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Immunoglobulin κ light chain gene promoter and enhancer are not responsible for B-cell restricted gene rearrangement

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ABSTRACT

We have produced transgenic mice which synthesize chimeric mouse-rabbit immunoglobulin (Ig) κ light chains following *in vivo* recombination of an injected unrearranged κ gene. The exogenous gene construct contained a mouse germ-line κ variable (Vκ) gene segment, the mouse germ-line joining (Jκ) locus including the enhancer, and the rabbit b9 constant (Cκ) region. A high level of V-J recombination of the κ transgene was observed in spleen of the transgenic mice. Surprisingly, a particularly high degree of variability in the exact site of recombination and the presence of non germ-line encoded nucleotides (N-regions) were found at the V-J junction of the rearranged κ transgene. Furthermore, unlike endogenous κ genes, rearrangement of the exogenous gene occurred in T-cells of the transgenic mice. These results show that additional sequences, other than the heptamer-nonamer signal sequences and the promoter and enhancer elements, are required to obtain stage- and lineage- specific regulation of Ig κ light chain gene rearrangement *in vivo*.

INTRODUCTION

Immunoglobulin (Ig) and T-cell receptor (TCR) variable region genes are assembled by somatic DNA recombination during B and T lymphocyte development from arrays of separate variable (V), diversity (D) and joining (J) germ-line gene segments (for review see 1, 2, 3). The rearrangement of variable region genes proceeds in a highly regulated fashion. Thus, during B-cell differentiation, recombination of Ig heavy chain V-D-J gene segments preceeds light chain V-J joining, the first step being heavy chain D-to-J recombination (reviewed in 4). While heavy and light chain gene rearrangements can occur on both sets of parental chromosomes, there is a phenomenon of allelic exclusion whereby only a single allele is functionally rearranged. Furthermore, assembly of Ig genes only occurs in B cells, even though similar recombination signal sequences (a conserved heptamer and nonamer motif separated by 12 or 23bp) flank all Ig and TCR component gene segments (see 1, 2). An exception to this rule is Ig heavy chain D-J rearrangements, which can be found in both B and T cells.

However this is an early event and may occur in a common lymphoid precursor before the separation of B and T cell lineages. Subsequent steps in the assembly of Ig variable genes are strictly B-lineage restricted.

The mechanisms which control Ig gene rearrangement can perhaps best be addressed in the whole animal by the study of transgenic mice carrying exogenous unrearranged genes. Previous studies, in which unrearranged chicken λ (5) and rabbit κ (6) light chain genes were introduced in transgenic mice have shown that the injected genes can be functionally rearranged even when integrated outside their normal chromosomal location. Rearrangement of the light chain transgenes however, was observed not only in spleen, but also in the thymus of certain transgenic mice lines. This may be due to the fact that target sequences involved in control of Ig gene rearrangement were either missing from the constructs, or are species specific. Furthermore, in the case of the rabbit κ gene, the level of transcription was low in spite of a high level of rearrangement. In order to examine whether the cis-regulatory elements responsible for transcriptional control of κ light chain genes govern tissue-specific gene rearrangement, we have produced transgenic mice carrying a chimeric unrearranged κ gene in which the variable region sequences are mouse encoded. In this paper, we show that unlike endogenous κ genes, the κ transgene is efficiently rearranged in both B and T cells. Furthermore, non germ-line encoded nucleotides (N-regions) were observed at the V-J recombination junction of the transgene. These findings show that the presence of a functional mouse promoter and enhancer are not sufficient to obtain a lineage-specific and developmentally regulated rearrangement of κ light chain genes *in vivo*. The results are discussed in terms of current models of Ig gene rearrangement.

MATERIALS AND METHODS

Construction of the chimeric unrearranged κ gene and generation of transgenic mice

The cloned mouse $V\kappa_{41}$ germ-line gene (7) and embryonic JκCκ locus (EcoRI-BamH1 fragment cloned in pBR322 ; 8) were gifts of Dr. Philip Leder (Harvard Medical School, Boston). The hybrid mouse-rabbit κ gene was constructed by inserting a 5.8 Kb EcoRI fragment containing the $V\kappa_{41}$ gene into the EcoRI site of the mouse JκCκ plasmid, situated 0.9 Kb 5' of the $J\kappa_1$ segment. The mouse Cκ gene was then replaced by the rabbit b9 Cκ gene by ligating a 4.1 Kb StuI-BamHI genomic fragment (9) to the XmnI site located in the mouse JκCκ intron, 3' of the murine enhancer sequence. The whole construct was cloned in the EcoRI-BamHI

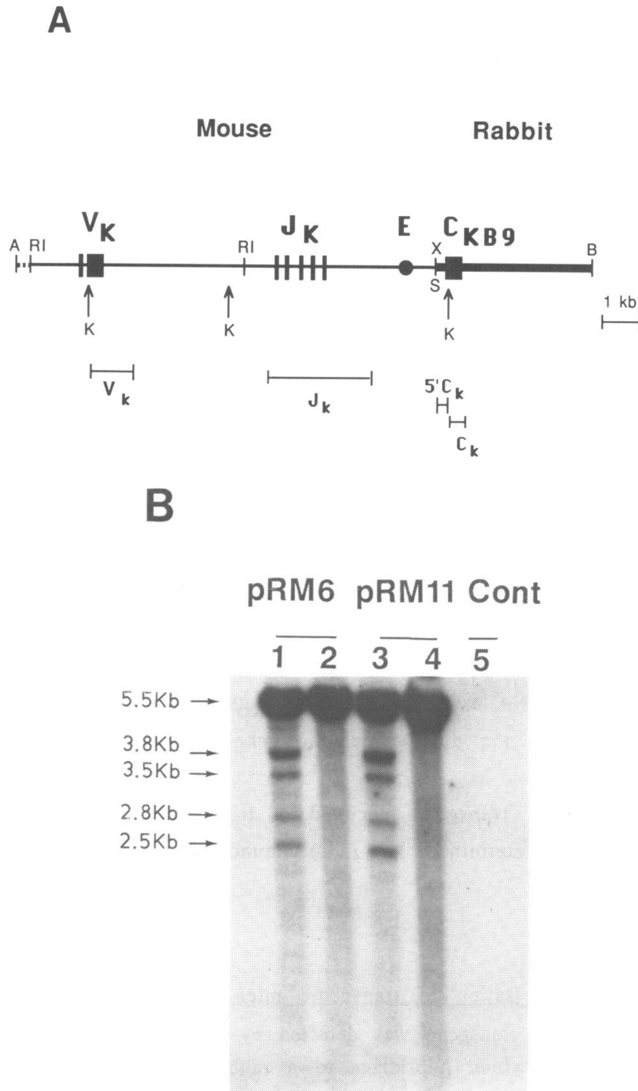


Figure 1. Structure and rearrangement of chimeric mouse-rabbit κ gene. (**A**) Map of pRM κ transgene. Mouse genomic sequences are depicted as a thin line, rabbit sequences a thick line and pBR322 sequences as a dashed line. Closed bars represent coding sequences. E, enhancer. The probes used are as follow : Vκ : 600 bp KpnI-XbaI genomic fragment ; Jκ, 3 kb HindIII fragment ; 5'Cκ : 200 pb StuI-KpnI fragment ; Cκ : 320 pb KpnI-AvaI fragment. Arrows indicate KpnI(K) sites. Restriction enzymes used in the construct are : A, AatII; B, BamHI ; RI, EcoRI ; S, StuI ; X, XmnI. (**B**) Southern blot analysis of KpnI-digested spleen (lanes 1, 3 and 5) and tail (lanes 2 and 4) DNA (10 μg) from pRM6 and pRM11 transgenic mice and control littermate (cont). The blot was hybridized with the 5'Cκ probe. Sizes of unrearranged and rearranged Jκ fragments are indicated.

sites of pBR322 (pRM plasmid). A 14.9 Kb AatII-BamHI fragment containing the mouse germ-line $V\kappa_{41}$ and $J\kappa$ segments and the rabbit b9 $C\kappa$ gene, was excised from the pRM plasmid (Figure 1A) and microinjected into fertilized (C57BL/6 x SJL/J)2 eggs as described (6).

Preparation of T cells

Purified T cell fractions were prepared from thymus of F_1 transgenic mice and control siblings by two successive incubations of thymus cell suspensions on plastic petri dishes precoated with affinity purified goat anti-mouse immunoglobulin antibodies (10). These antibodies were a kind gift of Dr C. Kanellopulos. The purity of the non-adherent T cell fraction was assessed by staining with fluorescein-labelled anti-mouse immunoglulin antibodies and analyzed using a FACScan (Becton and Dickinson, Mountain View, CA). The proportion of Ig bearing cells was reduced from 8.2 $\pm$ 2.9 % to 2.0 $\pm$ 0.9 % (Mean $\pm$ SD, n = 8) by this method.

DNA and RNA analysis

Preparation of genomic DNA, total and poly(A)$^+$RNA, as well as Southern and Northern blotting procedures were carried out as previously described (6).

cDNA cloning and sequencing

Double stranded oligo (dT)-primed cDNA was synthetized from pRM transgenic mice spleen poly(A)$^+$ RNA using the RNase H method of Gubler and Hoffman (11). DNA was sequenced by the dideoxynucleotide chain-termination method (12).

Radioimmunoassay

The presence of transgene encoded κ light chains in the serum of the transgenic mice was determined by radioimmunoassay as previously described (6).

RESULTS

Production and characterisation of transgenic mice containing a chimeric κ gene

Integration of the transgene was detected by the presence in tail DNA of a 5.5 Kb KpnI fragment which hybridizes to a rabbit specific 5' $C\kappa_{b9}$ probe and corresponds to the unrearranged $J\kappa$ locus of the construct (Figure 1A). Two transgenic mice (pRM6 and pRM11) containing 2-5 copies of the chimeric κ gene were obtained (Figure 1B). Since KpnI also cleaves within the $V\kappa_{41}$ gene, $V\kappa$-$J\kappa$ recombination of the transgene should generate novel 5'$C\kappa_{b9}$-hybridizing KpnI fragments of smaller size. As shown in Figure 1B, four additional bands of 3.8, 3.5, 2.8 and 2.5 Kb were seen in Kpn-digested spleen DNA of the transgenic mice. The lengths of these fragments match the size expected for joining of the $V\kappa$ gene to the $J\kappa_1$, $J\kappa_2$, $J\kappa_4$ and $J\kappa_5$ segments, respectively. A 3.3 Kb KpnI fragment

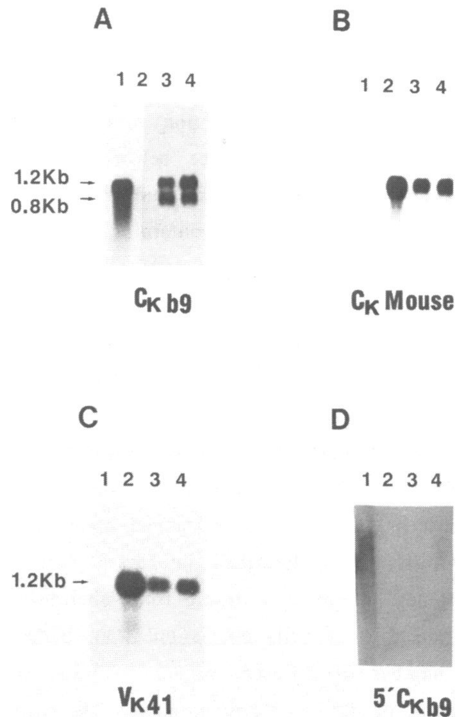


Figure 2. Detection of pRM encoded κ transcripts. Total RNA (10 µg) from spleen of pRM11 (lane 4) and pRM6 (lane 3) transgenic mice, or control littermates (lane 2) and poly(A)$^{+}$ mRNA (100 ng) from rabbit spleen (lane 1) was subjected to Northern blot hybridization analysis. Blots were hybridized with (A) the rabbit $C\kappa_{b9}$ probe (B) a mouse Cκ probe (9) (C) mouse $V\kappa_{41}$ probe (D) rabbit $5'C\kappa_{b9}$ probe. Probes are described in Figure 1A. Autoradiograms were exposed for 24 hrs (blots A, C and D) or 1 hr (blot B). Sizes of transcripts were determined by comparison with migration of RNA standards (Amersham).

corresponding to the rearranged $J\kappa_3$ segment could not be identified on these blots, indicating that as for assembly of endogenous murine κ genes (13), this J segment is either not utilized at all, or only at a very low frequency in the rearrangement of the pRM κ transgene. Based on densitometric scanning of autoradiographs, the rearranged bands represent 10-25% of the integrated pRM DNA, indicating that the exogenous κ gene is efficiently rearranged in transgenic mice spleen cells.

The κ transgene is efficiently transcribed in mouse spleen

Northern blot hybridization analysis was performed on RNA isolated from spleen of transgenic mice and control littermates. Using a rabbit Cκ probe, pRM encoded transcripts were detected in spleen of transgenic, but not control mice (Figure

2A). These transcripts are present at approximately 10-30% of the level of endogenous mouse κ mRNA as estimated from the relative intensity of hybridization signal obtained with the rabbit and mouse Cκ probes (Figure 2B), as well as from results of colony blot hybridization of LPS stimulated spleen cells (data not shown) and cloning experiments (see below). This represents a 10-100 fold increase in exogenous κ transcript when compared to the results obtained with a previous series of transgenic mice containing a wholely rabbit encoded unrearranged κ gene (6), and is probably due to the presence of mouse enhancer sequences in the present construct.

Transcripts of 1.2 Kb and 0.8 Kb were revealed with the rabbit Cκ probe in pRM spleen RNA (Figure 2A). The 1.2 Kb transcripts probably represent mature hybrid mouse-rabbit κ transcripts, since they also hybridize with mouse $V\kappa_{41}$ (Figure 2C) and Jκ probes (data not shown), but not to a DNA fragment from the Jκ-Cκ intron (Figure 2D). The 0.8 Kb transcripts hybridize only with the rabbit Cκ sequences and may be due to transcription of the unrearranged construct. Incomplete 0.8 Kb κ transcripts have previously been described in foetal liver pre-B cells (14) as well as in certain myelomas, where they result from aberrant splicing (15).

The κ transgene is rearranged in T cells but is not transcribed

DNA obtained from a number of different organs of pRM transgenic mice were analyzed for rearrangement of the chimeric κ gene. No rearrangement could be detected in nonlymphoid tissue such as liver, kidney or heart, whereas the same four rearranged Kpn fragments observed in spleen were present in the thymus (Figure 3A). In order to determine whether the rearrangement seen in the thymus was due to recombination of the pRM gene in T cells, T cells were isolated from the thymus of transgenic mice and control siblings. FACS analysis indicated that the thymocyte population obtained contained 97-99% non-Ig^{+} cells (see Materials and Methods), nevertheless the intensity of the rearranged bands was not decreased (Figure 3B). In contrast, there was no rearrangement of endogenous mouse κ genes in this cell fraction (Figure 3C). These data show that unlike the endogenous genes, the κ transgene can undergo V-J recombination in T cells.

Although the κ transgene is rearranged in T-cells, it does not seem to be transcribed (Figure 4A). The low level of pRM transcript detected in the thymus and purified T-cell fraction (approximately 10% and 1% of the level of spleen transcript, respectively) correlates with the quantity of B-cells present in these preparations as assessed by FACScan analysis and the amount of endogenous mouse κ transcript observed (Figure 4B).

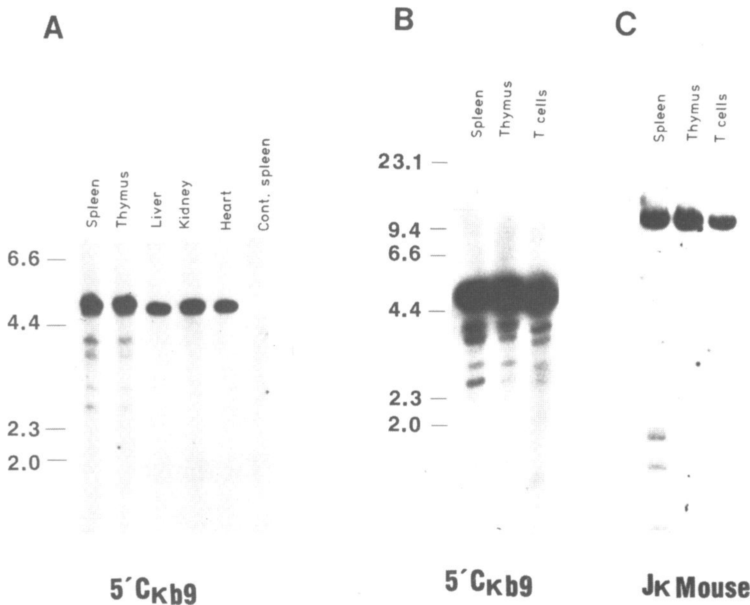


Figure 3. Tissue specificity of endogenous and pRM κ gene rearrangement. (**A**) Southern blot of KpnI-digested DNA (10 μg) from various tissue of pRM6 F_1 mice and spleen of control (cont) mice. The blot was hybridized with the rabbit 5'Cκ probe (Figure 1A). Numbers indicate the size (in Kb) of DNA molecular weight markers. (**B**) Rearrangement of pRM κ gene in spleen, thymus and purified T cells. T cells were prepared from thymus of pRM6 F_1 offspring as described in Materials and Methods. Southern blot hybridization analysis was performed as in (**A**). The results shown were obtained with DNA from 3 pRM6 F_1 mice and the experiment was repeated twice. (**C**) Rearrangement of endogenous κ genes in spleen, thymus and T cells. DNA was prepared as in (**B**), digested with KpnI and BstEII and hybridized with a mouse Jκ probe containing $J\kappa_4$ and $J\kappa_5$ sequences (560bp AvaI-BstEII fragment). Germ-line (12 kb) and rearranged $J\kappa_1$ (1.8 kb) and $J\kappa_2$ (1.4 kb) segments only are visible on the blot (13).

N region insertions at the recombination junction of the κ transgene

In order to define the structure of the rearranged pRM κ transgene, a cDNA library was prepared from spleen of pRM transgenic mice and $C\kappa_{b9}$ positive clones, representing full length 1.2 kb chimeric mouse-rabbit κ transcripts, were sequenced. In all the clones analysed, recombination had occurred between the $V\kappa_{41}$ gene and either the $J\kappa_1$, $J\kappa_2$, $J\kappa_4$ or $J\kappa_5$ segments. The sequence of the recombination junctions are shown in Figure 5A. There was a high degree of variability in the exact site of joining resulting in a variable loss of bases from the joined V and J coding sequences. In some clones as many as 7 nucleotides were

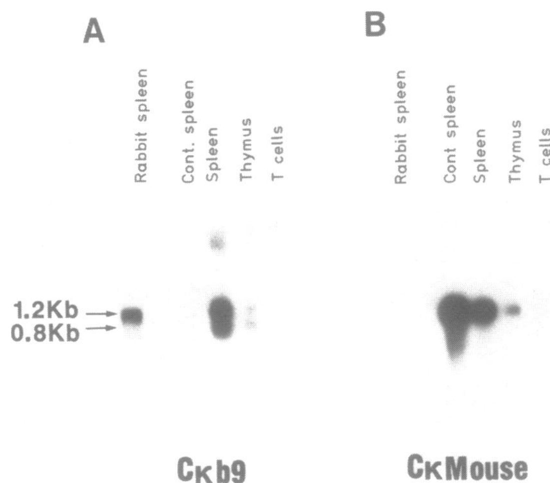


Figure 4. Tissue specificity of transcription of endogenous and pRM κ genes. Total RNA (10 μg) from spleen of control mice (Cont. spleen), spleen, thymus and purified T cells of pRM6 F_1 offspring and 100 ng of poly(A)$^+$ mRNA from rabbit spleen was subjected to Northern blot hybridization analysis. The blots were either hybridized with a rabbit $C\kappa_{b9}$ probe (**A**) or mouse Cκ probe (**B**).

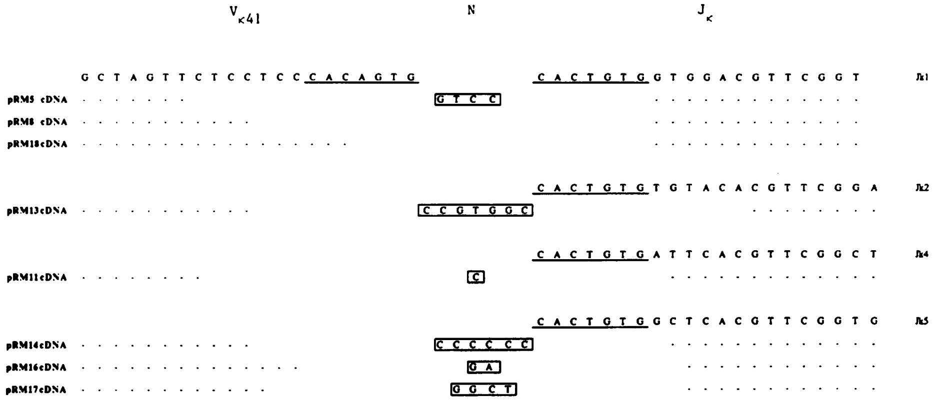
removed from the V sequence. We also observed the insertion of non-germ line encoded nucleotides (N regions) at the V-J junction of over half of the clones sequenced. The number of extra bases varied from 1 to 7 and in 1 clone consisted of a stretch of at least 6 C's. Although the deletions and N-region insertions found at the V-J joint of the κ transgene are more extensive than typically observed for light chain gene recombination, such junctional variability is commonly found in Ig heavy chain V-D and D-J rearrangements.

The sequence of the J-C junction of the pRM transcripts (Figure 5B) showed that in all cases splicing had occurred correctly between the rabbit Cκ gene and the rearranged mouse Jκ segment. The results are therefore consistent with the synthesis of chimeric mouse-rabbit κ light chains in the pRM transgenic mice.

Production of chimeric κ chains

The presence of transgene encoded κ light chains in the serum of pRM mice was tested by a direct RIA binding assay using anti-rabbit $C\kappa_{b9}$ allotypic antibodies. Both founder mice as well as all pRM positive F_1 offspring tested had detectable, but low amounts of exogenous κ light chains in the serum (Figure 6A). The κ chains were associated with endogenous mouse μ and γ1 heavy chains (Figure 6B), indicating that they are secreted in the serum as complete immunoglobulin molecules. There was also evidence of mixed antibody molecules containing both

A



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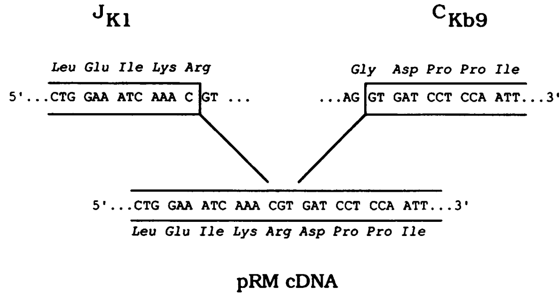

Figure 5. Nucleotide sequence of V-J joints (A) and J-C splicing (B) of pRM κ transcripts. pRM κ cDNA was synthesized and sequenced as described in Materials and Methods. (A) Nucleotide sequence of V-J junctions of pRM κ cDNA are compared to mouse germ-line $V\kappa_{41}$ (7) and Jκ (8) sequences. Identical sequences are indicated by dashes. Nucleotide insertions are boxed. The heptamer of the recombination sequence is underlined. (B) Top, mouse $J\kappa_1$ (8) and rabbit $C\kappa_{b9}$ (9) germ-line sequences. Bottom, pRM cDNA sequence at the Jκ-Cκ splice site. Coding sequences are boxed and the derived amino acid sequences are shown above or below in italics.

endogenous and transgene encoded κ light chains in the serum of the pRM transgenic mice (Figure 6B). These results imply that functional rearrangement and expression of the κ transgene and the endogenous κ genes may not be mutually exclusive.

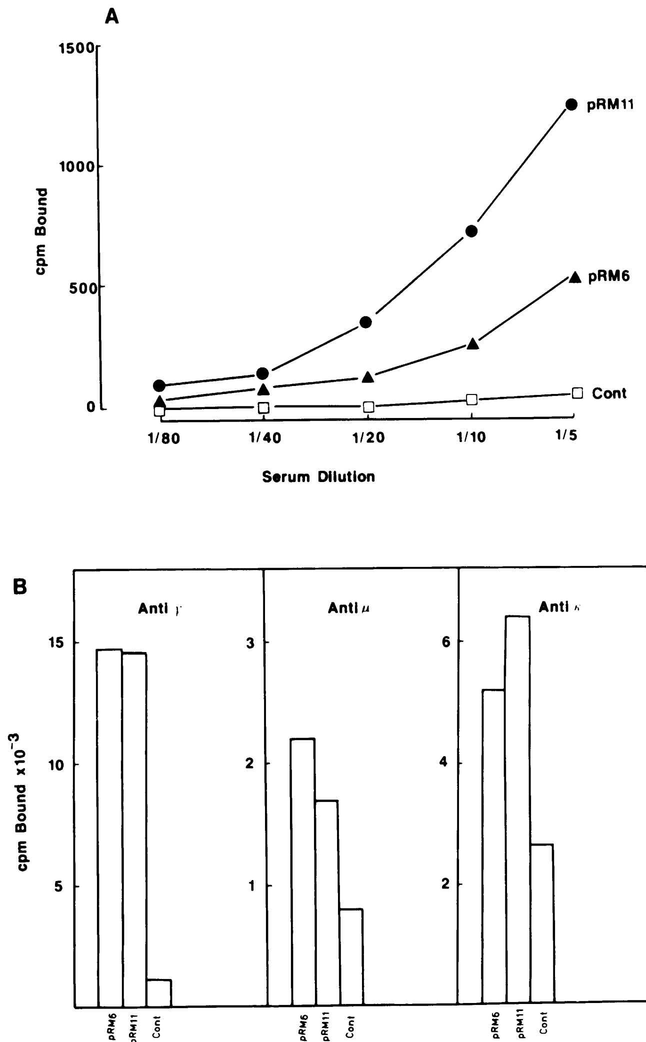

Figure 6. Production of chimeric pRM κ light chains. (**A**) Several dilutions of serum from transgenic (● ▲) and control (□) littermates were incubated on microtiter plates precoated with affinity purifed anti-b9 anti-allotype antibodies. Bound materials was revealed with ^{125}I-labelled anti-b9 antibodies. (**B**) Mouse γ1, μ or κ chains associated with pRM κ chains in sera (diluted 1:10) from transgenic mice pRM6 and pRM11 and a control mouse (cont) were detected as above but bound materials was revealed with ^{125}I-labelled polyclonal rabbit anti-mouse γ1 chain, rabbit anti-mouse μ chain and monoclonal rat anti-mouse κ chain (139-52-1) antibodies, respectively.

DISCUSSION

In this paper we describe the production of transgenic mice which actively rearrange and express a chimeric κ gene containing mouse germ-line V and J segments and the rabbit $C\kappa_{b9}$ gene. Rearrangement of the κ transgene was observed not only in B, but also in T cells of the pRM transgenic mice. These findings demonstrate that the recombination sequences flanking Ig variable region gene segments can be recognized and joined by T-cell receptor recombination enzymes *in vivo*. It has previously been reported that transfected T-cell receptor gene segments can recombine in a pre-B cell line (16). Taken together these results argue strongly in favour of a common recombination mechanism in B and T-cells.

If an exogenous κ gene can be actively rearranged in T cells of transgenic mice, why are endogenous light chain genes only assembled in B and not T cells ? Yancopoulos, Alt and colleagues have postulated that the tissue-specific rearrangement of Ig and TCR genes may be controlled by regulating the "accessibility" of the genes to the trans-acting recombination factors in B and T cells (16). It has been suggested that this regulation is mediated by transcription of the variable region gene segments (16,17,18,19). However, the question of causality between transcription and rearrangement of Ig genes remains unclear (6,20). Here we show that the cis-acting elements which regulate B-specific transcription of κ light chain genes do not control tissue-specific gene rearrangement, since the pRM κ transgene contains a functional mouse promoter and enhancer and yet is rearranged in T cells. Although the κ promoter and enhancer sequences do not appear to be responsible for restricting gene rearrangement to B cells, it remains to be determined whether these elements are required for rearrangement of κ light chain genes within the B cell lineage *in vivo*. Other sequences have been proposed as candidates for the control of Ig gene rearrangement. In particular, gel retardation experiments have revealed two tandemly repeated sequences located 5' of the mouse $J\kappa_1$ heptamer-nonamer recombination sequence, which bind a pre-B specific nuclear protein (21). However, since this region is also present in our pRM construct it cannot be the sole element responsible for the lineage- specific regulation of κ light chain gene rearrangement.

Results obtained from cDNA sequencing of the pRM transgenic κ transcripts indicate that rearrangement of the exogenous gene may occur prior to endogenous κ gene assembly in the transgenic mice. We found a high degree of junctional variability at the V-J joint of the κ transgene and in particular

addition of extra nucleotides (N-regions) between the V and J coding sequences. Such nucleotide insertion and variability in the precise site of recombination is commonly observed in D-J and V-DJ junctions of Ig heavy chains, but does not normally occur at light chain V-J joints (22). N-region insertions are thought to result from the *de novo* addition of nucleotides catalysed by the enzyme terminal deoxyribonucleotide transferase (16,17,23). Terminal transferase activity is high in the early stages of B cell development when heavy chain gene rearrangement occurs and apparently decreases in cells rearranging κ light chain genes. Nevertheless, this enzyme has been shown to be capable of stimulating the addition of N-regions during the recombination of transfected Vκ-Jκ gene segments in pre-B cell lines (24). If the N-region insertions observed in the rearranged κ transgene are indeed due to terminal transferase activity, then one must assume that assembly of the transgene occurs early in the developement of B cells, before the normal onset of light chain gene rearrangement. Since V-D-J recombination activity is present at the earliest stages of B cell differentiation, even in pluripotent progenitor cells (20), it is possible that κ transgene rearrangement occurs in a common lymphoid precursor before the separation of B and T cell lineages. This would explain why rearrangement of the κ transgene is found in both lymphoid cell types.

The differences observed between the rearrangement of endogenous and the exogenous κ gene may be due to a number of factors. Firstly, it is conceivable that the presence of multiple (2-5) copies of the transgene or its site of integration may alter the "accessibility" of the κ gene to recombination. Neither of these factors, however, were found to affect the rearrangement of a rabbit κ gene in a larger series of transgenic mice (6). Alternatively, the fact of artificially bringing the V and J segments in closer proximity may increase the frequency of recombination, particularly in the early stages of lymphoid development where a bias for recombining proximal gene segments has been observed (25). However, it seems unlikely that this alone would result in such a major change in the pattern of rearrangement. Transfection studies with exogenous rearrangement substrates have shown that the conserved heptamer-nonamer sequences bordering germ-line variable region gene segments are sufficient to cause site-specific rearrangement in pre-B cells (26,27). The results of this study shows that these minimal sequences do not allow regulated rearrangement *in vivo*. Likewise, the presence of a functional mouse promoter and enhancer in the chimeric κ gene construct is not sufficient to obtain B-cell restricted rearrangement. Additional cis-elements are probably therefore

required for regulated temporal- and lineage-specific rearrangement of κ light chain genes.

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