Structure of Germ Line Immunoglobulin α Heavy-Chain RNA and Its Location on Polysomes

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We describe the structure of the major germ line RNA transcribed from unrearranged immunoglobulin α heavy-chain genes in immunoglobulin M-expressing cells of the I.29 μ B-cell lymphoma, a cell line capable of switching to immunoglobulin A expression upon lipopolysaccharide treatment. This germ line α RNA has a small open reading frame that does not include the C α domain, and this RNA appears to be present on polysomes in I.29 μ cells.

Immunoglobulin class switching is effected by a DNA recombination event that replaces the C μ gene with one of the other heavy (H)-chain constant-region (C $_H$) genes located 3' to the C μ gene. How the specificity of this event is controlled is unknown. However, it has been shown that the immunoglobulin M-expressing (IgM⁺) cells capable of switching to specific antibody classes have the corresponding unrearranged C $_H$ genes in an accessible or active chromatin state, as demonstrated by the fact that these specific C $_H$ genes are hypomethylated and are transcriptionally active (21, 24). Furthermore, RNAs transcribed from specific unrearranged C $_H$ genes are induced in spleen cells before switching in response to lipopolysaccharide (LPS) or lymphokines, which induce switching to these same C $_H$ genes (1, 4, 14, 17, 19). The function of transcription of germ line C $_H$ genes is unknown. In this report, we describe the structure of the major RNA transcribed from unrearranged C α genes in IgM⁺ cells of the I.29 μ B-cell lymphoma, a cell line capable of switching to IgA⁺ upon LPS treatment (20).

By RNA blotting experiments, we previously determined that the predominant 1.7-kilobase (kb) RNA transcribed from unrearranged immunoglobulin C α genes in IgM⁺ I.29 μ cells and in spleen cells treated with a supernatant from a T $_{H2}$ cell line (which has interleukin-4, interleukin-5, and other lymphokines) is encoded by an I α segment located 5' to the α switch region and also has C α exons but has no V $_H$ or J $_H$ gene segments and is polyadenylated (19, 21; J. Stavnezer, unpublished data) (Fig. 1). Two larger RNAs present in I.29 μ cells also hybridize with a probe for the membrane domain of the immunoglobulin α chain (19). Additional smaller species hybridize with the C α probe but not with the I α or α_m probe (19) except that occasionally a 1.3-kb species is detected with the I α probe (P. Shockett and J. Stavnezer, unpublished data).

To more precisely determine the structure of RNAs transcribed from unrearranged immunoglobulin C α genes, a λ gt11 cDNA library was prepared from poly(A)⁺ RNA from I.29 μ cells. Plaque lifts of the library were hybridized with three different probes: a cDNA probe for the C α region (15), a subclone of this cDNA that encoded the C $_{H1}$ domain of C α ,

and a genomic DNA fragment encoding the α membrane domain (23). Of the 10⁶ clones, 600 (0.06%) hybridized with at least one of these probes. The restriction enzyme maps and nucleotide sequences of the cloned cDNAs that were analyzed indicated that the predominant 1.7-kb RNA detected by the I α and C α probes has an exon encoded 2 kb 5' to the beginning of the tandem repeated α switch sequences spliced to the normal splice acceptor for the C α domain, encodes the three C α exons, and is terminated near the normal polyadenylation site for mRNA encoding the secreted immunoglobulin α H chain. Figure 1B shows the splicing diagram of this RNA. The polyadenylation sites of

FIG. 1. (A) Two lanes from blots of I.29 μ poly(A)⁺ RNA hybridized with the *Bam*HI-*Hind*III probe, I α , and a cDNA probe encoding the C α domain (15), showing the various species of germ line α RNAs. **(B)** Map of the germ line C α gene in IgM⁺ cells of the I.29 μ B-cell lymphoma. The probes were labeled by random priming (5). Enzyme sites: B, *Bam*HI; BgII, *Bgl*II; E, *Eco*RI; H, *Hind*III; S, *Sac*I. ■, Exons present in spliced germ line RNAs. The splice sites were determined by nucleotide sequencing.

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A.

<u>AATAAA</u> CTCTGTGCTCATCGAAAGTATCCCTGCACTTCCAAAAAAAAA	mRNA end
AATAAA CTCTGTGCTCATCGAAAGTAAAAAAAAA	clones 10-1, 2-7
AATAAA CTCTGTGCTCATCGAAAAAAAAA	clone 11A3

B.

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cagccagaccacagggcagacatgacgtggaggcaagcgccacacagtgagggtggaacaggaagtgggtgagggacccacctagggccagacacac      100

      HhaI
      |
agtcattgcagccccctcagcgctcaCTCTGACCCATCCACAGGCAATCACACAGAGAAAAACAGCTTCCAGCCAAGGCTTCGCCAGTCTTAAGCTCT      200
      |
      MspI
      |
CTACCATAGGGAAGATAGCCTTTAGCTATTATGAGAATGGATCTTCAGTGTGGGACCACTGGCCACAGGAGGCTGGCTGTTCCCTATGAAGGACACT      300
      |
      M R M D L Q C G T T W P Q E A W L F P Y E G H
      |
CAACAGCATTGAGCAGGTGGGTCCCGGTACCATCTGGACTCCTCTGCTCATTGGTACACTGAGGAGCCTCAACCAGGCCAGGCATGGTTGAGATAGAGAT      400
S T A L S R W V P V P S G L L C S L V H T E R
      |
      MspI
      |
AGAGATAAGATAGAAAGCATTAAACAAGAAGGAGAAGGTGATTACAGGAGCAAGAGCCAGAGGAATACAGGACATGATCACAGGCACAAGGCGGTGTCGTC      500
      |
      MspI
      |
TGAGCTGCAGAGAAGAGAAAAAAGGATGAAACCCAGCTTCTTGAAAGCAGCAACAGGAGACTCCCAGGCTAGACAGAGGcaaggattattagg      600

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FIG. 2. Nucleotide sequence of germ line α RNA. (A) Nucleotide sequences of 3' ends of three cloned germ line α cDNAs, starting at the polyadenylation signal sequence (overlined AATAAA), compared with the sequence of mRNA for α H chain (23). (B) Nucleotide sequence of the I α exon of the 1.7-kb germ line α RNA (in capital letters) and flanking sequences (in lowercase letters). The sequence of the splice donor differs from the consensus for 5' splice sites, which is AG/GTAAGT (18). The two initiation sites identified for the 1.7-kb α RNA are indicated by arrows. Both sites are initiated with the sequences GTC and are preceded by GCPyCA. The predicted amino acid sequence of the ORF is given below the nucleotide sequence, and the initiator ATG is underlined. The nucleotides complementary to the 70- and 17-nucleotide primers used for primer extension experiments are overlined. The 5' end of the cDNA that encoded 131 nucleotides of the I α exon is indicated (▼). This sequence has been deposited in GenBank (accession number M29011).

these cDNAs were located 15 and 20 nucleotides 5' to that reported for immunoglobulin α H-chain mRNA (23) (Fig. 2A). The sequences of other cDNAs, which hybridized with the α_m probe, indicated that the normal splice donor and acceptor sites used to produce mRNA encoding membrane-bound α H chains were utilized in germ line α RNAs containing α_m sequences.

The transcriptional initiation sites of the predominant germ line α RNAs were located by RNase protection and primer extension experiments. The 5' border of the I α exon was located by RNase protection experiments using three different probes (RNA transcripts of the *Bam*HI-*Msp*I, *Msp*I-*Hind*III, and *Bam*HI-*Hind*III fragments) (Fig. 3D). Figure 3C shows the RNase-resistant fragments produced when a labeled transcript complementary to the *Bam*HI-*Msp*I segment was hybridized with RNA from I.29 μ (lane M) or yeast (lane Y) cells. Two RNase-resistant fragments of 201 and 138 nucleotides were detected after hybridization with I.29 μ cell RNA, indicating that the 5' border of the I α exon was located at two sites 138 and 201 nucleotides 5' to the *Msp*I site.

These two sites were shown to be initiation sites by primer extension experiments. A DNA fragment, labeled at its 5' end at the *Msp*I site (position 326 in Fig. 2B), was used to prime cDNA synthesis by reverse transcriptase on RNA from I.29 μ cells. The lengths of these extension products, 138 and 201 nucleotides (Fig. 3A), indicated that the 5' borders of the I α exon determined by RNase protection corresponded to the transcription initiation sites. Thus, germ

line α RNAs initiate at two sites located 63 nucleotides apart. Primer extension experiments using an oligonucleotide complementary to the 3' end of the I α exon (Fig. 2B) produced only one product, 397 nucleotides in length (Fig. 3B). This product corresponded to the 5'-proximal initiation site. It is possible that the 5'-distal initiation site was not detected with this primer because of inefficient extension. As is also true for analogous RNAs transcribed from the μ , γ_{2b} , and γ_1 genes (11, 13; M. Xu and J. Stavnezer, submitted for publication), there are no TATA or CCAAT boxes located immediately upstream of the initiation sites for the germ line α RNA.

The sizes of the I α exon determined by these experiments, 460 and 397 nucleotides, plus the size of the three C α exons (1,073 nucleotides) (23), would produce a germ line α RNA of 1.7 kb, assuming a 200-nucleotide poly(A) tail. This size corresponds to that of the predominant 1.7-kb germ line α RNA detected on RNA blots.

The nucleotide sequence of the I α exon indicated the presence of an open reading frame (ORF) that would be initiated by the most 5' Met codon in this exon and would encode a 43-amino-acid polypeptide whose termination site would be within this exon. The predicted amino acid sequence of the ORF is indicated below the nucleotide sequence in Fig. 2B. The nucleotide sequence surrounding the initiator AUG codon should form a good translation initiation site, according to the data of Kozak (8, 9).

To determine whether the germ line 1.7-kb α RNA can be translated in I.29 μ cells, we looked for its presence on

FIG. 3. Determination of 5' initiation sites for germ line α RNAs. (A) Products of a primer extension experiment using a 70-nucleotide DNA fragment complementary to a portion of the I α exon of the germ line α RNA to prime cDNA synthesis by reverse transcriptase from total cell RNA from I.29 μ (lane M) or yeast (lane Y) cells electrophoresed on a DNA-sequencing gel. The products of a dideoxynucleotide-sequencing reaction using the same primer were electrophoresed alongside as a marker. (B) Primer extension experiment using a 17-nucleotide DNA primer complementary to the 3' end of the I α exon of germ line α RNA. The oligonucleotide was hybridized with 100 μ g of total cell RNA from I.29 μ (lane M), IgA⁺ (lane A), or yeast (lane Y) cells. (C) RNase protection experiment in which a 1,030-nucleotide ³²P-labeled RNA, transcribed by T3 polymerase from a Bluescribe plasmid containing the *Bam*HI-*Msp*I I α fragment, was hybridized with 40 μ g of total cell RNA from I.29 μ (lane M) or yeast (lane Y) cells. RNase-resistant products were denatured in formamide and electrophoresed on a sequencing gel alongside a dideoxynucleotide-sequencing ladder. Lane P, Probe alone; lane Y, RNase-resistant product after hybridization with yeast RNA; lane M, RNase-resistant products after hybridization with I.29 μ RNA. (D) Probes and primers used for these experiments and summary of results. Boxes below the I α exon indicate primers used in primer extension. Arrows indicate primer-extended products from the two primers.

polysomes. Polysomes were displayed by sucrose gradient centrifugation of cytoplasmic extracts from I.29 μ cells either untreated or treated for 2 days with LPS (Fig. 4A and B). RNA from individual fractions of the gradients was phenol extracted and electrophoresed through formaldehyde-agarose gels. The gels were blotted and hybridized with the I α (*Bam*HI-*Hind*III) probe (Fig. 4D). The 1.7-kb germ line α RNA sedimented in the polysome region of the gradient, in fractions containing tri-, di-, and monosomes from cells treated (Fig. 4B) or not treated (Fig. 4A) with LPS, although extracts from untreated cells also contained 1.7-kb α RNA that sedimented at the top of the gradient. LPS treatment appeared to result in an increase in the amount of the 1.7-kb α RNA in the cytoplasm and also increased the proportion of the α RNA associated with ribosomes, suggesting that LPS stimulated translation of this RNA. The apparent location of this RNA on polysomes having one, two, or three ribosomes is appropriate for an mRNA having an ORF of 129 nucleotides and 5' untranslated regions 42 and 105 nucleotides in length (16, 22). When these blots were melted and reprobbed with a cDNA for λ light-chain mRNA, λ mRNA was found to cosediment with polysomes containing three to six ribosomes (Fig. 4E), which is consistent with the larger size of its ORF (approximately 714 nucleotides). Unlike the case for

the germ line α RNA, the proportion of λ mRNA located in the polysome region of the gradient was not significantly altered by LPS treatment. This apparent association of the 1.7-kb α RNA with polysomes was disrupted by treatment of the cytoplasmic extracts with EDTA, which has been demonstrated to dissociate mRNA (in the form of ribonucleoprotein) from polysomes (10, 25) (Fig. 4C).

The sequences of the 5', or I, exons of RNAs analogous to the 1.7-kb germ line α RNA that are transcribed from the μ and γ_{2b} genes have been interpreted to indicate that these RNAs could not be translated (11, 13). However, both the μ and γ_{2b} RNAs do have small ORFs, although they appear to have poor translation initiation codons. This would reduce the efficiency of translation but may not preclude it (3, 6-9, 12). The structure of the germ line γ_1 transcript has recently been determined; it has an ORF with a potentially efficient initiator Met codon (Xu and Stavnezer, submitted).

In conclusion, transcription of unrearranged C_H genes appears to direct switch recombination. However, the function, if any, of transcription from unrearranged C_H genes remains unknown. Transcription itself might promote recombination. In addition, the transcripts themselves or a protein encoded by them might also promote switching. The fact that the germ line transcripts that have been character-

FIG. 4. UV absorption profiles of polysomes from I.29 μ cells after sedimentation through sucrose gradients (A to C) and blots of the fractionated polysomal RNA hybridized with a probe for the I α exon (D) and for mRNA for immunoglobulin λ chains (E). Cells were cultured for 2 days in the absence or presence of LPS (50 μ g/ml); cycloheximide (2.5 μ g/ml) was added, and flasks were returned to the 37°C CO₂ incubator for 15 min. Cells were pelleted, washed in cold Puck solution containing cycloheximide (50 μ g/ml), and suspended at 1×10^7 to 2×10^7 cells per ml in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4)–10 mM KCl–3 mM MgCl₂–0.5% (vol/vol) Nonidet P-40–50 μ g of cycloheximide per ml for lysis. After 5 min on ice, lysed cells were centrifuged at $16,000 \times g$ for 10 min. Polysomes were displayed by centrifugation at 35,000 rpm through 15 to 50% sucrose gradients in 50 mM HEPES (pH 7.4)–10 mM KCl–3 mM MgCl₂ in the SW41 rotor for 2 h at 4°C. To release mRNA from polysomes, EDTA (25 mM) was added to the cytoplasmic extract before centrifugation on sucrose gradients containing 25 mM EDTA (pH 7) (10, 25). Sedimentation is from right to left, as indicated by the arrow. The positions of polysomes containing two and three ribosomes are indicated by numbers on the polysome profiles in panels A and B. Polysomal RNA from untreated cells (A), from LPS-treated cells (B), and from LPS-treated cells whose polysomes had been dissociated in EDTA (C) was loaded on lanes of RNA gels aligned beneath the corresponding fractions from the gradient. In panel C, the top two fractions of the gradient were pooled and the RNA was loaded in one lane. The blots were first hybridized with a ³²P-labeled RNA probe for sense RNA transcribed from the *Bam*HI-*Hind*III I α fragment. The probe was removed, and the blots were hybridized with a random-primed cDNA probe for λ mRNA (2).

ized have similar structures argues that their transcription does have a function and is not simply an accidental by-product of the accessibility of the C_H gene.

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