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The immunoglobulin κ locus contains a second, stronger B-cell-specific enhancer which is located downstream of the constant region

Kerstin B.Meyer and Michael S.Neuberger

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

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The description of cell lines capable of transcribing immunoglobulin heavy or light chain genes in the apparent absence of an active enhancer has led us to look for novel enhancers in the immunoglobulin gene loci. Here we show that there is a second B-cell-specific enhancer in the mouse κ locus and that this is located 9 kb downstream of C_κ. This enhancer is some 7-fold stronger than the κ-intron enhancer and shows striking sequence homologies to the lymphotropic papovavirus, IgH and κ-intron enhancers. The location of the κ3′ enhancer between C_κ and the RS element means that it is deleted in some B cells that express λ light chains.

Key words: enhancer/B cell/immunoglobulin κ/transcriptions/RS element

Introduction

The immunoglobulin gene loci constitute one of the most extensively studied systems for the analysis of cell-type-specific gene expression in mammalian cells. Cellular enhancer elements were first identified in the major introns of the immunoglobulin heavy (IgH) and κ light chain loci (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Neuberger, 1983; Picard and Schaffner, 1984; Queen and Stafford, 1984). These enhancers are sufficient to direct lymphoid-specific expression of a heterologous gene. However, in addition to the enhancers, the promoters located at the 5′ ends of the variable (V) gene segments are also found to be lymphoid specific (Grosschedl and Baltimore, 1985; Mason *et al.*, 1985; Picard and Shaffner, 1985). Currently, much effort is focused on the factors that bind to these transcription elements. Presumably, factors bound at the enhancer interact with factors bound at the promoter to recruit RNA polymerase II and ensure the formation of an active transcription complex.

However, this picture of immunoglobulin gene transcription being solely regulated by a V gene promoter coupled to a J-C intron enhancer is an over-simplification. For example, the κ-intron enhancer is not active in the plasmacytoma cell line S107 as this cell lacks the enhancer-binding factor NF-κB. Nevertheless, S107 cells actively transcribe their endogenous κ gene but they did not express transfected κ genes (Atchinson and Perry, 1987, 1988). An attractive way of explaining this discrimination between the endogenous and transfected genes is to propose that there is more than one enhancer in the region of the κ locus and that this second enhancer(s) does not require NF-κB for its activity.

A somewhat analogous situation has been found in the heavy-chain (IgH) locus. Several cell lines have been described that actively transcribe their IgH genes although the IgH enhancer has been deleted (Klein *et al.*, 1984; Wabl and Burrows, 1984; Aguilera *et al.*, 1985; Eckhardt and Birshtein, 1985). This has resulted in considerable discussion concerning the role of the IgH enhancer in the regulation of immunoglobulin gene expression (Klein *et al.*, 1985; Zaller and Eckhardt, 1985; Grosschedl and Marx, 1988). The most attractive explanation for continued transcription of the IgH loci in these enhancer-deleted cell lines is similarly provided by postulating the existence of more than one enhancer in the IgH locus.

A further line of evidence pointing towards the existence of as yet unidentified transcription elements in the immunoglobulin gene loci comes from the analysis of transgenic mice. Although mice that carry immunoglobulin transgenes express these transgenes in their lymphoid tissues, the level of expression is often found to be significantly less than that of the endogenous loci. This contrast is all the more striking in view of the fact that the transgene is usually present in multiple copies. This relatively low level of expression is most easily explained by proposing that the endogenous locus is linked to a transcription element(s) that is not included in the transgene. We have therefore looked for further enhancers in the immunoglobulin loci and here describe a strong B-cell-specific enhancer that is located 3′ of the mouse C_κ exon.

Results

An enhancer is located 9 kb downstream of C_κ

We reasoned that if there were a second enhancer implicated in κ gene transcription, then this enhancer would most probably be located 3′ of the J_κ segments. The germline V_κ genes, at least in man, are scattered over some several thousand kilobases upstream of J_κ; much of the DNA upstream of J_κ is deleted in κ-expressing cell lines (Klobeck *et al.*, 1987). To test for enhancer activity downstream of C_κ, we exploited the fact that the human β-globin gene in plasmid pβ800 (Figure 1B) is only a weak transcription unit in transfected cells unless it is provided with an exogenous enhancer element. DNA fragments from the germline mouse κ clone L1 (Steinmetz *et al.*, 1979) were subcloned into plasmid pβG800 with the fragments (*Bam*HI fragments A–D; see Figure 1A) being placed 800 bp upstream of the β-globin start site. The DNA from the resulting constructs was introduced into MPC11 myeloma cells by calcium phosphate co-precipitation along with a human α2-globin plasmid that provided an internal reference. The amount of β- and α-globin mRNA produced by the transfected cells was then measured in ribonuclease protection assays.

As can be seen from Figure 1C, little mRNA is produced from the β-globin gene in the absence of an exogenous enhancer; however, provision of the SV40 enhancer strongly

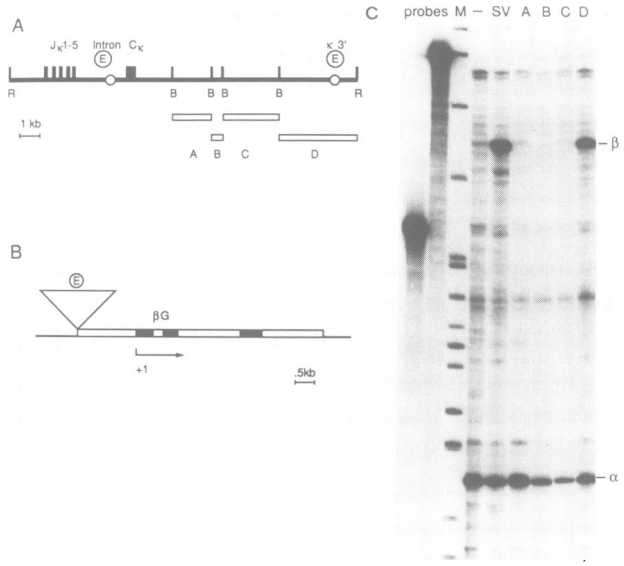

Fig. 1. Identification of an enhancer 3′ of C_κ. (**A**) A map of the mouse κ locus depicting the fragments assayed for enhancer activity. The two κ enhancers are indicated (κ-intron E and $\kappa 3'$ E) and restriction sites are abbreviated: B, *Bam*HI, *Eco*RI. (**B**) Schematic representation of the pβ800 plasmid used for enhancer assays. (**C**) Ribonuclease protection assays of β- and α-globin mRNA in MPC11 cells transfected with either pβ800 (−) or with derivatives in which either the SV40 enhancer or one of the fragments A−D have been cloned in at −800. Bands corresponding to correctly initiated β-globin transcripts are indicated as are the α-globin transcripts encoded by the co-transfected reference plasmid.

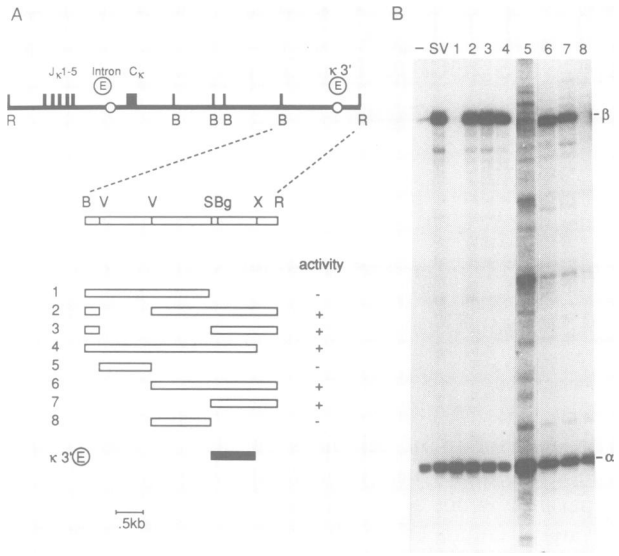

Fig. 2. Location of $\kappa 3'$ enhancer. (**A**) A map showing the fragments assayed for enhancer activity. Restriction sites are abbreviated as in Figure 1 except for Bg, *Bgl*II; S, *Sac*I; V, *Eco*RV; X, *Xba*I. The bars depict the κ locus fragments included in each construct. (**B**) Ribonuclease protection assay of enhancer activity. MPC11 cells were transfected with the *a*2-globin reference plasmid plus either plasmid pβ800 containing no enhancer (−) or with derivatives that included either the SV40 enhancer (SV) or the κ DNA fragments 1−4 that are depicted in panel (A) at position −800. **Lanes 5−8** were obtained by transfection with pβ128 derivatives in which κ DNA fragments were cloned into position −128 (the pβ128 plasmid is identical to pβ800 except that it includes 128 bp rather than 800 bp of β-globin 5′-flanking DNA). The positions of correctly initiated α- and β-globin transcripts are indicated.

activates transcription. As regards the DNA fragments from the κ locus, we find a strong stimulation of β-globin transcription using fragment D but no such stimulation using fragments A, B or C. Thus, inclusion of fragment D on the plasmid but at a distance from the β-globin gene activates transcription from the β-globin promoter. We deduce the existence of a transcription enhancer element within fragment D and refer to this enhancer as the $\kappa 3'$ enhancer to distinguish it from the κ intron enhancer located in the J_κ-C_κ intron that has been identified previously (Queen and Baltimore, 1983; Picard and Schaffner, 1984; Queen and Stafford, 1984).

To delineate the $\kappa 3'$ enhancer element further, we assayed a number of deletions of fragment D (constructs 1−4; Figure 2A) as well as some individual sub-fragments (constructs 5−8). Enhancer activity was found in constructs 2, 3, 4, 6 and 7 but not in constructs 1, 5 and 8 (Figure 2A,B). This suggests that the enhancer is located within the 0.8 kb *Sac*I−*Xba*I fragment, a prediction that was confirmed by subcloning this fragment into an enhancerless β-globin vector (Figure 4).

The $\kappa 3'$ enhancer contains regions homologous to the IgH, κ-intron and LPV enhancers

The sequence of this *Sac*I−*Xba*I fragment was determined and is presented in Figure 3. Comparison with the sequences of other known enhancers revealed several striking homologies. The most extensive homology detected was with the enhancer region of the human lymphotropic papovavirus. There are two distinct regions of homology, a 17 out of 18 match and an 11 out of 12 match; both these elements are repeated within the origin region of the viral genome (Pawlita *et al.*, 1985).

The other impressive homology is with a region around the E3 element of the IgH enhancer. Motifs (elements E1, E2, E3 and E4) within the IgH enhancer have been identified on the basis of *in vivo* footprinting data (Ephrussi *et al.*, 1985). The $\kappa 3'$ enhancer contains a segment showing a 12 out of 14 homology to the region of the IgH enhancer that has been shown to bind NF-μE3 (Sen and Baltimore, 1986a; Peterson and Calame, 1987). Furthermore, the regions that flank either side of the E3 element in the IgH enhancer are also found to be present adjacent to the E3-like element of the $\kappa 3'$ enhancer but in a reshuffled form (Figure 3). Significant homologies are also found between the κ-intron and $\kappa 3'$ enhancers. There are three separate 10 out of 11 matches: one overlaps the κ-intron E3 element, another overlaps the κ-intron E1 and the third homology includes within it a 7 out of 8 match to the octanucleotide element (Falkner and Zachau, 1984; Parslow *et al.*, 1984) that has been identified in V gene promoters. In addition, sequence comparisons reveal an 8 out of 10 match to the NF-κB binding site, a sequence homologous to the consensus for the interferon response element (Samanta *et al.*, 1986; Porter *et al.*, 1988; Reid *et al.*, 1989) and a homology to the conserved upstream sequence of the MHC class II genes, a sequence that may play a role in induction by γ-IFN (Boss and Strominger, 1986). Several segments that show similarity to regions of the SV40 enhancer are also found (Figure 3).

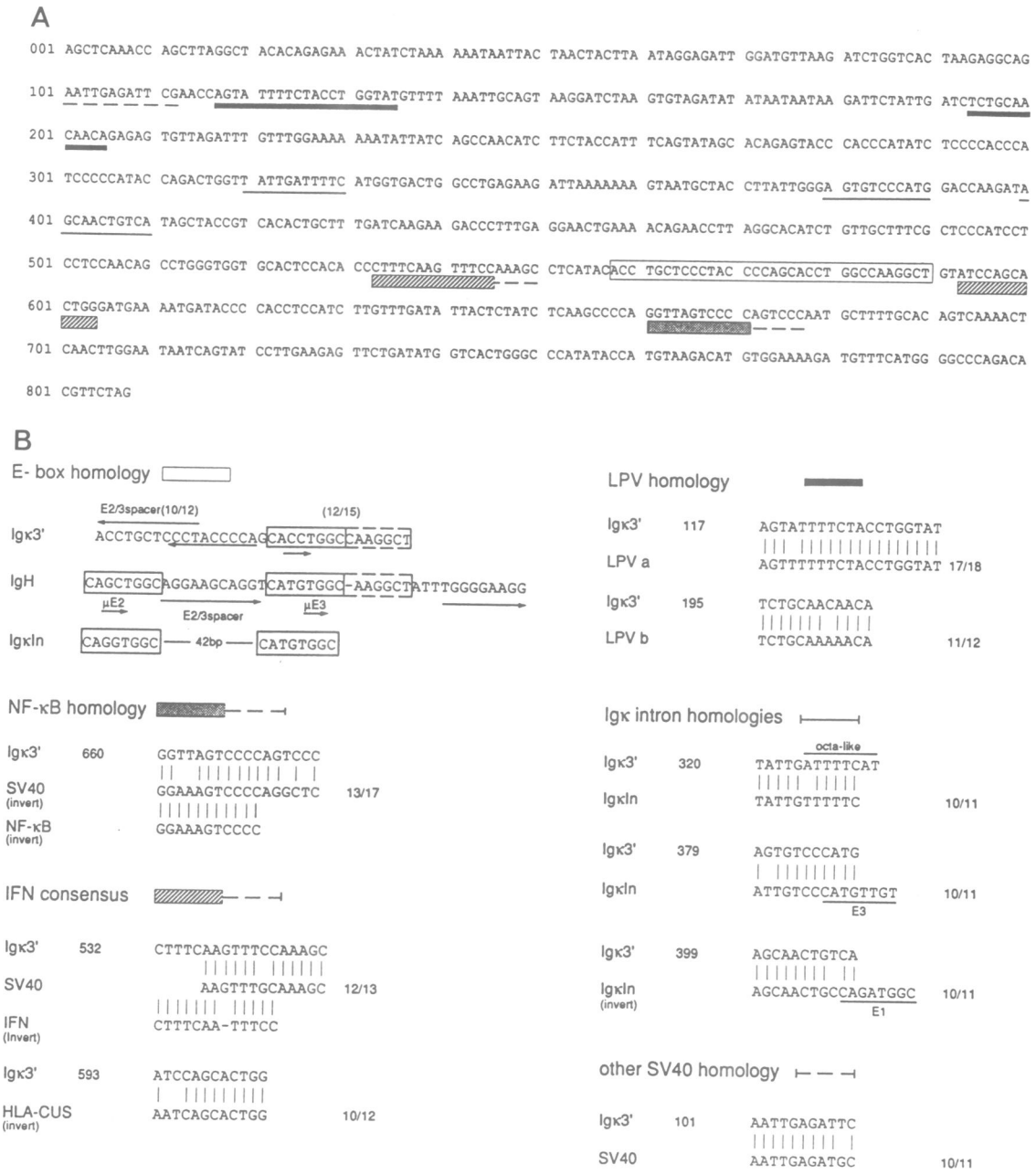

Fig. 3. Sequence of the κ3′ enhancer. (**A**) The sequence of the *Sac*I−*Xba*I enhancer fragment with boxes and lines indicating homologies that are shown in (B). (**B**) Sequence homologies are shown to regions of the IgH, LPV, κ-intron and SV40 enhancers as well as to the IFN response element (Samanta *et al.*, 1986; Reid *et al.*, 1989) and the conserved upstream sequence (CUS) of HLA-DQβ that is implicated in γ-IFN induction (Boss and Strominger, 1986). The E-boxes marked in the IgH comparison refer to the nomenclature used by Ephrussi *et al.* (1985). In the comparison of the κ3′ enhancer with the E2/3 region of the IgH enhancer, the dashed box shows the extended homology to μE3 and the arrows denote the inverted repeat of the μE2/3 spacer as well as the region that flanks the 3′ side of μE3.

The κ3′ enhancer is B cell specific

The homologies between the κ3′ enhancer and other known enhancers led us to believe that it would be specifically active in lymphoid cells. To test this, we assayed the activity of the enhancer in two non-lymphoid cell lines (NIH 3T3 and HeLa) as well as in three different lymphoid cell lines (the plasmacytoma MPC11, the B-cell line WEHI231 and the thymoma EL4). The α-globin internal reference was included and mRNA levels quantitated by ribonuclease protection assays as before. While the SV40 enhancer was active in all these lines, the κ3′ enhancer was only active in the plasmacytoma and B cell lymphoma but not in fibroblast or T cell lines (Figure 4). In other experiments (not shown), we have found that the enhancer is also inactive in another T cell lymphoma, BW5147. Thus, the κ3′ enhancer is B cell specific.

The κ3′ enhancer is stronger than the κ-intron enhancer

The κ-intron enhancer is known to be a weak enhancer. We compared the activities of the κ3′, κ-intron, IgH and SV40 enhancers in transient transfection assays in the MPC11 myeloma. The results are presented in Figure 5 and were quantitated by densitometry with each signal being

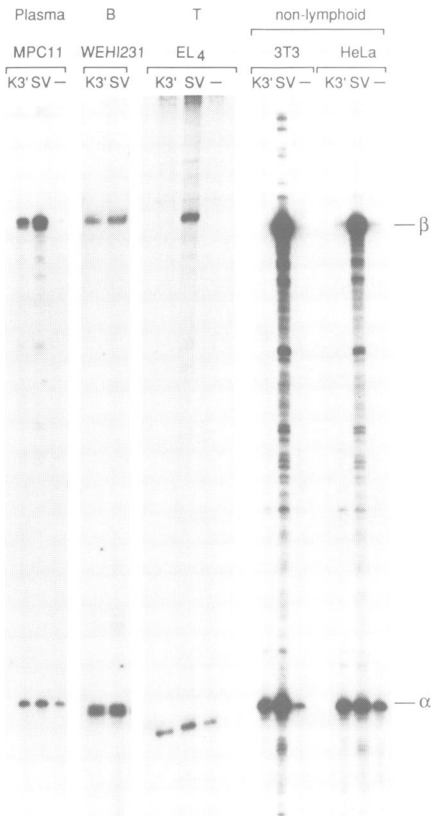

Fig. 4. Cell type specificity of the $\varkappa 3'$ enhancer. Cell lines were transfected with the α-globin reference plasmid along with a pβ128 derivative that included at position −128 either no enhancer (−), the SV40 enhancer (SV) or the 808-bp *Sac*I−*Xba*I fragment containing the $\varkappa 3'$ enhancer.

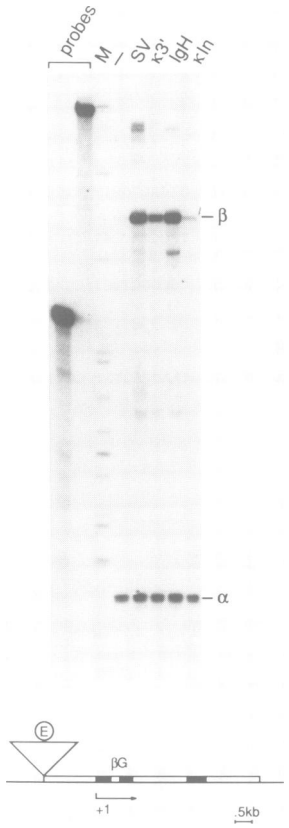

Fig. 5. The $\varkappa 3'$ enhancer is stronger than the $\varkappa$-intron enhancer. MPC11 cells were transfected with the α-globin reference plasmid plus either pβ128 containing no (−), the SV40 (SV), the $\varkappa 3'$ or $\varkappa$-intron enhancer at position 128 or with a pβ800 derivative containing the IgH enhancer at −800. Globin transcripts were mapped by ribonuclease protection assays.

normalized with respect to the α-globin mRNA levels. We find that $\varkappa 3'$ enhancer is 7-fold stronger than the $\varkappa$-intron enhancer but some 2-fold weaker than the SV40 enhancer. Direct comparison between these three enhancers and the IgH enhancer is not possible from these experiments as the IgH enhancer was placed at −800 with respect to the β-globin start site, whereas the other three enhancers were all assayed at −128. Nevertheless, our results are consistent with those of Picard and Schaffner (1984) that the $\varkappa$-intron enhancer is some 20-fold weaker than the IgH enhancer and furthermore we clearly establish that the $\varkappa 3'$ enhancer is nearly an order of magnitude stronger than the $\varkappa$-intron enhancer when assayed in MPC11 cells.

The $\varkappa 3'$ enhancer is deleted upon RS rearrangement

The location of the $\varkappa 3'$ enhancer places it between the $C_\varkappa$ exon and the RS (rearranging sequence) element (Figure 6B). Cells that express λ light chains are usually found to have aberrant rearrangements involving the RS element on at least one of their $\varkappa$ alleles; this can lead to deletion of $C_\varkappa$ (Durdik *et al.*, 1984; Moore *et al.*, 1985). One would expect the $\varkappa 3'$ enhancer to be deleted in these lines if RS rearrangement occurred by a simple looping-out mechanism. To confirm this, we carried out a Southern blot analysis of various λ-expressing cell lines. The blot (Figure 6A) revealed that several of the cell lines (HOPC1, BCL-1 and CH-1) retained at least one RS allele in germline configuration and, therefore, as is to be expected, retained at least one copy of the $\varkappa 3'$ enhancer. The case of I29 is difficult to interpret as the mouse strain from which this lymphoma derives shows considerable restriction map differences from the BALB/c strain (Stavnezer *et al.*, 1985) and the difference in the sizes of restriction fragments could therefore reflect polymorphism rather than rearrangement. However, the MOPC315 myeloma does not retain an RS allele in germline configuration and has entirely deleted the $\varkappa 3'$ enhancer from its genome (Figure 6A,B). This strongly suggests that the RS rearrangement is itself responsible for the loss of the enhancer and that RS recombination does indeed lead to enhancer deletion.

Discussion

In this work we have demonstrated the existence of an enhancer downstream of $C_\varkappa$. This enhancer is B cell specific and is some 7-fold stronger than the $\varkappa$-intron enhancer. Thus the mouse immunoglobulin $\varkappa$ locus contains at least one other *cis*-acting transcription activator element in addition to the $V_\varkappa$ promoter and $\varkappa$-intron enhancer. Such a multiplicity of transcription elements scattered over a large region is reminiscent of the findings of Grosveld *et al.* (1987) who have identified elements flanking the human β-globin gene that form erythroid-specific DNase I-superhypersensitive sites and which are sufficient when inserted into a β-globin mini-locus to specify high-level, position-independent expression in transgenic mice. Thus, the existence of multiple activating sequences in the region

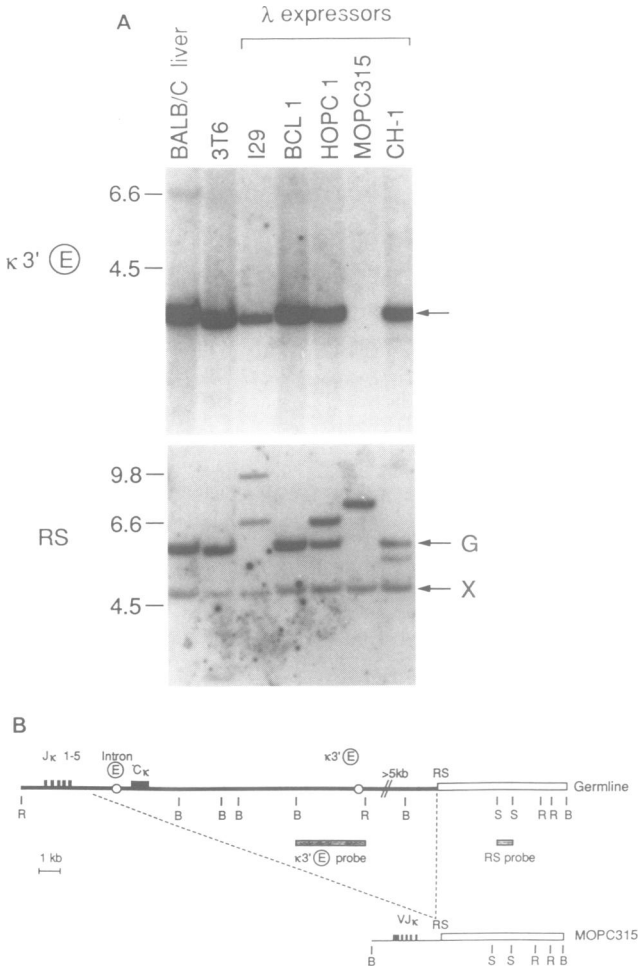

Fig. 6. The κ3′ enhancer can be deleted on RS rearrangement. (**A**) Southern blot of DNA from BALB/c mouse liver, NIH 3T3 fibroblast cells and five mouse λ-expressing lymphoid cell lines probed first for the κ3′ enhancer (**upper panel**) and then reprobed for RS (**lower panel**). In this reprobing, G indicates the germline RS band of BALB/c whereas × is a cross-hybridizing band. The genomic DNA was digested with *Eco*RI+*Bam*HI and the probe for the κ3′ enhancer was a 3 kb *Eco*RI−*Bam*HI fragment whereas the probe for RS was the rs0.8 probe of Moore *et al.* (1985). (**B**) A map of the mouse κ locus showing the RS element as well as the rearranged RS allele of plasmacytoma MOPC315. The map of this rearranged RS is taken from Durdik *et al.* (1984). Restriction sites are abbreviated as in other figures except that S indicates a *Sau*3AI site, although the only such sites depicted are those that were used to generate the rs0.8 fragment. The interrupted line indicates the postulated simple looping-out that could have given rise to the MOPC315 RS rearrangement.

of the immunoglobulin loci may also explain why many immunoglobulin transgenes are less transcriptionally active than the endogenous loci; the transgenes may not include the full complement of activating sequences. Indeed, we have found only weak transcription activity in transgenic mice that harbour a κ transgene that extends only 1.2 kb downstream of the C_κ exon (M.J.Sharpe *et al.*, unpublished observations).

We see no reason why other loci encoding lymphocyte cell surface receptors apart from κ should not also contain multiple enhancer elements. In fact, enhancers have been identified 3′ of the T cell receptor C_α and C_β genes, although no J-C intron-enhancer has been found in TcRβ and the existence of an intron enhancer in the TcRα locus is controversial (Luria *et al.*, 1987; Krimpenfort *et al.*, 1988; McDougall *et al.*, 1988; Winoto and Baltimore, 1989). The existence of enhancers at the 3′ ends of the immunoglobulin gene loci may also have implications for the activation of proto-oncogenes by chromosomal translocations in leukaemias and lymphomas. Thus in the case of Burkitt's lymphomas and mouse plasmacytomas, the c-*myc* gene is frequently translocated into the IgH locus. However, this does not normally link c-*myc* to the IgH intron-enhancer but, rather, to the 3′ end of the IgH locus (Dunnick *et al.*, 1983; Neuberger and Calabi, 1983; Gerondakis *et al.*, 1984). The existence of a 3′ enhancer in the IgH locus analogous to that described here for the κ locus could give a role to the translocation event in the genesis of the neoplasia. Similarly, the κ3′ enhancer could play a role in the translocations involving the κ and *pvt*-1 loci (Cory *et al.*, 1985). Finally in this context, as discussed in the Introduction, multiple enhancers could well explain the transcription activity of loci that lack an active J-C intron enhancer.

An unambiguous identification of the DNA elements responsible for the lymphoid-specific transcription activity of the κ3′ enhancer must await a detailed dissection and mutational analysis. Nevertheless, the sequence of the enhancer already reveals several striking homologies. In particular, there are extensive matches to segments of the IgH and lymphotropic papovavirus enhancers. There are two separate homologies to the enhancer region of this B-cell-specific virus. These homologies are repeated within the viral enhancer and are present within a fragment that is sufficient to direct lymphoid-specific expression (Mosthaf *et al.*, 1985). The homology to the IgH enhancer covers a 32 bp region of the κ3′ enhancer that shows great homology to the E2/E3 region of the IgH enhancer, but in a scrambled and partially inverted form. This is particularly interesting as analysis of the IgH enhancer has revealed that the region around E2/E3 is sufficient to confer lymphoid-specific transcription activation (G.Cook and M.S.Neuberger, in preparation). The piecemeal homology of the two enhancers leads one to speculate that, by analogy to what has been demonstrated for the SV40 enhancer, the E2/E3 region of the IgH enhancer may, in fact, be composed of a tight cluster of binding sites for multiple factors.

The location of the κ3′ enhancer between C_κ and the RS element means that it is likely to be deleted—at least in one allele—in many B cells that express λ light chains. Indeed, we have shown that both copies of the 3′ enhancer are deleted in MOPC315. From the position of the enhancer, it is possible to imagine that it might play a role in regulating RS rearrangement. It will therefore be particularly interesting to discover at what stage the enhancer is activated during early differentiation along the B cell lineage. In this context, two further sequence homologies within the κ3′ enhancer deserve comment: the homology to the consensus for the IFN response element and the 8 out of 10 match to the NF-κB binding site (Sen and Baltimore, 1986b). Treatment of a pre-B cell line with either γ-IFN or phorbol ester has been shown to result in induction of expression of a rearranged κ gene (Briskin *et al.*, 1988; Lee *et al.*, 1988). Whereas phorbol ester induction is mediated through NF-κB, induction by γ-IFN works by a different pathway. It will clearly be of interest to determine the activity of the κ3′ enhancer in pre-B cell lines and ascertain whether it plays a role in the induction of κ gene expression at this stage of ontogeny.

Materials and methods

DNA and plasmids

The phage λ clone L1 (Steinmetz *et al.*, 1979) containing the germline mouse κ locus was a gift from H.Zachau and plasmids pβ128, pβ800 and πSVHPα2 (Treisman, 1985) were from R.Treisman. A pβ800 derivative with a 1 kb *Xba*I fragment containing the IgH enhancer at −800 and a pβ128 derivative with the SV40 enhancer at −128 were gifts from K.Weston. A pβ128 derivative containing a 481 bp *Sau*3AI fragment spanning the κ-intron enhancer at position −128 was a gift from Graham Cook. The plasmid containing the RS probe (rs0.8 of Moore *et al.*, 1985) was provided by E.Selsing.

Cell lines, cell culture and DNA transfection

Myelomas HOPC1 (Weigert *et al.*, 1970) and MOPC315 (Eisen *et al.*, 1968) and the CH-1 B cell lymphoma (Lanier *et al.*, 1978) were obtained from the American Type Culture Collection, MD, USA. The BCL1 line was given to us by Ellen Vitetta, I29 by Roberto Sitia, HeLa by Richard Treisman and MPC11 BU4 by B.Wasylyk. The WEHI231, BW5147, NIH 3T3 and EL4 cells were from our laboratory collection and their origin is as previously described (Mason *et al.*, 1985). All cells were grown in DMEM/10% FCS/50 μM 2-mercaptoethanol except that RPMI medium was used for CH-1, I29 and BCL1. The MPC11, BW5147, HeLa and NIH 3T3 cells were transfected by calcium phosphate coprecipitation (Graham and van der Eb, 1973) using 20 μg of test plasmid and 5 μg of πSVHPα2 internal reference. Transfection of WEHI231 and EL4 was achieved by use of DEAE−dextran as previously described (Mason *et al.*, 1985). Cells were harvested 36−42 h after transfection.

Preparation and analysis of RNA and DNA

Total cytoplasmic RNA was prepared and ribonuclease protection assays (Melton *et al.*, 1984) carried out as previously described (Mason *et al.*, 1985). DNA from mammalian cells for analysis by Southern blotting was prepared by phenol extraction of whole cell lysates made using lithium dodecyl sulphate. For sequencing of the $\kappa 3'$ enhancer, subclones were generated in M13mp18 or M13mp19 and sequenced by the chain termination method of Sanger *et al.* (1977) except that the extension was catalysed by Sequenase (United States Biochemical Corporation). The region was sequenced on both strands

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