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Immunology

Molecular characterization of germ-line immunoglobulin A transcripts produced during transforming growth factor type β-induced isotype switching

(polymerase chain reaction)

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ABSTRACT **The addition of transforming growth factor type β to lipopolysaccharide-stimulated murine B-cell cultures enhances isotype switching to IgA and induces the appearance of two sizes of α mRNA transcripts. One of these is the same size as mRNA for secreted IgA but the other, which is 300–400 base pairs (bp) shorter, does not correlate in size with any form of productive α mRNA. Both sizes of transcript were shown to contain germ-line sequences 5′ to the α switch region, suggesting that the longer transcripts included both germ-line and productive forms of α mRNA, whereas the shorter transcripts were only germ-line α mRNA. We isolated cDNA clones corresponding to the shorter, 1.3-kilobase (kb), transcript by using an anchored polymerase chain reaction and a specific primer for the constant region. Analyses of these cDNA clones show that the short transcript consists of a 126-bp exon located ≈1.5 kb 5′ to the α switch region spliced to the first exon of the α constant region locus. Furthermore, a minor fraction of the longer, ≈1.7 kb, transcripts also contains this exon. These results demonstrate that transforming growth factor type β-mediated isotype switching to IgA is preceded by transcriptional activation of the heavy-chain locus.**

The process of isotype switching in B cells appears to be directed rather than random. Analyses of isotype expression by B-cell clones suggest that surface IgM (sIgM)-expressing B cells are not restricted in their potential to switch to other isotypes (1). However, certain cytokines have been shown to influence the pattern of isotypes secreted. For example, the addition of interleukin 4 (IL-4) to cultures of lipopolysaccharide (LPS)-stimulated B cells enhances the production of both IgG1 and IgE and decreases the production of IgG3 and IgG2b (2–5). Furthermore, IL-4 increases the proportion of B-cell clones secreting IgE, demonstrating that it acts to induce isotype switching (6). Recently, we have shown that the addition of transforming growth factor type β (TGF-β) to LPS-stimulated B-cell cultures specifically enhances IgA secretion concomitant with a decrease in the secretion of other isotypes (7). The precursors of IgA-secreting cells in these cultures are $sIgA^-$, suggesting that TGF-β stimulates isotype switching to IgA (8).

Heavy-chain class switching is accomplished by a recombinational event between switch (S) regions located immediately 5′ to each heavy-chain locus (C_H) (C, constant region). This recombination, S–S joining, brings a specific variable region (V_H) adjacent to a new C_H (9). Studies with B-cell lines demonstrated that the C_H to which recombination occurs is transcriptionally active prior to recombination (10, 11). The I.29 lymphoma switches preferentially to either IgA or IgE after stimulation with LPS. Prior to LPS stimulation, both α and ε transcripts containing sequences 5′ to the respective switch region spliced to the C_H gene are detected (12). Similar germ-line γ2b transcripts are detected in the 300-18P pre-B-cell line, which switches spontaneously to γ2b (11). Secondary heavy-chain loci are also transcriptionally active prior to heavy-chain class switching in normal B cells. The addition of IL-4 to LPS-stimulated B-cell cultures induces the appearance of both γ1 and ε transcripts containing germ-line sequences prior to the appearance of productive V_H-containing transcripts (12–15).

In LPS-stimulated B-cell cultures, TGF-β induces the appearance of two α transcripts; one is the same size as αs mRNA (α mRNA using the 3′ end for secreted IgA), while the other is ≈400 base pairs (bp) shorter and presumably does not encode a complete IgA molecule (8). The present study demonstrates that the shorter, 1.3-kilobase (kb) transcript contains a sterile exon, consisting of germ-line sequence located 5′ to S_α spliced to the first exon of the C_α gene. In addition, a fraction of the 1.7-kb transcripts also contains a sequence from this sterile exon. Thus, transcriptional activation of the heavy-chain locus is associated with TGF-β-mediated isotype switching to IgA in normal B cells.

MATERIALS AND METHODS

Animals. Female BALB/c mice were purchased from Simonsen Laboratories (Gilroy, CA) and used when they were 8–12 weeks old.

Cell Preparation and Culture. B-cell preparation and cultures were performed essentially as described (4). B cells prepared from spleen were cultured at 1×10^6 cells per ml in 24-well tissue culture plates. All cultures were stimulated with *Salmonella typhosa* LPS (8 μg/ml) (Sigma) and the indicated cytokines.

Cytokines. Porcine TGF-β was purchased from R & D Systems (Minneapolis) and used at a final concentration of 2 ng/ml. Recombinant murine IL-2 expressed in *Escherichia coli* was purified to >95% purity by Schering. Recombinant IL-2 with a specific activity of 1.25×10^8 units/mg where 1 unit is defined as the concentration that supports one-half maximal growth of the T-cell line HT2 was used at a concentration of 10 ng/ml. The concentrations of IL-2 and TGF-β were optimal for IgA production as determined by titration both individually and together (7).

RNA Isolation and Northern Blot Analyses. Total RNA was prepared from single cell suspensions by the guanidine isothiocyanate/cesium chloride method (16). $Poly(A)^+$ RNA was isolated on oligo(dT)-cellulose (Collaborative Research).

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Abbreviations: sIg, surface immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; TGF-β, transforming growth factor type β; PCR, polymerase chain reaction; S, switch; C, constant; V, variable; J, joining; D, diversity.

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Either 10 μg of total or 3 μg of poly(A)$^+$ RNA per lane was electrophoresed in a 1% agarose gel containing formaldehyde and was transferred to Nytran filter membranes (Schleicher & Schuell). Prior to hybridization with a second probe, blots were boiled in 0.1× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS and exposed to film to determine whether the previous probe had been removed.

Probes. The probe for C_α sequences was the *Bst*EII fragment of pαJ558 (ref. 17; kindly provided by Philip Tucker, University of Texas, Dallas), which contains all of $C_\alpha 1$, $C_\alpha 2$, and part of $C_\alpha 3$, and was labeled by the random primer method (18). The SXα1 probe was a 70-mer synthesized from the sequence of pSXα1 and was labeled by fill-in polymerization (19).

Cloning and Sequencing. The anchored polymerization chain technique (20) was used for cloning. cDNA was prepared by using 3 μg of total RNA from day three LPS-stimulated B-cell cultures containing TGF-β and IL-2, an oligo(dT) primer and Abelson murine leukemia virus reverse transcriptase (21). The reverse transcription product was phenol/chloroform extracted and precipitated twice with ethanol. The cDNA was tailed with dGTP using terminal deoxynucleotidyltransferase (Pharmacia) in a 20-μl reaction mixture containing 150 mM sodium cacodylate, 2 mM cobalt chloride, 1 mM dGTP, 0.1 mM dithiothreitol incubated 30 min at 37°C. The tailed cDNA was phenol/chloroform extracted and ethanol precipitated. The entire product was amplified by using *Thermus aquaticus* DNA polymerase (IBI) in four 50-μl reaction mixtures with the standard buffer. The C_α primer contained an *Xba* I site and base pairs 76–100 of C_α (5′-ATTAGCTCTAGGGAAAAGGGAAGTAATCGTGAATCAGGC-3′). For the poly(dG) end, a 1:9 mix of a primer containing the restriction sites for *Pst* I, *Xba* I, and *Sph* I and a short C tail (5′-GGCGCCCTGCAGCGGTCTAGAGCATGCC-3′), and the same sites with a long C tail (5′-GGCGCCCTGCAGCGGTCTAGAGCATGCCCCCCCCCCCCCCC-3′) were used. Amplification was performed for 30 cycles; the first 5 cycles used an annealing temperature of 45°C and the subsequent cycles used an annealing temperature of 55°C. The amplification mixes were pooled, phenol/chloroform extracted, ethanol precipitated, and loaded onto a 5% acrylamide gel. A band at ≈220 bp was isolated, cut with *Xba* I and *Sph* I, and ligated into pUC18. For screening, DNA was isolated from individual colonies and cut with *Xba* I and *Sph* I. If the insert was present, DNA was sequenced by the dideoxynucleotide chain-termination method (22) using both the "-40" primer (United States Biochemical) and the M13 reverse primer (New England Biolabs). The location of SXα1 was determined by sequence homology with p5′S_α (ref. 12; kindly provided by Janet Stavnezer).

RESULTS

TGF-β Induces Two Distinct α mRNA Transcripts. The addition of TGF-β to LPS-stimulated B-cell cultures enhances IgA secretion 5- to 7-fold (7). Although IL-2 alone causes at best a 2- to 3-fold enhancement of IgA secretion in these cultures, it acts synergistically with TGF-β to cause approximately a 50-fold enhancement in IgA secretion. Furthermore, the addition of TGF-β to LPS-stimulated B-cell cultures resulted in the appearance of two distinct sizes of α mRNA transcripts by day 2 of culture (8). Significantly, in TGF-β-containing cultures, an increase in detectable sIgA$^+$ cells does not occur until day 3 of culture, and increased IgA secretion does not occur until between days 4 and 5 of culture (8), demonstrating that the appearance of both transcripts precedes TGF-β-mediated effects on IgA expression. On day 1 of culture, a 1.7-kb C_α transcript was found in cultures both with and without TGF-β (Fig. 1). Normal spleen cells prior to culture also contain αs mRNA (23), suggesting that the day

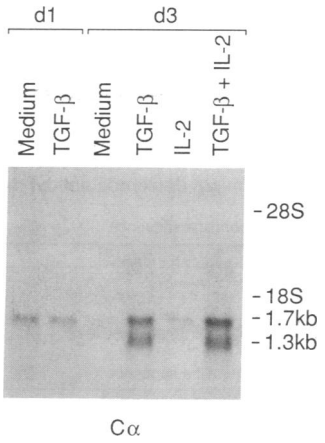

FIG. 1. TGF-β induces two distinct α mRNA transcripts. Total RNA from the indicated populations was electrophoresed and blotted to nitrocellulose filters. Prior to transfer, the gel was stained with ethidium bromide to ensure that comparable amounts of RNA were electrophoresed. The blot was hybridized with a probe specific for C_α.

1 α mRNA transcripts were not a consequence of *in vitro* induction. Furthermore, TGF-β enhancement of the steady-state level of α mRNA was not detected until day 2 after stimulation (7). On day 3, two sizes of α mRNA transcripts were present only in TGF-β-containing cultures; one of 1.7 kb, which is the same size as productive αs mRNA transcripts from the IgA secreting myeloma MOPC 315, and one of 1.3 kb, which does not correlate with the size of any known form of productive α mRNA transcript. The steady-state level of both forms of α mRNA in TGF-β-containing cultures appears to be comparable regardless of the presence of IL-2. IL-2 alone does not appear to induce the 1.3-kb transcript or significantly affect the steady-state level of α mRNA. This is consistent with our earlier findings in which S1 nuclease protection was used to determine the steady-state level of C_α-containing mRNA (7). Although the 1.7-kb band could contain productive transcripts as well as germ-line transcripts, the 1.3-kb band is too short to encode complete IgA molecules, suggesting that it consists solely of germ-line transcripts.

Isolation of cDNA Clones for Germ-Line α mRNA Transcripts. To characterize the transcripts containing C_α sequences, mRNA was prepared from cultures stimulated with LPS in the presence of TGF-β and IL-2 for 3 days. Since the sequence of the 3′ end of the C_α transcripts was known, it was possible to isolate clones containing the unknown 5′ exon(s) by using a recently described technique for polymerase chain reaction (PCR) amplification of cDNAs with an unknown 5′ end (20). The basic protocol is outlined in Fig. 2 and is described in detail in *Materials and Methods*. The first-strand cDNA is tailed with dGTP so that a dCTP-containing primer can be used for amplification. However, the specificity of the amplification appears to be greater when a 9:1 mix of two primers, one consisting of restriction sites (the anchor) without a dCTP tail and one consisting of the anchor with a dCTP tail, respectively, is used (ref. 20; data not shown). To minimize the length of the sequence to be amplified, primers were prepared from within the first exon of C_α. To facilitate cloning, an *Xba* I restriction site was added to the C_α primer. After PCR amplification, an ≈220-bp fragment was isolated and cloned into pUC18. To characterize the transformants, DNA was isolated from individual colonies and digested with *Xba* I and *Sph* I enzymes, which should cut in the oligonucleotide primer sequences. Approximately 30% of the colonies contained an insert of the appropriate size. Six of the positive clones were sequenced and all contained the iden-

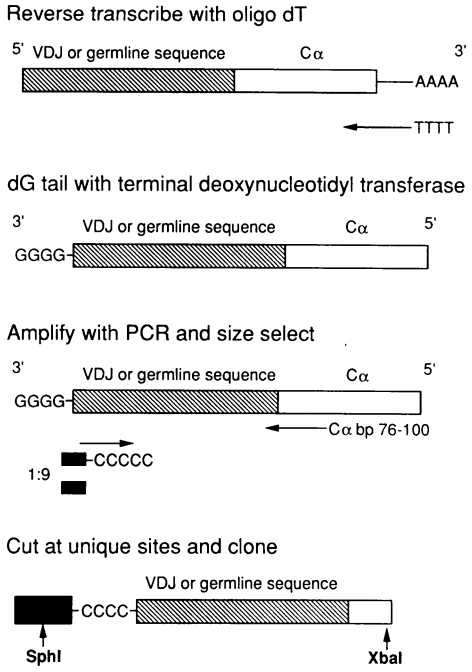

FIG. 2. Protocol for cloning. Details of the protocol are elaborated in *Materials and Methods*. (*A*) cDNA was prepared from total RNA from B cells stimulated with LPS plus TGF-β and IL-2 for 3 days with an oligo(dT) primer. (*B*) The cDNA was tailed with dGTP by using terminal deoxynucleotidyltransferase. (*C*) Amplification of the desired sequence was performed by PCR with the indicated primers. Solid box represents the restriction site containing anchor. (*D*) The amplified sequence was isolated, cut with restriction enzymes as indicated, and ligated into pUC18. (*E*) Clones containing the insert were sequenced.

tical sequence spliced to the first 100 bp of C_α, suggesting that this is the predominant if not the only α mRNA transcript present in the 1.3-kb band. In addition, at least two of the six clones have different lengths of dCTP tail, indicating that they were independently derived.

The sequence of one of these clones, pSXα1, is shown in Fig. 3. SXα1 contained no joining (J) region sequence 5′ to C_α, indicating that it was not derived from an incomplete cDNA containing a truncated VDJ (D, diversity) region. Stop codons are present in all three translation reading frames, although only those in frame with C_α are shown in Fig. 3. In addition, there are translation initiation codons in frame with C_α. Since these transcripts use sequences from unrearranged α loci, they are regarded as germ-line transcripts. However, since any putative product derived from transcripts containing SXα1 in lieu of VDJ would not yield a complete IgA molecule, SXα1 is regarded as a sterile exon.

Since pSXα1 hybridized to the 5′ S_α fragment indicated in Fig. 4 (data not shown), its location within that fragment was mapped by sequence comparison. The 5′ end of a 130-bp sequence homologous to SXα1 was located 485 bp 5′ to the *Hin*dIII end of the probe. pSXα1 and the 5′ S_α probe were derived from different strains of mouse. Consequently, the differences in sequence could reflect allelic differences between the two strains. Thus, pSXα1 starts ≈1.5 kb 5′ to S_α (Fig. 4). Since all of the detectable α mRNA in these cultures used the secreted 3′ end (7), the size difference between the two C_α-containing transcripts, 300–400 bp, must be due to differences in the 5′ end. The difference between a typical VDJ (≈450 bp) and SXα1 (126 bp) can account for the observed size difference between the two sizes of C_α-containing transcripts.

SXα1 Is Represented in α mRNA. To demonstrate that SXα1 was present in the 1.3-kb α transcript, a 70-mer synthetic oligonucleotide prepared from the sequence was hybridized to a Northern blot of poly(A)$^+$ RNA from B cells stimulated for 3 days with LPS either alone or with TGF-β (Fig. 5*A*). The SXα1 probe hybridized with a 1.3-kb band and to a much lesser extent with a 1.7-kb band in mRNA from TGF-β-containing cultures. The sterile exon was not detectable in mRNA isolated either from cultures stimulated with LPS alone or from the IgA-secreting myeloma MOPC 315. The same blot was washed and rehybridized with a C_α probe that hybridized to two bands of the same size. The hybridization of C_α to the 1.7-kb band was more intense than to the 1.3-kb band, suggesting that only a small fraction of 1.7-kb transcripts contain sequence from SXα1.

DISCUSSION

The generation of B cells expressing secondary, non-IgM, isotypes occurs as a consequence of S–S recombination (9). However, in both B-cell lines and normal B cells, secondary C_H loci appear to be transcriptionally active prior to recombination. For example, in the pre-B-cell line 300–18P, which switches spontaneously to γ2b, transcripts containing sequence 5′ to $S_{\gamma 2b}$ spliced to $C_{\gamma 2b}$ have been found (11). These transcripts, which have multiple start sites and cannot encode functional IgG2b molecules, can be considered sterile transcripts. In LPS-stimulated B cells induced to switch to either γ1 or ε by the addition of IL-4, putative sterile transcripts containing sequences 5′ to the respective S region in lieu of VDJ are present prior to the appearance of productive γ1 or ε transcripts (12–15). Similar sterile or germ-line C_α transcripts were initially described in the B-cell line I.29 (12). This cell line, which switches preferentially to IgA or IgE after stimulation with LPS, produces at least six different α transcripts. Three of these transcripts contain sequences

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A G T C C T A A C T C T C T A C C A T A G G G A A G A T A G  30
C C T T T A G C T A T T A T G A G A A T G G A T C T T C A G  60
T T G G G A C C A C C T G G C C A C A G G A G G C C T G G C  90
T G T T C C C C T A T G A A G G A C A C T A A C A G C A T T 120
G A G C A G a g t c t g c g a g a a a t c c c a c c a t c t 150
a c c c a c t g a c a c t c c c a c c a g t c c t g t g c a 180
g t g a t c c c g t g a t a a t c g g c t g c c t g a t t c 210
a c g a t t a c t t c c c t t t
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FIG. 3. Sequence of pSXα1. Capital letters are the sequence of SXα1. Lowercase letters are the sequence of C_α. Only start and stop codons in-frame with C_α are underlined.

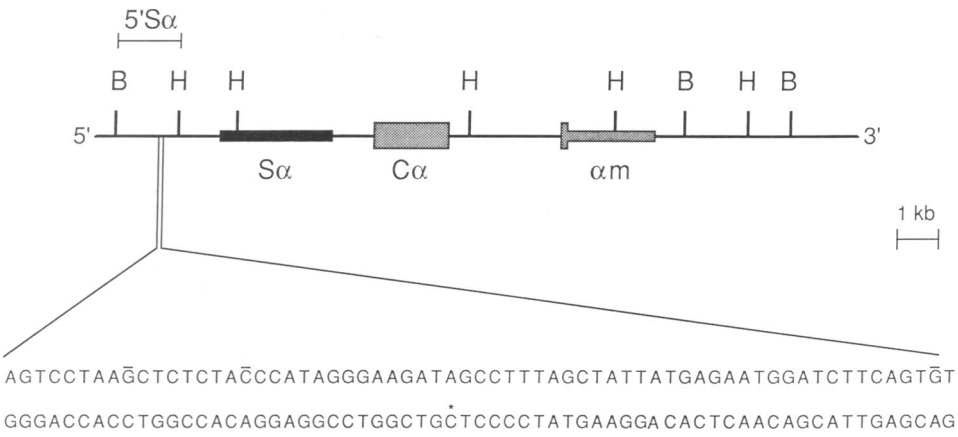

FIG. 4. Mapping of SXα1. Diagram represents the germ-line region. The location and sequence of the region homologous to SXα1 are shown. The 5′ S_α probe is indicated. SXα1 starts 485 bp 5′ to the *Hin*dIII end of 5′ S_α. Solid line over a nucleotide indicates it is not present in SXα1 and an asterisk indicates a difference in sequence. B, *Bam*HI; H, *Hin*dIII.

located 5′ to S_α spliced to C_α. The predominant transcript in this cell line is the same size as productive αs mRNA and could be comparable to the 1.7-kb transcript observed in LPS cultures stimulated with TGF-β. In addition, there are three short C_α transcripts whose start sites are unknown. The physiological relevance of any of these transcripts in the process of isotype switching in normal B cells is uncertain.

Although TGF-β was originally characterized as a factor capable of inducing anchorage-independent growth of fibroblasts (24), it has subsequently been shown to affect the proliferation and/or differentiation of a wide variety of cell types (25). In regard to the immune system, TGF-β inhibits proliferation and differentiation of B cells (26, 27) as well as proliferation and cytokine secretion by T cells (28–31). In contrast, TGF-β enhances IgA secretion in LPS-stimulated B-cell cultures by stimulating isotype switching to IgA (7). Two major C_α transcripts are induced by TGF-β; one is the same size as productive αs mRNA and the other is 300–400 bp shorter. Both sizes of transcript hybridize to a probe, p5′ S_α, containing a germ-line sequence located 5′ to S_α, suggesting that the larger transcript may contain both germ-line and productive α mRNA, whereas the smaller transcript is exclusively germ-line α mRNA (data not shown). The smaller, 1.3-kb transcript consists of a sterile exon, ≈126 bp long, spliced to the first base pair of the first exon of C_α. The same sterile exon appears to be present in a fraction of the 1.7-kb transcripts, although it is unclear whether the size difference between the transcripts is accounted for by additional sequence spliced 5′ or 3′ to SXα1. Whether the remainder of the 1.7-kb transcripts observed prior to day 4 represents productive (VDJ) transcripts or a different germ-line transcript is not clear. It is interesting to note that not only are fewer different sizes of germ-line α transcripts detected in normal B cells than in the I.29 cell line, but the 1.3-kb transcript, which accounts for a considerable proportion of germ-line α mRNA transcripts in normal cells, is at best a minor component of the complement of transcripts in the cell line.

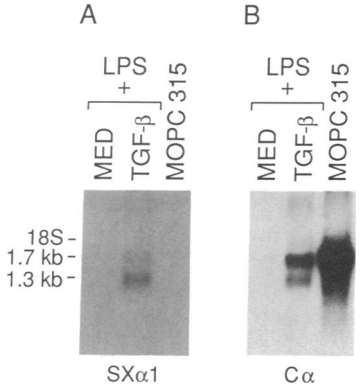

FIG. 5. SXα1 is represented in C_α transcripts. (*A*) Poly(A) RNA from B cells stimulated for 3 days as indicated or total RNA from the IgA-secreting myeloma MOPC 315 was electrophoresed and blotted. Blot was hybridized with a 70-mer-containing sequence from SXα1. (*B*) The same blot was washed and hybridized with a C_α probe.

The correlation between the appearance of germ-line or sterile C_H transcripts and switching to those loci has led to a model for the regulation of isotype switching (32). According to this model, switching is regulated by the accessibility of S regions to a recombinase. Although the mechanistic role of germ-line transcripts is unclear, there are at least three possibilities. The appearance of these transcripts could merely be a consequence of increased accessibility of the S region. Alternatively, transcription through the S region could maintain accessibility to the recombinase. The third possibility is that the transcripts themselves play a role in directing isotype switching. The effect of cytokines, such as IL-4 and TGF-β, could be to open specific regions of the heavy-chain locus causing the induction of sterile transcripts or to initiate transcription from open regions, thereby maintaining accessibility. Regardless of whether transcriptional activation of the locus or the germ-line transcripts themselves direct the process of switching, an understanding of the role cytokines play in this process potentially provides a tool for regulating isotype switching.

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