A novel germ-line J<sub>K</sub> transcript starting immediately upstream of J<sub>K</sub>1

Lise Leclercq*, Petra Butkeraitis and Michael Reth*

Institute of Genetics, University of Cologne, Weyertal 121, D-5000 Köln 41, FRG

ABSTRACT

Germ-line transcripts of the immunoglobulin (Ig) and T cell receptor loci are thought to be involved in the control of V gene rearrangement by rendering these loci accessible to the recombinases. We have analyzed the transcriptional activity of germ-line K alleles in two bone marrow-derived Abelson-murine leukemia virus transformed pre-B cells: 300-19, a null cell line, and P8 a μ-producing line. We found a novel germ-line J<sub>K</sub> transcript starting immediately upstream of J<sub>K</sub>1 and spliced to C<sub>K</sub>. The potential role of this transcript in the opening of the Ig K locus as well as in the ordered usage of J<sub>K</sub> segments is discussed.

INTRODUCTION

Germ-line transcripts of immunoglobulin (Ig) loci as well as of T cell receptor (TcR) loci have been described in different tissues and cell lines (1—6). No clear biological function has yet been assigned to these transcripts but their tissue specificity as well as the time in ontogeny at which they appear suggest that they may play a role in the opening of a given locus. Indeed according to the accessibility model the transcriptional activity of a Ig or TcR locus renders it accessible to the recombinase system (reviewed in 7).

More specifically, with respect to the B cell lineage, transcripts of the Ig K light chain locus, have been mostly studied after stimulation of cells with lipopolysaccharide (LPS). Early studies of the B cell lymphoma 70Z/3 showed that LPS not only induces transcriptional activation of the productively rearranged K allele (8) but also the transcriptional activation and alteration in the chromatin structure of the unrearranged (germ-line) K allele (9,10). The transcriptional activity of the IgK locus in its germ-line configuration has been so far monitored by the presence of an 8-kb message, which is transcribed from a promoter located 3.5 kb upstream of J<sub>K</sub>1 (5,11). A 1.1-kb processed derivative of this long transcript has been described (12) which can be detected with a probe (K° probe) containing the promoter of the 8-kb transcript.

The presence of the 8-kb germ-line K transcript was also analyzed in Abelson-murine leukemia virus (A-MuLV) transformed pre-B cells (6). This analysis focused on pre-B cells which carry a V<sub>H</sub>D<sub>H</sub> rearrangement on one or both IgH alleles and whose IgK alleles were both in germ-line configuration. We have studied two bone marrow-derived A-MuLV transformed pre-B cell lines: 300-19, a null cell with an early D<sub>H</sub>1/D<sub>H</sub>1 genotype and P8, a μ-producing derivative of 300-19, carrying V<sub>H</sub>D<sub>H</sub> re-rangements on both alleles (13). Southern blot analysis reveals that most of the 300-19 and P8 cells carry the K locus in germ-line configuration. However, in P8 the K alleles are actively rearranging because some P8 subclones carry V<sub>K</sub> to J<sub>K</sub> rearrangements. In addition small amounts
Figure 1: LPS induces germ-line J_K transcripts in early pre-B cells. (a) map of the whole K locus, showing the different probes mentioned in our study. R = Eco RI, H = Hind III, P = Pvu II, Av = Ava II, X = Xba I, He = Hae III, Bg = Bgl II. (b) 70Z/3, 300-19, P8 and EXC5 were cultured with (+) and without (−) LPS overnight and polyA⁺ RNA was isolated. PolyA⁺ samples were first assayed with a β2m probe to assess the integrity of the RNA as well as to standardize the amount of material used in the protection assay. The AHJ_K
of K chain can be detected in protein extracts of P8 (13). Using these two cell lines we wished to establish how early in ontogeny the K locus is transcriptionally active.

In this study we could identify a novel germ-line J\_K transcript starting immediately upstream of J\_K1, and spliced to C\_K. We report the structure of this transcript and discuss its potential role in the opening of the Ig K locus as well as in the ordered usage of J\_K segments.

**MATERIALS AND METHODS**

**Cells and cell culture**

70Z/3 is a murine lymphoma with a phenotype intermediate between pre-B cell and immature B cells (14). 300-19 and P8 are bone-marrow-derived A-MuLV-transformed pre-B cells which have been extensively characterized (13). EXC5 is a subclone of the murine thymoma EL4 (15). 70Z/3, 300-19, P8 and EXC5 were propagated in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 2 x 10^{-5} M 2-mercaptoethanol. For all experiments exponentially growing cells were used. When indicated cells were grown overnight in the presence of 10 μg/ml LPS (Difco, Detroit, MI).

**Probes**

The AHJ\_K probe was constructed by subcloning the 450-bp Hind III Ava II fragment spanning the J\_K1 segment and containing part of the J\_K2 segment, into SP65. The HHC\_K probe was constructed by subcloning the 400-bp Hae III Hae III fragment containing intron sequence as well as most of the C\_K exon into SP64. The HP5'J\_K probe was constructed by subcloning the 300-bp Pvu II Hind III fragment 5' of J\_K1 into SP64. The β2m probe was constructed by subcloning a 360-bp Pst fragment spanning part of the β2m exon (16) into SP64. This probe was used to control for equal amounts of RNA samples. The restriction fragments subcloned into the transcription vectors were in reverse orientation with respect to the SP6 promoter. 32P-labelled RNA probes derived from the transcription of the linearized plasmids using SP6 RNA polymerase (Promega, Madison, WI) and [32P] UTP according (17). To screen the cDNA library the AHJ\_K insert was recovered from the plasmid by double digestion with EcoRI and Hind III and was nick-translated by random priming, using the Boehringer Mannheim kit (Mannheim, FRG).

**RNA analyses**

PolyA\(^+\) RNA was prepared from cell lines by SDS lysis-protease K digestion and oligo(dT) cellulose batch absorption (18). RNA mapping experiments with the complementary-strand RNA probes were done as described (17). Sample from mapping experiments were analyzed on polyacrylamide/urea sequencing gels. Markers were provided by an Hpa II digest of pBR322. For Northern blotting polyA\(^+\) RNA was denatured in formamide and formaldehyde, electrophoresed through 1% formaldehyde-agarose gels (19), blotted overnight to Nytran membranes (Schleicher and Schuell) and cross-linked to the membrane by UV irradiation according to the manufacturer's instruction. Pre-hybridization and hybridization with 32P-labelled RNA probes were carried out as described (17). After probe was hybridized to 2 μg of polyA\(^+\). M, end-labelled marker DNA fragments of pBR322 digested with Hpa II; P, undigested probe. The fragment labelled 118 points to the processed germ-line transcript starting upstream of J\_K1; the fragment labelled 37 corresponds to the spliced V\_K1U\_K1C\_K transcript of 70Z/3. (c) Scheme of RNA mapping using complementary-strand RNA probes. The template used for the synthesis of the AHJ\_K RNA probe is shown at the top. Fragments of the RNA probe which were RNase resistant after hybridization are shown below, with their size in nucleotides.
Figure 2: Germ-line transcripts of the JK locus are spliced to the CK locus. (a) The same polyA+ samples (2 μg) as those used in Fig. 1 were hybridized to the HHC_K probe. M, end-labelled marker DNA fragments of pBR322 digested with Hpa II; P, undigested probe. Note that the size difference between the band corresponding to full protection and the band corresponding to the probe (P) before digestion is accounted for by polylinker sequences introduced upon cloning of the probe. (b) Scheme of RNA mapping using complementary-strand RNA probes. The template used for the synthesis of the HHC_K probe is shown at the top. Fragments of the RNA probe which were RNAase resistant after hybridization are shown below, with their size in nucleotides.

hybridization membranes were washed at 65°C in 0.1×SSC 3 to 5 times (20 min. each), 0.1% SDS and then autoradiographed at −70°C using Kodak XAR-5 film and intensifying screens. The sizes of the RNA components were estimated using a polyA+ RNA ladder (9.5, 7.5, 4.4, 2.4, 1.4 and 0.3 kb; BRL, Gaithersburg, MD) which was run in a parallel track and was also transferred to the Nytran membrane. Before prehybridization of the membrane the marker lane was cut off and the marker bands visualized by staining with methylene blue (19).
Figure 3: Northern blot analysis of JK germ-line transcripts. PolyA+ RNA from 70Z/3, 300-19, P8 and EXC5 (4 µg/lane) was hybridized with the AHJK probe. The filter was exposed for five days. The size of the transcripts (in kb) is indicated on the left, and was estimated by comparison to a control lane containing size marker RNA molecules (see Materials and Methods). For purpose of comparison, note that the AHJK probe will detect a mature VJKJC transcript with only roughly one tenth of its length whereas it will detect germ-line JK transcripts more efficiently.

cDNA cloning and sequencing
PolyA+ of LPS-stimulated P8 cells was prepared as described in the preceding section; it was then subjected to one more round of oligo-(dT) cellulose chromatography and used to construct a cDNA library in the bacteriophage vector λgt10 (Promega) according to (20,21). The library was screened with the nick-translated AHJK probe. Positive clones were plaque-purified, and inserts were subcloned into EcoRI-digested pUC19. 32P sequencing by the dideoxy method was performed according to Boehringer Mannheim protocol using the M13/pUC universal sequencing primer.

RESULTS
Germ-line transcripts of the JK locus are present in early pre-B cells
Because the JK1 segment seems to be used preferentially during the first VK to JK joint (22,23), we decided to monitor germ-line transcripts of the Ig K locus in a riboprobe protection assay using the JK1-containing probe AHJK. AHJK spans the region going from the Hind III site 80 bp 5' of JK1 to the Ava II site within the JK2 segment (see Fig. 1a,c). In the absence of LPS stimulation no protected band was visible in the lanes containing polyA+ RNA of 70Z/3, 300-19 and P8 (Fig. 1b). However, upon LPS stimulation of 70Z/3 one could detect a 37-bp fragment corresponding to the protection of the JK1 sequence by the mature VKJK1CK transcript of 70Z/3. This 37-bp fragment is not visible in the protection assay using 300-19 and P8 RNA, indicating the absence of mature VKJK1CK message in these lines. Upon LPS stimulation of 300-19 and P8 one could detect two bands: one band of 450 bp corresponds to full protection of the probe (most likely reflecting the presence of run-through transcripts and/or of transcripts starting upstream

6813
Figure 4: Structure of the cDNA clone containing a germ-line \(J_K\) transcript spliced to \(C_K\). The genomic mouse sequence upstream of \(J_K\) is shown at the top (35). Homology with the corresponding human sequence (36) is depicted by a dash and starts refer to introduction of gaps in the sequence. The third line is the sequence of the cDNA clone. The sequence of the two binding sites KI and KII (24) for the K locus protein (KLP) is underlined. The conserved TATA-like motives are overlined. The \(J_K\) segment is boxed and the beginning of the \(C_K\) exon in the cDNA clone is underlined with stippled bars. The arrows point to the two initiation sizes mapped with the HP5'\(J_K\) RNA probe. The Hind III site and using the \(J_K\) splicing donor site); the other band, of 118 bp could correspond to protection of the probe by a processed transcript starting upstream of \(J_K\) and being spliced at the \(J_K\) splice donor site (Fig. 1c). Protection of these bands was specific for B cell-derived RNA since no band was visible in the assay using RNA of the thymoma EXC5. Beside the specific protected bands, digestion products are visible in all lanes, below the 67-bp marker.

Germ-line transcripts of the \(J\) locus are spliced to the \(C\) locus
In order to further characterize the transcript yielding the 118-bp fragment upon protection of the AHJ\(J_K\) probe, we used another riboprobe, called HHC\(C_K\), covering part of the \(C_K\) exon and intron sequences 5' of this exon (Fig. 2b). Upon LPS stimulation one can detect two bands in the lanes containing RNA of 70Z/3, 300-19 and P8: a 403-bp fragment indicating the presence of run-through transcripts and an abundant 265-bp fragment corresponding to protection by transcripts containing a spliced \(C_K\) exon. Given the genotype of the different cell lines as well as the protein data mentioned in the introduction, the 265-bp fragment most likely corresponds to the mature \(V_KJ_KC_K\) message in the case of 70Z/3 and to a germ-line transcript in which \(J_K\) or another \(J\) segment is spliced to \(C_K\) in the case of 300-19 and P8 (Fig. 2a).

In the absence of LPS stimulation the spliced germ-line \(J\) transcripts are detected in P8 although weakly, but not in the more immature 300-19 line. Thus expression of the different \(C_K\) transcripts is either induced by LPS in the case of 70Z/3 and 300-19 or dramatically increased in the case of P8. LPS treatment does not induce any K transcript in EXC5 (Fig. 2a).

Northern blot analysis of \(J\) germ-line transcripts
In order to elucidate the size of the spliced germ-line K transcript we used the AHJ\(K\) probe in a Northern blot analysis. As can be seen in the Northern blot shown in Fig. 3, after
Figure 5: Mapping of the initiation site of the 0.8 kb germ-line JKL transcript. PolyA+ (2 μg) of P8 and EXC5 cells cultured overnight with LPS were hybridized to the HP5'JK probe. tRNA was included as a control for complete digestion of the probe. M, end-labelled marker DNA fragments of pBR322 digested with Hpa II; P, undigested probe. The arrows point to the two major protected fragments of the HP5'JK probe.

LPS stimulation, several RNA species hybridize with the AHJK probe. In 70Z/3 the probe detects mostly a 1.2-kb message, corresponding to the VKJK1CK mature transcript, which yielded the 37-bp fragment in the protection assay (Fig. 1b). In 300-19 and P8 the most abundant species is a 0.8-kb transcript. Less abundant species of 1.2 kb, 5 kb and 8 kb are also visible. The 8-kb transcript may be the species described previously in 70Z/3 and other pre-B cell lines; however, it was not present in our isolate of 70Z/3. The 5-kb transcript is either a splicing derivative of the 8-kb transcript or an unspliced precursor of the 1.2- and 0.8-kb species. No transcript is detected in EXC5 confirming the protection assay data.

Whereas it is clear that the 1.2-kb species corresponds to the mature transcript in 70Z/3, the 0.8-kb and 1.2-kb species in 300-19 and P8 must be (mostly) germ-line transcripts given the genotype and the protein data of these two cell lines.

Structure of the most abundant germ-line JK transcript
To analyze the JK germ-line transcripts in more detail, we constructed a cDNA library with polyA+ RNA of LPS-stimulated P8 cells and screened it with the AHJK probe. A
0.7-kb cDNA clone was isolated and sequenced. The cDNA started 86 bp upstream of \( J_K1 \) (Fig. 4). It contained a \( J_K1 \) segment accurately spliced to \( C_K \) in the same way as the \( V_K J_K1 \) complex is spliced to the \( C_K \) exon. The corresponding transcript was polyadenylated at the normal polyA-additional site 3' of \( C_K \). Finally, using the insert of this cDNA clone as a probe on a Northern blot, we detected the 0.8-kb message and more faintly the 1.2-kb message in polyA\(^+\) RNA of LPS-stimulated P8 cells (data not shown).

To map the potential transcription initiation sites of the germ-line \( J_K \) transcripts, we carried out a protection assay of a uniformly labeled probe, called HP5'\( J_K \), corresponding to the 300-bp Pvu II Hind III fragment, immediately upstream of the Hind III site of the AH\( J_K \) probe (see Fig. 1a). Protection of the HP5'\( J_K \) probe with polyA\(^+\) RNA of LPS-stimulated P8 cells generated two major fragments of about 25 and 35 bp (Fig. 5). The fragments were specifically protected by RNA of P8 and not by tRNA or RNA of the thymoma EXC5. In addition, in the P8 lane some run-through transcripts present in much smaller amounts were able to fully protect the probe and may be derived from the \( K^o \) promoter.

Because the sequence upstream of \( J_K1 \) did not contain any splice site consensus sequence, the two fragments identified presumably map the starts of the transcript about 115 and 105 bp upstream of \( J_K1 \). The localization of the initiation sites is consistent with a spliced 0.8-kb germ-line \( J_K1 \) transcript provided the addition of a 100-bp polyA tail at the normal polyA additional site 3' of \( C_K \). Both initiation sites lie within or immediately upstream of the KII binding site for the K locus protein (KLP; 24). This B cell-specific protein recognizes a DNA sequence upstream of \( J_K1 \) in a region which is very conserved between mouse and human (24). The potential role of this protein in the regulation of \( V_K \) to \( J_K \) rearrangement in lymphoid cells by targeting the recombinase system to the \( J_K \) locus has been discussed (24).

**DISCUSSION**

The preceding results have described two novel species of germ-line transcripts of the \( J_K \) locus, respectively 0.8 and 1.2 kb long. One of them, the 0.8-kb species, has been cloned; RNA mapping and sequence data demonstrated that this transcript initiates upstream of \( J_K1 \), is spliced to \( C_K \) and then polyadenylated at the normal polyA additional site, 3' of \( C_K \). There are two major sites for the \( J_K \) transcript, and several TATA-like motives are found 5' of the presumed starts (two of them are conserved between mouse and human). Multiple start sites have also been reported for germ-line \( C_\mu \) transcripts (25) and germ-line \( C_{\gamma2b} \) (26). The 0.8-kb transcript does not contain an AUG start codon followed by an open reading-frame and thus presumably does not code for a protein.

The structure of the 1.2-kb transcript remains speculative. Although this transcript has not been cloned, a structure consistent with the size and with the protection assays would be the following: a transcript initiating at the same position as the 0.8-kb message, which, instead of selecting the most 5' splice donor site (the \( J_K1 \) donor site) would use the next one available to splice \( J_K2 \) to \( C_K \). However, because of recent data (27,28), showing that the most 5' \( J_K \) splice donor site is exclusively selected in vivo, confirmation of the structure of the 1.2-kb transcript will require sequence data.

So far analysis of germ-line transcripts of the \( K \) locus has mostly focused on an 8-kb transcript starting at a promoter located 3.5 kb 5' of \( J_K1 \). This 8-kb transcript, which is only present in the nucleus, can be processed and yield a 1.1-kb derivative hybridizing to the \( K^o \) probe (5,6,12). Although the exact structure of this 1.1 kb derivative has not
been determined, the fact that it hybridizes to the K° probe—which spans the promoter region of the 8 kb transcript—clearly distinguishes it from the species we have been describing in our study using the AHJK probe. We have been able to detect this 1.1-kb message after LPS stimulation in 300-19 and P8, using the K° probe, but for yet unknown reasons it is present in these cells in much smaller amounts than the 0.8-kb and 1.2-kb species which we detect with the AHJK probe (data not shown).

None of the transcripts described in this study were abundant and furthermore they could mostly be detected only after LPS stimulation of the cells. Since we have analyzed steady-state RNA, we can not at this stage determine whether the low abundancy of the germ-line JK transcripts reflects a low transcription rate or RNA unstability. However, we regard it as unlikely that LPS opens the JK locus. Indeed, in the absence of LPS stimulation both 300-19 and P8 display a DNAse I hypersensitive site 5' of JK1 (D. Auch, unpublished data). LPS may rather act on the already open locus through K chain gene enhancer—binding proteins such as NF-KB (29,30). In any case the LPS-mediated transcriptional activity of the JK locus is not sufficient to induce the rearrangement of the K locus, as indicated by the fact that the frequency of K gene assembly is not increased upon prolonged culture of pre-B cells in LPS (12).

We have mapped the initiation site of the 0.8-kb JK1 transcript within 100 bp upstream of the JK1 segment in a region which has a number of interesting features. First, this region (see Fig. 4) shows significant sequence homology in mouse and human. Indeed, sequence conservation is stronger upstream of JK1 than upstream of the other JK elements. Second, in pre-B cells and early B cells a DNA-binding protein, KLP, was found which binds to two sequences in this region, namely KI and KTJ (24). Interestingly, the initiation sites of the 0.8-kb transcript map to the KII site, which suggests a possible functional relationship between KLP and the germ-line JK transcripts we have described. KLP may be a transcriptional factor participating in the synthesis of the JK1 germ-line transcript. Alternatively, germ-line JK transcripts may interfere with the binding of KLP and thus modulate its yet unknown regulatory function.

KLP and/or transcription of the JK locus may target the recombinase to this region and explain the preferential usage of JK1 in the first VK to JK joint (see below). No specific binding sites were identified upstream of the other JK elements, and we could not identify discrete transcripts of other JK elements using appropriate probes (data not shown). Germ-line transcripts starting upstream of JK1 may therefore account for the preferential usage of JK1 observed in B cells (22,23). Targeting the recombinase first to the JK1 segment would enable the progeny of a pre-B cell to undergo multiple VK to JK joints on each allele. Such a mechanism would not only ensure the assembly of a productive VK to JK complex. It might also allow each pre-B cell to assemble and test several productive VKJK complexes until one of them produces a functional K light chain capable of binding the H chain with high enough an affinity to form an antibody molecule (31).

During ontogeny rearrangements of Ig genes proceed in an ordered fashion occurring first at the IgH locus then at the K locus and finally at the \( \lambda \) locus. This ordered process as well as transfection experiments (32) have suggested that the \( \mu \) chain plays a regulatory role in the onset of assembly of the K gene. Our studies compared 300-19, an early DJH/DJH null pre-B cell and P8, a \( V_HDJ_H^+/V_HDJ_H^- \) \( \mu \)-producing pre-B cell. To our surprise, we did not notice any major difference in the transcriptional activity of the K locus between 300-19 and P8. Our finding therefore seems to indicate that the K locus is already open early in ontogeny, regardless of the presence of a \( \mu \) chain. This is further
supported by recent data reporting the presence of germ-line transcripts of the K locus in scid mice: such transcripts could be detected with the K° probe in fetal liver of scid mice (33) whereas a CK probe was used to identify germ-line transcripts in scid pre-B cell lines (34). In both cases these transcripts were observed in the absence of a μ chain and of any external stimulation such as LPS. Thus, with respect to K gene assembly the signal induced by the μ chain (and perhaps other factors) seems to operate on a locus which is already open and in principle accessible to DNA binding factors such as the recombinase. How the signal induced by the μ chain activates rearrangement of the open K locus remains to be elucidated.

ACKNOWLEDGEMENTS
The authors thank Dr. H. Sommer for his advice and help in the construction of the cDNA library, and Dr. K. Rajewsky for critical reading of the manuscript. L.L. was on leave of absence of the Centre National de la Recherche Scientifique, Paris, and was supported in part by a fellowship of the Alexander von Humboldt Foundation.

REFERENCES