methods

Polyacrylamide and agarose electrophoresis, preparation of plasmid and phage DNAs, digestion with restriction enzymes, radiolabelling of DNA by nick translation, ligations and bacterial transformations were all carried out by standard procedures (Maniatis *et al.*, 1982, and references therein). All restriction enzymes, DNA polymerase I (Kornberg and Klenow) and T4 ligase were obtained from New England Biolabs.

Cells

BALB/c plasmacytomas were obtained from Litton Bionetics (MD) and inoculated into pristane-primed BALB/c mice until the tumor cells could be extracted for both DNA and RNA preparation (see below). The fetal liver-derived pre-B cell line 38B9 and the mouse fibroblast cell line (connective tissue) L929 (Sanford *et al.*, 1948) were gifts of Dr.F.W.Alt and Dr.R.Mishell, respectively. NZB myelomas were kindly provided by Dr.M.Weigert.

DNA analysis

Plasmacytoma DNA was obtained from inguinal and peritoneal tumors by Proteinase K digestion and phenol extraction according to established procedures (Maniatis *et al.*, 1982). DNA samples were digested to completion with *Eco*RI, separated in 0.8% agarose gels, blotted to nitrocellulose, and then analyzed by Southern hybridization (Southern, 1975; Wahl *et al.*, 1979). For the cloning of the 3.5-kb J_H allele, genomic DNA from M467 was digested with *Eco*RI and fractionated in a preparative agarose gel (0.6%). The fraction containing 3–4 kb DNA fragments was isolated and ligated to λ gtWES (Leder *et al.*, 1977) phage arms, packaged *in vitro* (Hohn, 1979), and screened with the J_{H1-4} probe by the method of Benton and Davis (1977). The cloned 3.5-kb J_H fragment was subcloned into the plasmid vector PUC8 (Vieira and Messing, 1982), mapped for the restriction sites and sequenced by the method of Maxam and Gilbert (1980). The 15-kb *Eco*RI fragment which was detected with the 5′ J_H probe, *Xho*I–*Xba*I, (see Figure 1) was isolated as above and cloned into the λ phage vector Charon 4A (Blattner *et al.*, 1977). The clone was analyzed by restriction enzyme mapping and DNA sequencing.

In vitro DNA cleavage

The preparation of the TG/CA endonuclease has been described elsewhere (Hope *et al.*, 1985). Substrate DNA fragments containing the sequences of interest were end-labelled with DNA polymerase I (Klenow) and [α-^{32}P]dNTPs (Sakano *et al.*, 1979). The end-labelled DNA substrates were incubated with the TG/CA

endonuclease purified from mouse fetal liver nuclei at 37°C for 10–20 min. The reaction mixture (15 μl) contained 10 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 20 mM NaCl, 1 mM EDTA and 10 μg sonicated salmon sperm DNA. The reaction product was precipitated with ethanol and separated in a 6% DNA sequencing gel using a G or C-reacted (Maxam and Gilbert, 1980) aliquot of the sample as a size marker.

Steady-state mRNA analysis

Dot hybridization of cytoplasmic RNA (White and Bancroft, 1982) was utilized to measure and compare the relative levels of heavy and light chain mRNA in several cell lines. Briefly, 1×10^7 cells were lysed with 0.1% (0.5% for L929 cells) Nonidet P-40. Nuclei were removed by centrifugation and the cytoplasmic fraction was denatured and blotted onto nitrocellulose with the use of a slot-blot manifold (BRL Hybri-Slot™). Identical blots were prepared and hybridized individually with the two probes (α,κ) essentially as described by Wahl *et al.*, (1979).

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