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Origin of transcription of a mouse immunoglobulin light chain gene

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Although the organization, the structure, and the somatic rearrangement of immunoglobulin genes have been studied in detail, there is no information regarding the precise origin of transcription of immunoglobulin genes. We have analyzed the 5′-flanking region of an expressed mouse V kappa light chain gene and the pre-mRNA transcript of that light chain gene, as it is expressed *in vivo*. Study of the pre-mRNA and of the DNA sequence by the procedure of "S1 mapping" establishes that the 5′ end of the pre-mRNA transcript is 25–26 nucleotides upstream from the initiation codon. A CATATA sequence is found 22–23 nucleotides upstream from the origin of transcription. Although several other TATA-like sequences are found further upstream, only a single origin of transcription can be documented. From the S1 protection experiments, the 5′ end of the mature mRNA was found to be the same as that of the pre-mRNA, indicating conservation of that sequence during RNA processing. Finally, a conspicuous pentanucleotide repeat CATTG-CATTG has been identified at the position of transcription initiation.

Key words: S1 mapping/origin of transcription

Introduction

Immunoglobulin genes are unusual among eukaryotic genes in that somatic rearrangement of germline DNA is essential for their expression into a functional protein (Tonegawa *et al.*, 1976). In the case of the kappa light chain genes, rearrangement of DNA joins a V kappa segment to one of four J kappa segments which are located 2–4 kb upstream from the C kappa segment (Sakano *et al.*, 1979) (Figure 1). This reorganization of the germline DNA was initially thought to be confined to a single chromosome and, as such, provided an explanation for the phenomenon of allelic exclusion (Seidman and Leder, 1980). We have shown, however, that the light chain kappa genes can undergo rearrangement on both chromosomes (Steinmetz *et al.*, 1979) and proposed an alternative explanation for allelic exclusion, namely that one of the two rearrangement events is "aberrant" and does not lead to the production of a functional immunoglobulin. The existence of non-functional immunoglobulin gene rearrangement has indeed been demonstrated in a number of other cases (Seidman and Leder, 1980; Perry *et al.*, 1980; Cory *et al.*, 1981). The fact that only one of the two alleles has undergone productive rearrangement provides the current model for allelic exclusion. An additional feature of interest concerning the expression of immunoglobulin genes is that transcription very likely occurs exclusively on V genes which have undergone V-C rearrangement (Storb *et al.*, 1981). This suggests that the DNA sequence alone, including the promoter signals in the 5′ region of the V gene, may not be the only elements involved in the control of immunoglobulin gene expression.

Early studies reported the existence of a very large kappa light chain mRNA precursor of 9 kb in length (Gilbert-Herbert and Wall, 1978; Gilbert-Herbert *et al.*, 1978; Perry *et al.*, 1979). This very large putative precursor molecule was thought to imply an origin of transcription located several kilobases upstream from the J segment cluster. Recent studies, however, have demonstrated that this large transcript is not related to the expression of functional V genes (Perry *et al.*, 1980). Transcription of a productively rearranged kappa locus has been seen to result in a pre-mRNA containing the V kappa/J kappa region, the C kappa region and the intervening sequence separating the two. In addition, the size of the precursor is dependent on which of the four J kappa segments has been involved in the rearrangement event (Perry *et al.*, 1980) (Figure 1). Although the events involved in rearrangement and transcription of kappa light chains have been broadly defined, a detailed knowledge of the sites involved in the initiation of transcription is still lacking. Studies of a number of eukaryotic structural genes have identified DNA sequences that are thought to be signals for transcription initiation. Sequences such as the Goldberg-Hogness box (Goldberg, 1979) and the CAAT box (Benoist *et al.*, 1980) are located 24–30 bp and 70–80 bp, respectively, upstream from the origin of transcription of many genes transcribed by RNA polymerase II (Goldberg, 1979; Ziff and Evans, 1978; Breathnach and Chambon, 1981). These conserved sequences are also found in the 5′-flanking region of the V kappa gene we have studied. In this paper, we report the position of the origin of transcription of a functionally rearranged kappa light chain gene expressed *in vivo*. In addition, we describe a conspicuous pentanucleotide repeat located within the region of transcription initiation. Finally, we present evidence that the 5′ terminus of the mature mRNA is coincident with the 5′ terminus of the pre-mRNA transcript.

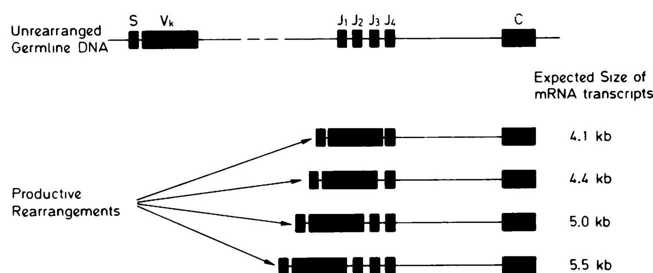

Fig. 1. Schematic representation of V-C rearrangement in the case of Ig light chain genes. The productive rearrangement of a V kappa gene segment from its germline configuration to a position adjacent to one of four J gene segments (J1-J4) results in a functional transcriptional unit. The size of pre-mRNA transcribed depends on which J segment is used in rearrangement, with J1, J2, J3, and J4 giving rise to 4.1, 4.4, 5.0, and 5.5 kb pre-mRNAs, respectively.

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Results

Hybridization of a V gene probe to poly(A)$^+$ RNA from myeloma T

The plasmacytoma tumour T used in this study contains both V kappa alleles in a rearranged state, with V-J joinings at J2 and J4 positions, respectively, for each of the two alleles (Steinmetz *et al.*, 1979; Altenburger *et al.*, 1980). In this work, we consider the transcription products from the functionally rearranged V kappa allele, which is located adjacent to the J4 segment. The V-J4 rearrangement event as well as the nature and size of the expected precursor mRNA is depicted in Figure 1. We analyzed this ~4-kb precursor using the technique of Northern blot hybridization (Alwine *et al.*, 1977). The V kappa probe used was the plasmid pSauA, which contains a 960-bp *Sau*3AI fragment (Figure 3) starting 580 bp upstream of the translation initiation codon and extending 380 bp into the coding region of the functionally rearranged V kappa allele of tumour T. This probe was shown to hybridize only to the functionally rearranged V gene of tumour T. Poly(A)$^+$ RNA from tumour T was fractionated by neutral sucrose gradient centrifugation. Three fractions of RNA were denatured, electrophoresed, and transferred to diazobenzyloxymethyl (DBM) paper for hybridization. The autoradiograph in Figure 2 presents the results of this hybridization. The 4-kb and 1.2-kb sized RNAs confirm the presence of precursor mRNA and mature mRNA respectively in tumour T. These sizes are in agreement with the transcription pattern expected in a V-J4 rearrangement. We also observe a band corresponding to RNA of 3.4 kb in length. Because this RNA is present in the poly(A)$^+$ fraction and reacts with a probe containing the 5′ region of the V kappa gene segment, it most likely contains sequences extending from the 5′ to the 3′ ends of the expressed allele. We suggest that this RNA is a splicing intermediate resulting from stepwise excision of the large intervening sequence. Such stepwise splicing has been reported for the rabbit β globin gene, where detectable levels of intermediate sized RNA molecules corresponding to partially excised intervening sequences are present (Grosveld *et al.*, 1981).

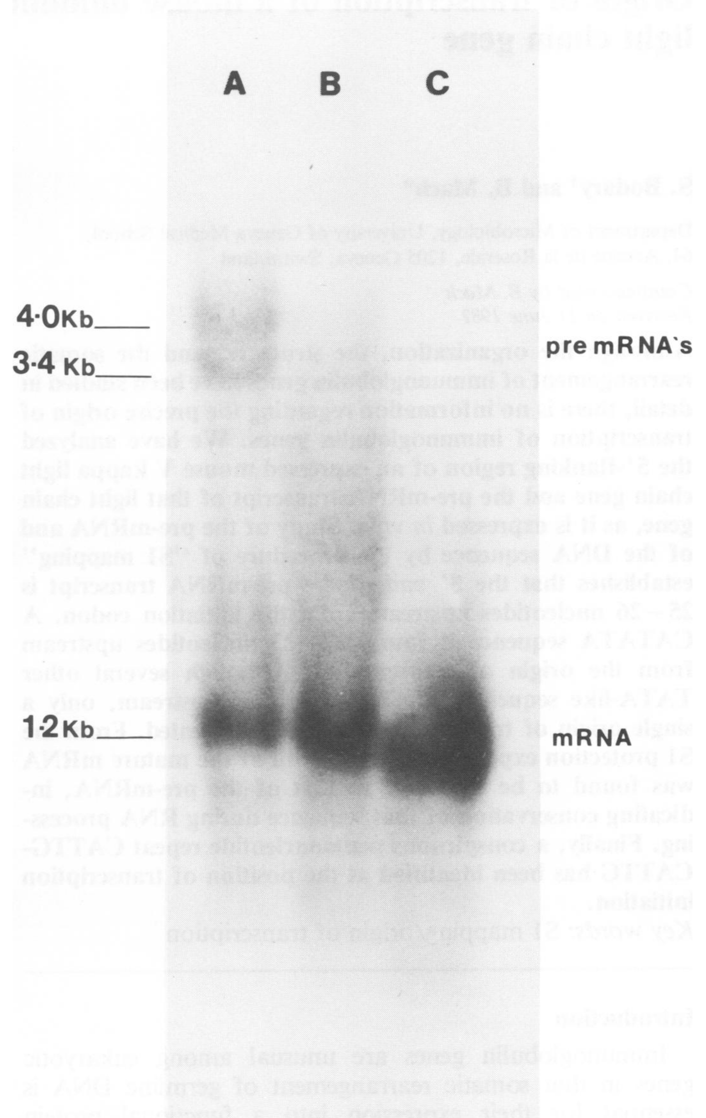

Fig. 2. Hybridization of V gene probe to poly(A)$^+$ RNA from myleoma T. Gradient fractionated poly(A)$^+$ RNA (**A**) 15 μg, (**B**) 15 μg, and (**C**) 24 μg was glyoxalated, electrophoresed in a 0.8% agarose gel, and transferred to DBM paper. The filter was hybridized overnight with ^{32}P-labeled nick-translated plasmid pSau3A, containing the 960-bp *Sau*3A fragment (Figure 2A) subcloned in pBR322. Sizes of RNAs were determined by running *Eco*R1 and *Hind*III digests of λ cI857 DNA in parallel as size standards.

S1 mapping of 5′ terminus of pre-mRNA

The DNA sequence of the region 5′ to the AUG initiation codon in the functionally rearranged V kappa allele reveals the presence of multiple Goldberg-Hogness boxes (Goldberg, 1979) (Figure 5). In order to establish the relationship between these putative promoters and the 5′ terminus of the pre-mRNA, the S1 nuclease mapping technique (Berk and Sharp, 1977) as modified by Weaver and Weissmann (1979) was used. The strategy is outlined in Figure 3a. The 960-bp *Sau*3AI fragment was labelled with ^{32}P and hybridized to two different gradient fractions of poly(A)$^+$ RNA from tumor T, treated with S1 nuclease, denatured and sized. Due to the presence of mature mRNA in the fraction expected to contain the pre-mRNA, we chose a probe which would distinguish between precursor and processed molecules. This is presented in Figure 3a, which shows that a mature mRNA would protect the DNA probe up to the small intervening sequence, while a pre-mRNA would hybridize across the intervening sequence protecting the DNA fragment to a position corresponding to its 5′ terminus. The hybridization was carried out under conditions of DNA probe excess.

Figure 3b shows an autoradiograph of a 5% acrylamide sequencing gel where S1-resistant DNA fragments were sized with respect to pBR322 digested with either *Alu*I or *Hinf*I restriction enzymes and labelled with ^{32}P. The film reveals two major bands that measure 212 and 403 – 404 nucleotides in length. The size of the 212-nucleotide band, found in both pre- and mature mRNA fractions, corresponds to the expected length of DNA that would be protected by mature mRNA. The more slowly migrating band (403 – 404 nucleotides), found only in fractions enriched for high mol. wt. RNA, corresponds to protection by pre-mRNA across the small intervening sequence of the DNA probe. The precision of sizing within this gel system was confirmed by running DNA of known mol. wt. in parallel with *Hinf*I and *Alu*I digests of pBR322. The size determined for a restriction fragment of 406 nucleotides (which corresponds in sequence to

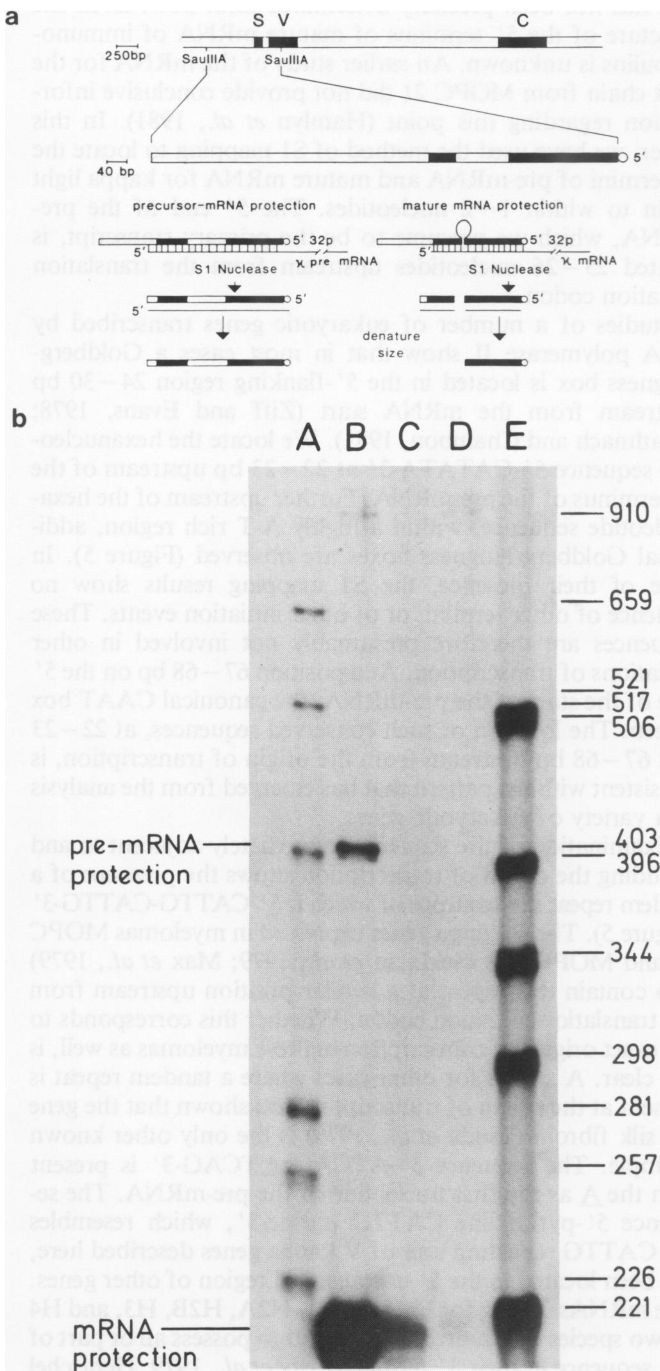

Fig. 3. S1 mapping of pre-mRNA. **(a)** The 960-bp *Sau*3A fragment used as probe is located within the DNA region containing the V kappa light chain gene of tumour T. The hybrids formed between pre-mRNA and mature mRNA and the labeled probe are shown together wtih the products of S1 nuclease digestion. **(b)** The probe was labeled with [γ-^{32}P]ATP to a specific activity of 4 x 10^6 c.p.m./μg and hybridized in ~2-fold molar excess to **(A)** high mol. wt. fraction of gradient purified poly(A)$^+$ RNA and **(B)** kappa light chain mRNA at 48°C. Because of the large amounts of mature mRNA remaining in the pre-mRNA fraction, which was present in low abundance, different amounts of S1-digested material were loaded on the gel. Approximately five times as many counts were loaded in slot **(B)** over slot **(C)**. Hybrids were digested with 5.6 units S1/100 μl for 40 min at 30°C. Lane **(D)** was probe DNA digested with 5.6 units S1 without hybridization to RNA. Lanes **(A)** and **(E)** are *Alu*I and *Hinf*I digested pBR322 labeled with [γ-^{32}P]ATP. Samples were denatured and loaded on a 5% acrylamide sequencing gel. Sizes of pBR322 markers are given to the right of the autoradiograph in base pairs.

the protected fragment), was in agreement with the length known from the DNA sequence. Within the limits of this technique, we assign the 5′ terminus of the V kappa pre-mRNA to the dinucleotide pair 5′-CA-3′ at position −25 to −26 in Figure 5.

S1 mapping of the 5′ terminus of mRNA

Having located the 5′ terminus of the pre-mRNA for an expressed V kappa gene, it was important to determine if the 5′ end was coincident with the 5′ terminus of the mature mRNA. The strategy chosen for this S1 nuclease mapping is outlined in Figure 4a. The 170-bp *Hae*III-*Sau*96I fragment which terminates within the signal peptide sequence was labelled with ^{32}P and cleaved into 100- and 70-bp fragments upon digestion with *Sph*I. The 100-bp fragment was recovered and the sequence determined using the Maxam and Gilbert technique (Maxam and Gilbert, 1980). The 100-bp fragment was also hybridized to poly(A)$^+$ RNA from tumour T, digested with increasing amounts of S1 nuclease, denatured, and run in parallel with the sequenced material on an 8% acrylamide sequencing gel. It is known that the fragments resulting from S1 digestion do not co-migrate exactly with sequenced DNA, but are retarded by 1.5 nucleotides (Tomizawa *et al.*, 1977; Sollner-Webb and Reeder, 1979). The points in Figure 4b indicate the location on the DNA sequence of the 3′ nucleotides of the probe that are protected from S1 digestion by the mRNA.

As is indicated in channels F and G of Figure 4b, increasing the concentration of S1 from 5.6 to 56 units/100 μl progressively digests across the hexanucleotide sequence ACCATT. This overlaps with the CA sequence at which the 5′ terminus of the pre-mRNA was mapped. When high concentrations of S1 are used (560 units/100 μl) (channel H, Figure 4b), some 80% of the ^{32}P of the probe is removed. However, a band corresponding in size to the smallest fragment protected under less harsh S1 conditions (channels F, G) remains. This aligns with the second T residue of the 5′-ACCAT<u>T</u>-3′ sequence. When another probe was used (results not shown), which was defined by the *Hinf*I sites indicated with closed circles in Figure 4a, corresponding results were obtained. We believe that the most plausible explanation for these multiple bands is that, under conditions of high S1 concentration, nibbling of both the 5′ and 3′ ends of the hybrid occurs. This agrees with the observation that a large amount of the 5′ ^{32}P of the probe is lost. On the basis of these digestion patterns we locate the start of the mRNA within the region ATT, at position 23−25 bp upstream from the first AUG translation initiation codon.

The codon ATG at position 0 in Figure 5 was chosen as the translation initiation codon, rather than the ATG three nucleotides downstream, on the basis of the observation that translation is generally initiated at the AUG proximal to the 5′ terminus of a mRNA (Baralle and Brownlee, 1978; Kozak, 1978). In addition, in the case of myeloma MOPC 41, the V kappa gene sequence of which shows a high degree of homology with tumor T (Seidman *et al.*, 1979), and which also has two ATG codons in a similar position, amino acid sequencing of the light chain precursor made *in vitro* (Burstein and Schechter, 1977) has shown that the first AUG is used as the initiation codon.

Discussion

In spite of the extensive analysis of the structure and expression of immunoglobulin genes, their origin of transcrip-

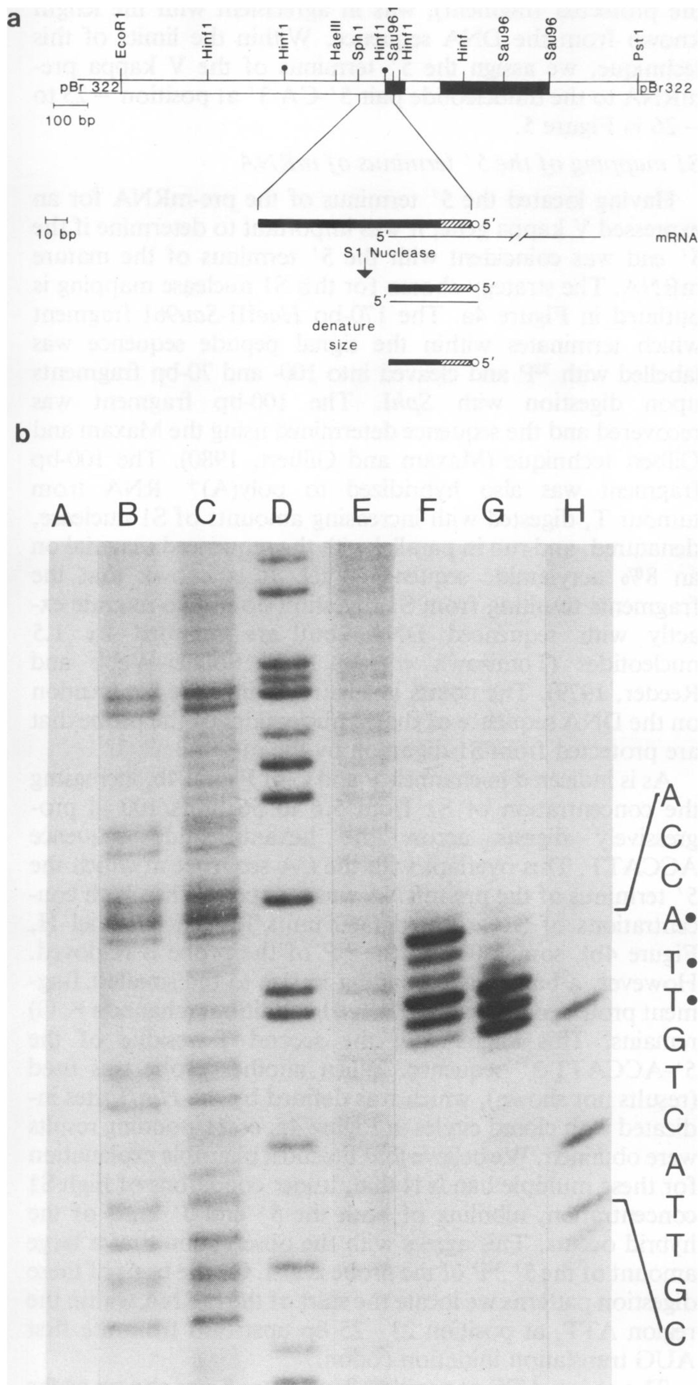

Fig. 4. S1 mapping of mature mRNA. **(a)** The 170-bp *Hae*III-*Sau*96I fragment was purified from the plasmid pR1-Pst shown in the figure, labeled at the 5′ termini with ^{32}P and digested with *Sph*I. The 100-bp *Sph*I-*Sau*96I fragment was hybridized with poly(A)$^+$ RNA, digested with S1 nuclease and sized on a sequencing gel. **(b)** The coding strand of the 100-bp kinased probe was sequenced according to Maxam and Gilbert (1980) and run in parallel with the products of S1 nuclease digested hybrids. **Lanes B, C, D,** and **E** correspond to chemical degradation of C, C+T, G, G+A residues, respectively. **Lane A** was loaded with probe DNA digested with 5.6 units S1 nuclease/100 μl without being hybridized to RNA. **Lanes F, G,** and **H** are probe hybridized to mRNA and digested with 5.6, 56, and 560 units S1 nuclease/100 μl. The sequence presented at the right of the figure corresponds to the mRNA sense strand. The differential migration of sequenced and S1 nuclease digested material has been taken into account in aligning the sequence and the points with the samples in **lanes F, G,** and **H.**

tion has not been precisely determined until now. Even the structure of the 5′ terminus of mature mRNA of immunoglobulins is unknown. An earlier study of the mRNA for the light chain from MOPC 21 did not provide conclusive information regarding this point (Hamlyn *et al.*, 1981). In this paper, we have used the method of S1 mapping to locate the 5′ termini of pre-mRNA and mature mRNA for kappa light chain to within 1–2 nucleotides. The 5′ end of the pre-mRNA, which we presume to be the primary transcript, is located 25–26 nucleotides upstream from the translation initiation codon.

Studies of a number of eukaryotic genes transcribed by RNA polymerase II show that in most cases a Goldberg-Hogness box is located in the 5′-flanking region 24–30 bp upstream from the mRNA start (Ziff and Evans, 1978; Breathnach and Chambon, 1981). We locate the hexanucleotide sequence 5′-CATATA-3′ at 22–23 bp upstream of the 5′ terminus of the pre-mRNA. Further upstream of the hexanucleotide sequence, within a highly A-T rich region, additional Goldberg-Hogness boxes are observed (Figure 5). In spite of their presence, the S1 mapping results show no evidence of other termini, or of other initiation events. These sequences are therefore presumably not involved in other initiations of transcription. At a position 67–68 bp on the 5′ side of the start of the pre-mRNA, the canonical CAAT box is seen. The location of such conserved sequences, at 22–23 and 67–68 bp upstream from the origin of transcription, is consistent with the pattern that has emerged from the analysis of a variety of eukaryotic genes.

Examination of the sequence immediately adjacent to and including the origin of transcription shows the presence of a tandem repeat the sequence of which is 5′-CATTG-CATTG-3′ (Figure 5). The V kappa genes expressed in myelomas MOPC 41 and MOPC 173 (Seidman *et al.*, 1979; Max *et al.*, 1979) also contain this repeat at a similar position upstream from the translation initiation codon. Whether this corresponds to the exact origins of transcription in these myelomas as well, is not clear. A search for other cases where a tandem repeat is present at the origin of transcription has shown that the gene for silk fibroin (Tsuda *et al.*, 1979) is the only other known example. The sequence 5′-ATCAGcATCAG-3′ is present with the A as the first nucleotide of the pre-mRNA. The sequence 5′-pyrimidine CATTC purine-3′, which resembles the CATTG repeating unit of V kappa genes described here, has been located in the 5′ untranslated region of other genes. The mRNAs coding for histones H1, H2A, H2B, H3, and H4 in two species of sea-urchin are found to possess all or part of this sequence at their 5′ termini (Levy *et al.*, 1979; Hentschel *et al.*, 1980). It is thus of interest to ask whether the presence of the sequences related to the pentanucleotide CATTG, found in immunoglobulin genes, may play a role in the regulation of expression of other eukaryotic genes. Whether this sequence is of significance to immunoglobulin gene expression, must await further studies involving deletions and *in vitro* transcription as well as precise mapping of the origin of transcriptions of other immunoglobulin genes.

Our results map the 5′ terminus for both the pre-mRNA and mature mRNA for a V kappa light chain within the tetranucleotides 5′-CATT-3′ (Figure 5). This defines a 5′ untranslated sequence in the kappa chain mRNA of 23–26 nucleotides in length. The identity of the sequence at the 5′ end of both the pre-RNA and the mature RNA strongly suggest conservation of the 5′ end of the pre-RNA during processing. This is frequently assumed to be the case in other

Fig. 5. Sequence of the non-coding strand of the genomic DNA surrounding the transcription initiation site of tumour T. Restriction enzyme recognition sites used in the mapping are located above the sequence. The ATG codon at position 0 is the putative translation initiation codon. Amino acids of the signal and part of the coding sequence are shown below the sequence. Solid and broken arrows indicate the 5′ ends of the pre-mRNA and mature mRNA, respectively. The tandemly repeated pentanucleotide at the origin of transcription is presented in bold faced type. The CAAT and Goldberg-Hogness sequences are boxed. Other sequences showing homology to transcription regulatory signals are underlined.

situations, without, however, an unequivocal demonstration. In this respect, it is of interest that in at least one case, a polymerase II transcript has been shown to be processed at its 5′ end during the course of RNA maturation (Murphy *et al.*, 1982).

The work reported in this paper establishes the precise position of the origin of transcription *in vivo* of an immunoglobulin light chain gene and relates it with specific promoter signals in the DNA sequence 5′ of the gene. One must remember, however, that in the particular case of immunoglobulin genes, signals within the DNA sequence may not be the only elements controlling gene expression. Indeed it appears that only the V genes which have been rearranged next to a J-C segment are being transcribed (Storb *et al.*, 1981), while all the non-rearranged V genes remain silent in spite of their identical 5′-flanking DNA sequence.

Materials and methods

DNA source, preparation, and labelling

DNA containing kappa light chain genomic sequences was obtained from the plasmid pR1-R1 the original cloning of which was described by Steinmetz *et al.*, 1979. Independent sequencing by Altenburger *et al.*, 1980, as well as M. Fiori, J. Gorski, and B. Mach (unpublished results) showed that this plasmacytoma was not MOPC 173, as initially indicated. This myeloma was called plasmacytoma "T" and its DNA, cloned in phage lambda and plasmids, serves as the source of DNA for the present study.

A number of V kappa genes containing fragments derived from pR1-R1 were subcloned into pBR322 for use in these studies. Restriction endonucleases were purchased from New England Biolabs and Bethesda Research Laboratories. DNA fragments were separated by acrylamide gel electrophoresis and purified by passage over DEAE-cellulose (Whatman DE 52) columns. The V kappa-containing plasmid pSauA used in Northern blot hybridizations was labelled with ^{32}P by nick-translation. DNA used in S1 mapping was treated with calf intestinal phosphatase (a gift of S.G. Clarkson) and labelled at its 5′ ends with ^{32}P using T_4 polynucleotide kinase (P-L Biochemicals). Uniquely labelled fragments were generated by cleavage with a second restriction enzyme, gel purified, and DNA to be sequenced was treated according to the method of Maxam and Gilbert (Maxam and Gilbert, 1980).

RNA preparation

Total RNA from myeloma T was isolated from frozen tumours by precipitation in 3 M LiCl, 6 M urea according to Auffray and Rougeon (1980). Poly(A)$^+$ RNA was prepared by two rounds of oligo(dT)-cellulose chromatography after boiling RNA in 1% SDS, 2 mM EDTA for 1 min. Further fractionation by centrifugation through a 5–20% sucrose gradient for 12 h at 40 000 r.p.m. was carried out to prepare samples enriched in high mol. wt. RNA for Northern blot hybridizations. The RNA used in S1 nuclease mapping of the pre-mRNA was carried through a second round of centrifugation in a formamide sucrose gradient (Pawson *et al.*, 1977). Total poly(A)$^+$ RNA was used for S1 mapping of the mature mRNA.

Northern blot hybridizations

Fractions of gradient-purified poly(A)$^+$ RNA, ranging in size from 12S to 45S, were treated with glyoxal and electrophoresed through a 0.8% agarose gel according to McMasters and Carmichael (1977). After electrophoresis, the gel was treated for 40 min in 50 mM NaOH and the RNA transferred to DBM paper (Schleicher and Schuell) by the method of Alwine *et al.* (1977). Hybridizations were essentially as described (Alwine *et al.*, 1977). The plasmid pSauA was nick-translated and hybridized in the presence of 10% dextran sulfate (Pharmacia 500) overnight at 42°C. The filter was washed extensively, dried, and exposed to X-ray film for 12 h (Laskey and Mills, 1977).

S1 nuclease mapping

Hybridizations of RNA to ^{32}P 5′-labelled DNA fragments were carried out in 0.4 M NaCl, 0.4 M PIPES (pH 6.4), 1 mM EDTA, 80% formamide (v/v), 3 μg intact pBR322 DNA, 6 μg tRNA at 42°C or 48°C for 14 h. While still at the hybridization temperature, DNA/RNA hybrids were diluted with cold S1 buffer (0.3 M NaCl, 0.05 M NaAc (pH 4.8), 0.5 M $ZnCl_2$, 20 μg/ml sheared calf thymus DNA) containing 5.6–560 units of S1 nuclease (P-L Biochemicals). Hybrids were immediately transferred to 30°C for digestion and incubated 40–60 min. The reaction was interrupted with the addition of EDTA (5 mM), SDS (0.10%), and tRNA (2 μg/ml) followed by extraction in phenol-chloroform. After deproteinization and ethanol precipitation, samples were denatured and electrophoresed through an acrylamide gel containing 8 M urea in 1 x TBE buffer. Gels were exposed to X-ray film at 4°C or −80°C when intensifying screens were used (Laskey and Mills, 1977).

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