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Immunology

Replacement of germ-line ε promoter by gene targeting alters control of immunoglobulin heavy chain class switching

(IgE/interleukin-4/homologous recombination)

LIXING XU, BEVERLY GORHAM, SUZANNE C. LI, ANDREA BOTTARO*, FREDERICK W. ALT*, AND PAUL ROTHMAN†

Departments of Microbiology and Medicine, and Department of Biochemistry and Howard Hughes Medical Institute, Columbia University College of Physicians and Surgeons, New York, NY 10032

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ABSTRACT **Recent work has shown that the ability of cytokines to direct immunoglobulin heavy chain class-switch recombination to particular heavy chain constant (C) region (C_H) genes correlates with the induction of specific germ-line C_H transcripts. To test the role of germ-line transcripts in class switching, we have used homologous recombination to mutate the immunoglobulin heavy chain locus of the 18.81A20 murine pre-B-cell line. In the parent cell line, the combination of interleukin-4 (IL-4) and lipopolysaccharide (LPS) induces germ-line ε locus transcription prior to class switching to ε. The heavy chain locus of the mutated cell line contains the immunoglobulin heavy chain enhancer and variable region gene promoter in place of the LPS/IL-4-responsive germ-line ε promoter. The mutant cell line constitutively transcribes the ε locus in the absence of IL-4. Strikingly, the mutant cell line also switches to ε in the absence of IL-4. This result demonstrates that, at least in the 18.81A20 cell line, germ-line ε transcription plays a direct role in class switching to the ε locus. In addition, the ability to change the pattern of class switching by altering transcriptional activity indicates that transcription of germ-line C_H is mechanistically important in regulation of class switching.**

Immunoglobulin heavy chain class switching is the process by which the constant region (C) of an antibody is changed, allowing the molecule to retain its antigen-binding specificity while changing its effector function. Most studies have found that class switching occurs by a recombination event between regions of repetitive DNA [termed switch (S) regions] that are present upstream of each heavy chain C region gene (C_H) except C_δ. This recombination deletes the intervening C_H genes from the chromosome and juxtaposes a different C_H gene directly 3′ of the rearranged *V-D-J* (variable–diversity–joining) gene segments (encoding the V region) of the heavy chain locus (reviewed in ref. 1). Certain mitogens and cytokines have been shown to alter the immunoglobulin isotypes produced by B cells both *in vivo* and *in vitro*. For example, the culture of murine B cells with the bacterial endotoxin lipopolysaccharide (LPS) and the T-cell lymphokine interleukin 4 (IL-4) induces the production of IgE (reviewed in ref. 2). The induction of IgE has been shown to be dependent on IL-4 both in transgenic mice (e.g., ref. 3) and in mice infected with parasites (e.g., ref. 4). In addition, mice homozygous for a disrupted IL-4 gene do not produce IgE (5).

The molecular mechanism by which cytokines such as IL-4 direct class switching remains unknown. However, recent work has shown that the ability of cytokines to direct class-switch recombination to particular C_H genes correlates with the induction of transcripts (termed "germ-line C_H transcripts") that initiate upstream of the switch recombination sequences of the involved C_H gene (reviewed in ref. 6). For example, concurrent treatment of murine splenic B lymphocytes or certain B-lineage cell lines with LPS and IL-4 induces expression of germ-line ε transcripts at the transcriptional level before the production of IgE (7–9). Germ-line transcripts have been identified at almost all murine and human C_H genes and are structurally conserved. Further evidence for the important role of germ-line C_H transcription has been provided by the identification of cytokine-responsive cis elements around the initiation regions of certain C_H transcripts (9–11).

The correlation between germ-line C_H transcription and subsequent recombination implicates transcription through specific *S* regions as a targeting mechanism for class switching. There are several models that could explain the mechanism by which germ-line transcription targets class switching (reviewed in ref. 6). One possibility is that there is a non-specific switch recombination machinery that is directed to specific regions by transcription, perhaps by modulating the accessibility of specific *S* regions. Another possibility, suggested by the conserved structure of these germ-line C_H transcripts, is that the transcripts themselves perform an essential role, either as RNA or by encoding small polypeptide molecules.

Although there is a strong correlation between the presence of germ-line C_H transcripts and subsequent switching, there is still no direct evidence that this activity is essential for class switching. To better define the importance of germ-line transcription in the process of class switching, we altered the endogenous heavy chain locus in an Abelson murine leukemia virus (Ab-MuLV)-transformed pre-B-cell line by homologous recombination. The gene targeting inserts the immunoglobulin heavy chain enhancer (E_μ) and variable region (V_H) promoter into the ε locus to ascertain whether constitutive transcription through the S_ε switch region is sufficient to target the ε locus for class switching.

MATERIALS AND METHODS

Cell Culture. The 18.81A20 Ab-MuLV-transformed cell line (12) and culture conditions (8) have been described.

DNA Construct. The "epsilon knock-out" (EPKO) construct used for targeting the heavy chain locus consisted of 10 kilobases (kb) of genomic ε region extending from the *Bam*HI

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Abbreviations: LPS, lipopolysaccharide; IL-4, interleukin-4; C, constant region; C_H, heavy chain C region; V, variable region; V_H, heavy chain V region; S, switch region; S_ε, ε heavy chain S region; C_ε, ε heavy chain C region; D, diversity region; J, joining region; Ab-MuLV, Abelson murine leukemia virus; E_μ, heavy chain gene enhancer; EPKO, epsilon knockout; *neo*, neomycin resistance gene.

*Present address: The Howard Hughes Medical Institute, The Childrens Hospital, Boston, MA 02115.
†To whom reprint requests should be addressed.

site 5′ of the germ-line ε promoter to the *Bam*HI site 3′ of C_ε. In the construct, the 1.1-kb *Pst* I fragment, which includes the promoter and 5′ exon of the germ-line ε transcript (I_ε) (9, 13, 14), was removed from the genomic ε region. Into this region we inserted a 1-kb fragment that contains the immunoglobulin V_H promoter and heavy chain enhancer (E_μ) (15). In addition, the neomycin-resistance gene (*neo*), expression of which is driven by the polyoma enhancer/herpes simplex virus-thymidine kinase gene (HSV-*tk*) promoter, was added. The *neo* gene was oriented so that transcription is directed away from the ε region. The HSV-*tk* gene was also added at the 5′ end of the construct.

Generation of Mutant Cell Lines. The EPKO construct was transfected by using electroporation into 18.81A20 cells, and individual G418/ganciclovir double-resistant clones were isolated (16) and analyzed for homologous integrants by Southern blotting.

DNA and RNA Blot Hybridization (Southern and Northern Blotting) and Preparation of Probes. Blotting techniques and preparation of probes were as described (13). The C_ε probe (C_ε117) is a cDNA clone (P.R., unpublished data) that contains the entire C_ε coding region. The 3′ C_ε probe (probe 1 in Fig. 1) was derived as a *Bam*HI–*Xba* I fragment from ε-6 phage clone (17), which was provided by T. Honjo (Kyoto University).

S1 Nuclease Protection Assay. An *Eco*RI–*Bst* II fragment derived from the CE6 cDNA was subcloned into an M13 phage vector (13), and a uniformly labeled single-stranded probe was prepared. Hybridization to RNA, S_1 nuclease digestion, and analyses on 5% polyacrylamide/7 M urea gels were performed as described (18).

Reverse Transcription–Polymerase Chain Reaction (RT–PCR) Assay. A 19-base-pair (bp) oligonucleotide (5′-CCTTTACAGGGCTTTAAGG-3′) complementary to a sequence in the C_H1 exon of the C_ε gene was used as a primer, and cDNA was synthesized by RT. The cDNA was amplified by PCR with the same C_ε oligonucleotide and an 18-bp oligonucleotide corresponding to the sequence (5′-GTAC-TACCTGCAGTTGAA-3′) of the V_H3660 nucleotide sequence (19). Reaction products were Southern blotted and probed with an end-labeled 17-bp oligonucleotide that corresponds to the sequence (5′-GGAACCCTCAGCTCTAC-3′) found in C_H1 of the germ-line C_ε gene; this latter sequence is 5′ to that represented by the oligonucleotide used in the PCR (13).

RESULTS

Replacement of the I_ε Region in a Pre-B-Cell Line. The EPKO construct (Fig. 1*A*) was transfected into 2×10^7 18.81A20 cells, and 65 individual G418- and ganciclovir-resistant clones were screened for the homologous recombinants. One clone, KO57, appeared to have integrated a single copy of the construct by homologous recombination as determined by Southern blotting analysis with a probe from the 3′ region of the ε locus (Fig. 1*B*). This was confirmed with a probe from the *neo* gene (Fig. 1*B*) and upstream probes (data not shown). When this cell was further subcloned, 11 of

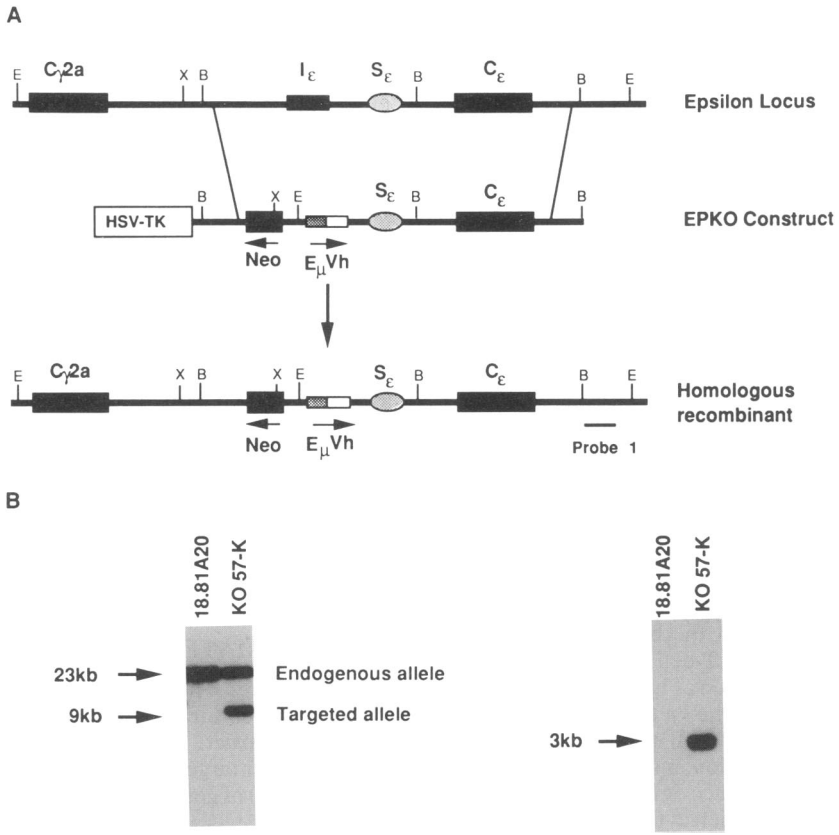

FIG. 1. Creation of mutant cell lines by gene targeting. (*A*) Scheme for replacement of the I_ε exon and promoter by the V_H promoter (V_H), C_H enhancer (E_μ), and *neo* gene. The wild-type ε locus is shown along with the targeting EPKO construct and locus after homologous recombination. B, *Bam*HI; E, *Eco*RI; X, *Xho* I. (*B*) Southern blot screening of transfected cells. (*Left*) Genomic DNAs from 18.81A20 and transfected cell clones are digested with *Eco*RI, run on 1% agarose gel, blotted, and probed with probe 1, which is 3′ of the C_ε region in the EPKO construct but 5′ of the *Eco*RI site. In 18.81A20, each allele gives a 23-kb band, while in KO57-K, one allele gives a 9-kb band—a size expected from homologous recombinant. (*Right*) DNAs are digested with *Xho* I and probed with the *neo* gene sequence. KO57-K gives only one band around 3 kb—a size expected if the 5′ portion of the EPKO construct is also integrated into the chromosome by homologous recombination. HSV-TK, herpes simplex virus thymidine kinase gene.

12 subclones gave the same pattern on Southern blots (data not shown). One of these subclones, KO57-K, was used for further studies.

Constitutive Expression of the Targeted ε Locus. Previous work has shown that the 18.81A20 cell line requires the presence of both LPS and IL-4 to produce germ-line ε transcripts (13). The induced transcripts initiate at the I_ε promoter and proceed through the S_ε region and the C_ε gene. These primary transcripts are then spliced to form mature transcripts, which consist of an I_ε exon, derived from sequences upstream of the S_ε region, spliced to the C_ε gene (13, 14). The targeted immunoglobulin C_H allele of the KO57-K mutant has the *neo* gene, the E_μ enhancer, and the V_H promoter in place of a 1.1-kb region of the germ-line ε locus, which contains the LPS/IL-4-responsive promoter and the I_ε exon.

To determine if this mutation has altered the transcriptional activity in this cell line, cells were placed in culture medium alone, with LPS, or with LPS/IL-4. RNA from these cells were analyzed by Northern blotting with a C_ε cDNA probe. As we have reported (13), the 18.81A20 line only produced the germ-line ε transcript (as indicated by the 1.7-kb C_ε-hybridizing band) when cells were cultured with LPS and IL-4 (Fig. 2, lanes 1–3). In contrast, KO57-K cells produced two C_ε-hybridizing bands, one of 1.7 kb and a predominant band of 7 kb (Fig. 2, lanes 4–6). Both of these transcripts were present at low levels constitutively (Fig. 2, lane 4). However, when cells were cultured with LPS alone or with LPS/IL-4, the levels of both transcripts were increased (Fig. 2, lanes 5 and 6). The C_ε-hybridizing transcripts produced by KO57-K mutant cells appear to represent transcription from the altered allele. The 7-kb transcript has the predicted size of an unspliced transcript initiating from the V_H promoter. The 1.7-kb C_ε-hybridizing band likely represents a spliced form of this primary transcript (see below). The RNA derived from cells cultured with both LPS and IL-4 appeared to contain a doublet of 1.7-kb C_ε-hybridizing transcripts. This likely represents a mixture of the 1.7-kb product from the targeted allele and the 1.7-kb germ-line ε transcript from the nontargeted allele (see below).

LPS and IL-4 Induces Germ-Line ε Transcript from the Unmutated Immunoglobulin Allele. We used an RNA protection assay to better define the transcriptional activity in these cells. In previous studies, we have used a single-stranded uniformly labeled DNA probe derived from a cDNA encoding the germ-line ε transcript (shown in Fig. 3*A*) to analyze the different C_ε-hybridizing transcripts produced by B lymphoid cells. As shown previously (13), the 18.81A20 cell line produces germ-line ε transcripts (which protect both the C_ε and I_ε portions of the probe) only after culture with LPS/IL-4 for more than 12 hr. After 6 days of culture with LPS and IL-4, these cells produced a small amount of a transcript that only protected the C_ε region of the probe (Fig. 3*B*, lane 6). These latter transcripts represent mature V-D-J-C_ε transcript produced after the switch to ε (13).

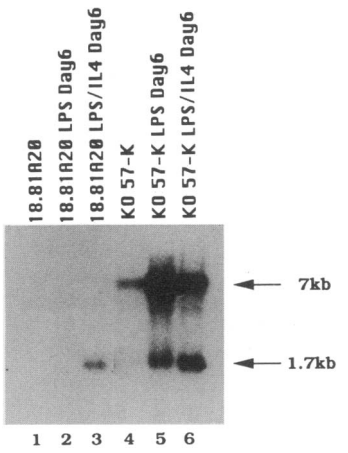

FIG. 2. Expression pattern of ε transcripts in 18.81A20 and KO57-K. The cell lines were cultured as indicated above the lanes, and total RNA was assayed by Northern blotting for C_ε hybridization transcripts with probe Cε117. The arrows indicate the size of C_ε-hybridizing bands.

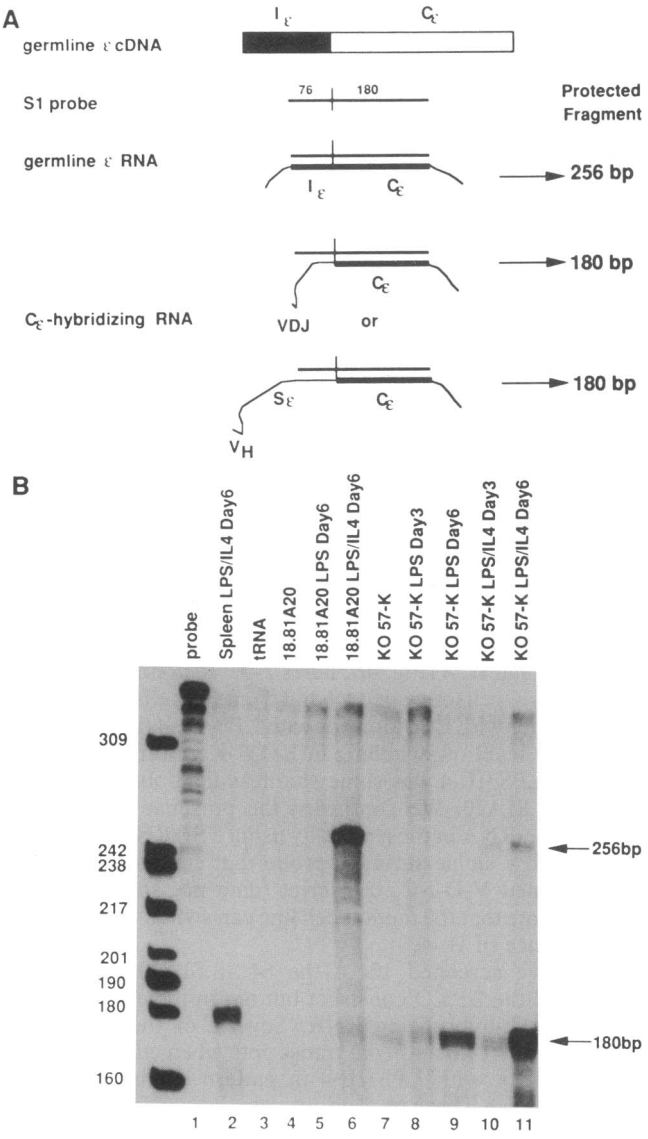

FIG. 3. Expression of ε transcripts in mouse splenocytes, 18.81A20, and KO57-K cells. (*A*) Diagram depicting the sizes of S1 nuclease-protected fragments of the 256-bp uniformly labeled probe derived from the 5′ end of the CE6 cDNA after hybridization to germ-line ε RNA or other C_ε-hybridizing RNA. (*B*) S1 nuclease protection analyses as in *A* with RNA from cells cultured under various conditions as indicated. Lanes: 1, markers (pBR322/*Msp* I); 2, probe alone; 3, BALB/c splenocytes cultured with LPS/IL-4 for 6 days; 4–6, 18.81A20 cells cultured as indicated; 7–11, KO57-K cells cultured as indicated.

A different pattern of transcripts was found in the KO57-K line. RNA from cells cultured alone produced no germ-line I_ε transcripts (the I_ε exon has been removed from the altered allele) but did produce transcripts that protect the C_ε region of the probe (Fig. 3*B*, lane 7). These transcripts were induced by culture with LPS (Fig. 3*B*, lanes 8 and 9). These data, in combination with that presented above (Fig. 2), indicate that these transcripts most likely represent a mixture of several species including the 7-kb transcript initiating from the V_H

promoter and any form of this RNA that spliced an upstream region to C_ε. In addition, if KO57-K switches to ε, a V-D-J-C_ε mRNA would also be represented by this band (see Fig. 3*A*). When KO57-K was cultured with LPS and IL-4, a new band, which corresponds in size to protection of both the I_ε and C_ε regions of the probe, was induced (lanes 10–11). This represents induction of germ-line ε transcripts from the nontargeted allele.

KO57-K Can Class Switch to C_ε Without IL-4. The above analyses revealed that the KO57-K cell transcribes the ε locus without IL-4. To determine whether this altered transcriptional activity has changed the pattern of switching to ε, we used a PCR method to assay for V-D-J-C_ε transcripts (shown in Fig. 4*A*) (13). Total RNA, from the experiments outlined above, was used to generate cDNA by using an oligonucleotide from the 5′ end of the C_ε gene as a primer and reverse transcriptase. This cDNA was amplified by PCR using the C_ε oligonucleotide and an oligonucleotide from the V_H3660 gene used in the productive *V-D-J* rearrangement previously described in this cell line (12). The PCR product was then blotted and probed with an oligonucleotide from the C_ε gene 5′ (internal) to the one used in the previous steps. When RNA from the 18.81A20 line was assayed, we only observed a band when the cells were cultured for 6 days with both LPS and IL-4 (Fig. 4*B*, lanes 1–3). In contrast, assay of the KO57-K cells revealed a band from the cells cultured for 6 days either with LPS alone or with LPS/IL-4 (Fig. 4*B*, lanes 4–6). The level of switching in KO57-K cultured with LPS alone or LPS/IL-4 was somewhat less than observed in the parent 18.81A20. We confirmed the presence of a mature V-D-J-C_ε mRNA in these cells by using a S_1/RNA protection study with a single-stranded probe that contains the previously cloned V-D-J-C_ε transcript (data not shown). These data indicate that the mutant cell line can switch to C_ε without the presence of IL-4.

We have screened 10 of the 64 18.81A20 clones that integrated the EPKO construct but not in the endogenous ε locus for C_ε-hybridizing RNA. Several of these cells expressed the 1.7-kb and 7-kb transcripts when cultured alone, with LPS, or with LPS/IL-4 (a pattern similar to that of KO57-K cells). These transcripts were derived from cells that had integrated the EPKO construct intact. However, the level of 1.7-kb and 7-kb transcripts in these cells was less than that of KO57-K, which is likely because the construct(s) were integrated outside the heavy chain locus in these cells. When we assayed these cells for the ability to switch to C_ε, as determined by the presence of V-D-J-C_ε RNA, they switched when cultured with both LPS and IL-4 but not with LPS alone (data not shown). Therefore, it is unlikely that V-D-J-C_ε RNA is produced by the trans-splicing of RNA, suggesting that class-switching in KO57-K occurs via DNA recombination.

DISCUSSION

We have replaced the I_ε exon and IL-4-responsive promoter element with the V_H promoter and E_μ enhancer in an Ab-MuLV-transformed pre-B-cell line, using homologous recombination. Our data indicate that this mutation causes the cells to transcribe the ε locus in the absence of IL-4. The remarkable finding of our experiment was the presence of V-D-J-C_ε transcripts in the KO57-K cell line cultured in the presence of LPS alone. This is evidence that switching to ε can occur (if the ε region is being actively transcribed) in the absence of IL-4. This would suggest that, at least in the 18.81A20 cell line, IL-4 directs switching to ε through its role as an inducer of germ-line ε transcription and not by the induction of S_ε-specific recombination factors. In addition, the ability to change the control of switching by altering transcriptional activity indicates that germ-line C_H transcription is mechanistically important in class switching.

Although we are unsure which allele of KO57-K we have mutated, it appears that we have targeted the productive allele. Unlike the parent line, which requires culture with LPS and IL-4 to switch to ε, we have found V-D-J-C_ε transcripts that contain the productive V-D-J with LPS alone in the KO57-K line. If our integration occurred in the nonproductive allele, then KO57-K would have to be switching by interchromosomal recombination. However, the culture of KO57-K with LPS and IL-4 does not affect the level of V-D-J-C_ε transcripts. If the switching seen with LPS alone were secondary to interchromosomal recombination, then switching on the productive allele should not be altered and there should be a higher level of V-D-J-C_ε transcripts with LPS and IL-4 (due to both intrachromosomal switching on the wild-type productive allele and interchromosomal rearrangement to the targeted allele). The absence of increased levels of V-D-J-C_ε transcripts with the addition of IL-4 implies that the targeted allele contains the productive *V-D-J* rearrangement.

The conserved structure of germ-line C_H transcripts implies a functional role for these transcripts. Our homologous integrate removed the germ-line I_ε exon and replaced it with the E_μ element and V_H promoter. Our finding that this cell line can switch to ε demonstrates that the sequences within the I_ε

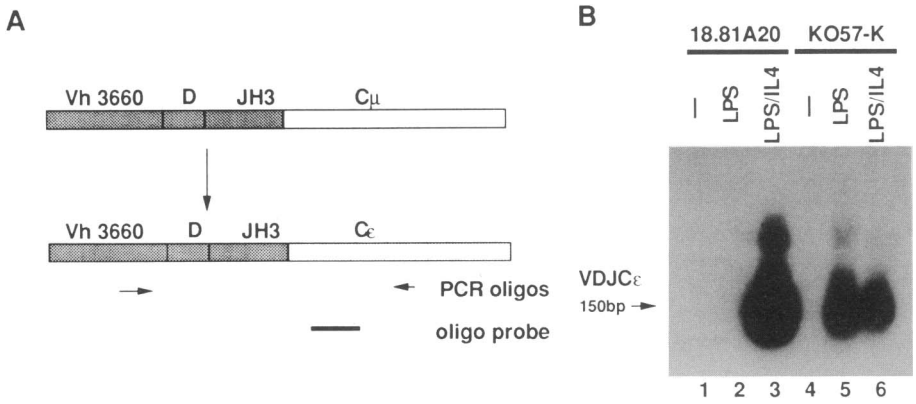

FIG. 4. Expression of V-D-J-C_ε transcripts by 18.81A20 and KO57-K cells. (*A*) This map indicates positions of oligonucleotides used in the PCR experiment. Oligonucleotides derived from the germ-line sequence of V_H3660 and the Ch1 domain of C_ε were used to amplify cDNA. The location of the internal oligonucleotide (oligo) probe used for the hybridization is indicated. (*B*) Products of the PCR analysis as outlined in *A* and performed on total RNA of the 18.81A20 or KO57-K cells cultured without additional agents (lanes 1 and 4), in the presence of LPS alone for 6 days (lane 2 and 4) or with LPS/IL-4 for 6 days (lanes 3 and 6) were assayed by Southern blotting for hybridization to the internal C_ε oligonucleotide probe. The arrow indicates the expected size of the hybridization band.

exon are not absolutely required for switching and does not support models in which the I exons of germ-line transcripts encode functionally essential small polypeptides (20). Although the mutant cell line has clearly altered the regulation of switching in these cells, our results also indicate that switching to C_ε in KO57-K appears to be lower than in 18.81A20 parent cell line. We have previously noted that subclones of Ab-MuLV-transformed pre-B-cell lines normally vary in their capacities to class switch. The difference between the switching seen in 18.81A20 and the mutant KO57-K cells might be a reflection of this normal variation. However, this difference might also indicate that the mutation has decreased the ability of the targeted allele to switch to C_ε. This might indicate that some sequences that were removed by the mutation perform functions that were not totally replaced by the inserted DNA. Therefore, although our data shows that sequences within the I_ε region are not essential for class switching to ε, it is still possible that they perform a function that influences the level of switching in normal B cells.

The ability to demonstrate that switching in the KO57-K cells occurs via DNA recombination and not by the proposed trans-splicing mechanism (21) is difficult because of the low level of switching in Ab-MuLV-transformed pre-B-cell lines (e.g., ref. 22). The level of switch recombination to ε in the 18.81A20 parent line cultured with LPS/IL-4 is below the level of detection of methods that have been used to examine switch recombination in normal splenic cells (e.g., ref. 23). We have attempted an alternative approach to this question by asking whether a cell that produces the same RNA species as KO57-K, but does not contain the same mutation within the C_H locus, produces V-D-J-C_ε transcripts (as would be expected if switching occurred by trans-splicing). The results described above suggest that switching in KO57-K does not occur by trans-splicing. Finally, the E_μ element, which our mutation placed within the ε locus, in addition to possessing core enhancer elements also contains elements that can act as a matrix attachment site (24) and as a locus control region (R. Grosschedl, personal communication). These other functions may also be important in directing switching to specific C_H loci.

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