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Immunology

Formal proof that different-size Lyt-2 polypeptides arise from differential splicing and post-transcriptional regulation

(T-lymphocyte differentiation antigen/expression vector/transfection)

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ABSTRACT **We recently isolated the gene and a cDNA clone for the mouse T-cell surface antigen Lyt-2 and showed that Lyt-2 is homologous to the human Leu-2 (T8) antigen and that the gene encoding it is a member of the immunoglobulin gene superfamily. By screening a mouse thymus cDNA library with the Lyt-2 cDNA clone, we isolated two classes of cDNA clones, α and α', which differ by 31 base pairs. Comparison of the α cDNA with genomic sequence data indicates that there are five exons encoding Lyt-2: a fused leader/immunoglobulin variable region-like exon, a spacer region exon, a transmembrane exon, and two cytoplasmic exons. The α' cDNA clones lack the first of the two cytoplasmic exons and have a direct splice from the donor splice site of the transmembrane exon to the acceptor of the second cytoplasmic exon. This splice changes the reading frame for the second cytoplasmic exon, causing a stop codon shortly after the splice so that the α' cDNA clone codes for a peptide 25 residues shorter than the α cDNA-encoded peptide. We have constructed expression vectors with α and α' cDNAs and have shown that L-cell transfectants of these produce Lyt-2 polypeptides of the predicted sizes and that these associate as homodimers on the cell membranes. We found the two species of mRNA corresponding to α and α' cDNAs at equal levels in thymus RNA by using S1 nuclease analysis. Although lymph node T cells have only the α form of Lyt-2 protein, S1 nuclease analysis shows that lymph nodes have about 20% α' mRNA relative to α. Thus, Lyt-2 is regulated at RNA processing, translational, and/or post-translational steps.**

The murine lymphocyte membrane glycoprotein differentiation antigen, Lyt-2, is expressed on immature T cells as well as certain subpopulations of mature T cells, namely the cytotoxic T cell (CTL) and suppressor subsets (1–3). The Lyt-2 antigenic determinant is found on two polypeptides from thymus cells of mass 38 kDa (Lyt-2-α) and 34 kDa (Lyt-2-α'), usually disulfide-linked to a 30-kDa Lyt-3 polypeptide encoded by a closely linked gene (4–7). The Lyt-2,3 peptide seems to serve an auxiliary role in antigen recognition (8, 9), as antibodies to Lyt-2 or Lyt-3 will block the lysis of target cells by many cytolytic T cells (10–14).

Recently, Zamoyska *et al.* (15) isolated and sequenced two cDNA clones that they postulated code for the 38- and 34-kDa Lyt-2 chains and that arise by differential RNA splicing of the cytoplasmic domain-encoding regions. Also, Luescher *et al.* (16) found from studies with "inside-out" vesicles prepared from thymus cells that the cytoplasmic domains of Lyt-2-α and -α' differed in size by 1 to 4 kDa and suggested that the two chains of Lyt-2 resulted from some form of post-transcriptional processing.

We confirm the findings of Zamoyska *et al.* and go on to show that each type of cDNA, after insertion into expression vectors and transfection into L cells, results in production of either 38-kDa Lyt-2-α or 34-kDa Lyt-2-α' peptides. These are found on the cell membrane as homodimers. In transfectants of the entire *Lyt-2* gene, α and α' chains are both made, but in contrast to thymocytes, they are found on the cell membrane as both types of homodimer and as the α/α' heterodimer.

MATERIALS AND METHODS

Enzymes and Cells. Restriction enzymes were obtained from New England Biolabs and Boehringer Mannheim. Mouse strains BALB/c, C57BL/6, and SJL/J are maintained in the Herzenberg laboratory mouse colony. Cytotoxic T-cell line CTL-108 was obtained from John Russell (17).

Transfection, Flow Analysis, and Cell Sorting. Thymidine kinase (TK)-defective L cells (LTK^-) were transfected with 20 μg of Lyt-2 cDNA plasmid, 1 μg of chicken TK gene, and 10 μg of carrier human DNA by the calcium phosphate precipitation method (18). After selection in hypoxanthine/aminopterin/thymidine medium, TK^+ cells were stained with monoclonal rat anti-Lyt-2 antibody (53-6.7) (19), and Lyt-2^+ cells were sorted by using a fluorescence-activated cell sorter (FACS) as described (20).

Immunoprecipitations and Gel Electrophoresis. Cells were labeled externally with ^{125}I by using lactoperoxidase (21) or were labeled metabolically with [^{35}S]methionine (22). Labeled cells were solubilized in 1% Nonidet P-40, and the lysates were preabsorbed with *Staphylococcus aureus* Cowan I strain and then immunoprecipitated with the monoclonal rat anti-Lyt-2 antibody, followed by protein A-Sepharose bound rabbit anti-mouse IgG or monoclonal rat anti-mouse κ chain antibody. Electrophoresis in polyacrylamide gels containing $NaDodSO_4$ was performed (22) under reducing or partially reducing conditions. For some gels, reduction and alkylation were first carried out.

Reduction and Alkylation. Cell lysates were reduced with 10 mM dithiothreitol at room temperature for 45 min and subsequently alkylated with 20 mM iodoacetamide at room temperature for 20 min. The materials were extensively dialyzed against 0.01 M phosphate in normal saline (pH 7.2) (23).

RNA Extraction and S1 Nuclease Protection. Total RNAs were extracted as described (24). The ^{32}P-labeled single-stranded probe was synthesized from M13 mp template based on Burke's procedure (25). These probes also were used to detect the exon-specific sequences. Annealing of probe fragments with RNAs and S1 nuclease (Pharmacia P-L Biochemicals) digestions was performed as described by Favaloro *et al.* (26).

DNA Sequence Analysis. DNA sequencing was performed by the dideoxynucleotide chain-termination technique of Sanger *et al.* (27) as modified by Messing (28).

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Abbreviations: CTL, cytotoxic T lymphocytes; TK, thymidine kinase; bp, base pair(s).

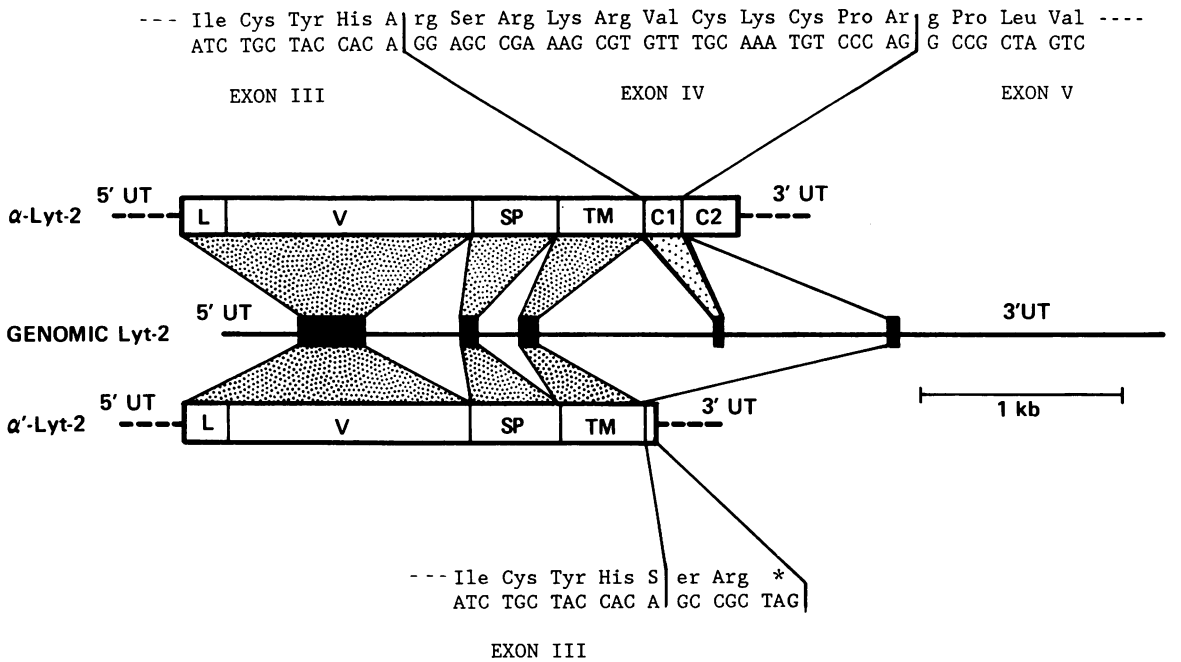

FIG. 1. Genomic structure of the Lyt-2 gene and comparison of the sequences of α and α′ cDNA. Partial sequences of the α and α′ Lyt-2 cDNA are shown. The splicing pattern, as determined from sequencing of genomic Lyt-2 (unpublished data) and the relative location of genomic exons, is shown. There are five exons (I–V) in the *Lyt-2* gene; they encode leader/Ig variable-like region (L and V) (exon I), spacer region (SP) (exon II), transmembrane region (TM) (exon III), and cytoplasmic regions C1 and C2 (exons IV and V).

RESULTS

Two Types of Lyt-2 cDNA Result from Differential Exon Usage. Screening of a C57BL/6 mouse thymocyte cDNA library (courtesy of H. Gershenfeld and I. Weissman) and sequence analysis revealed two types of cDNAs for Lyt-2. The previously characterized Lyt-2 cDNA (α) (29) is distinguished from the second cDNA type (α′) in that the α′ form lacks 31 base pairs (bp) at the end of the predicted transmembrane domain (Fig. 1). This effects a shift in the reading frame for α′ cDNA, as compared with α cDNA, so that the peptide encoded by α′ cDNA has only 2 intracytoplasmic residues versus 27 residues for α cDNA. The predicted molecular weights of the two molecules, after leader peptide cleavage, are 24.7 kDa (α) and 21.7 kDa (α′).

Comparison of exon/intron data from the 5.4-kilobase *Hin*dIII fragment, which includes the entire *Lyt-2* gene (29), shows that α′ cDNA lacks the first of two exons coding for intracytoplasmic polypeptide. These two exons are exons IV and V of the *Lyt-2* gene, whereas exon I encodes a fused leader/Ig variable region-like exon, exon II encodes a spacer region, and exon III encodes a hydrophobic transmembrane domain. Boundaries between intron and exon predicted by alignment of the two cDNAs to the genomic sequence conform to consensus splice sequences for mammalian mRNAs (30) (Table 1). The universal donor G-T–acceptor A-G sequences are found in this case.

In order to determine the relative frequency of α and α′ cDNA, we examined 31 Lyt-2 cDNA clones obtained from the mouse thymocyte cDNA library with exon-specific probes. We identified 15 cDNA clones positive for exons III, IV, and V, and 10 that, though positive for exons III and V, lack exon IV. Thus, the alternatively spliced α and α′ cDNA clones are represented approximately equally in this library.

α and α′ cDNA Expression Vectors Confirm the Origin of 38- and 34-kDa Lyt-2 Polypeptides. To confirm that α and α′ cDNAs encode the 38- and 34-kDa Lyt-2 polypeptides, respectively, we made expression vectors with the two types of cDNA (Fig. 2) and transfected these into mouse L cells. Immunoprecipitation of ^{125}I-surface-labeled and ^{35}S-biosynthetically labeled transfectants showed that α cDNA codes for the 38-kDa peptide, α′ cDNA codes for the 34-kDa peptide, and the genomic clone codes for both 38- and 34-kDa peptides (Fig. 3).

By comparison of reduced and nonreduced material separated on NaDodSO$_4$/PAGE, we can see that α and α′ cDNAs transfected separately form homodimers of 76 and 68 kDa, respectively (Fig. 3 *Left*). When both are present in the same cell, as in the genomic transfectant, both homo- and heterodimers of α and α′ chains are formed, except when Lyt-3 is also present, such as in normal thymocytes. When Lyt-3 is present, heterodimers of α or α′ peptides with Lyt-3 are made to the exclusion of homodimers of α or α′ or of heterodimers of α with α′. Lyt-3, as previously observed (21),

Table 1. Comparison of the *Lyt-2* nucleotide sequences around splice junctions with consensus splice site sequences

Exon	Donor		Intron	Acceptor		Exon
Transmembrane (III)	CACA	GTAAGT	... ...	CCTTGTCTAG	GGAGC	Cytoplasmic 1 (IV)
	*	******		**** ** **	**	
Cytoplasmic 1 (IV)	CCCAG	GTGAGT	... ...	TCTCTTTCAG	GCCG	Cytoplasmic 2 (V)
	***	******		**********	*	
Consensus splice sequence	5'–CAG A	GTGAGT A	... ...	Y_6NCAG	GT–3' G	

Y, unspecified pyrimidine nucleoside; N, unspecified nucleoside; *, identity to consensus splice sequence.

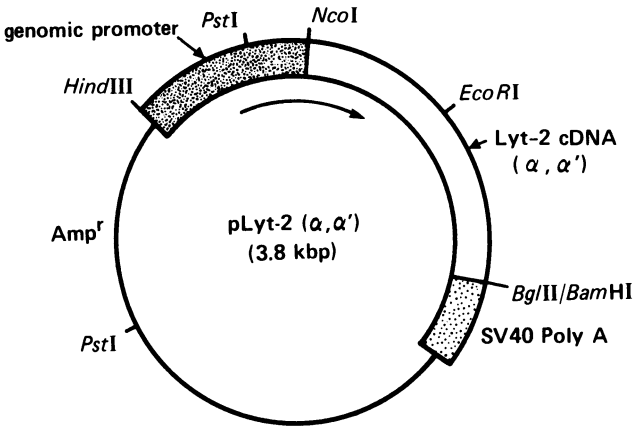

FIG. 2. Construction of cDNA expression vectors. pLyt-2-α' comprises the *Hin*dIII–*Nco* I fragment [Lyt-2 genomic promoter (unpublished data)], *Nco* I–*Bgl* II fragment (α' cDNA), and the *Bam*HI–*Hin*dIII fragment [simian virus 40 (SV40) polyadenylylation site, bacterial origin of replication, and ampicillin-resistance (Amp^r) gene from Okayama–Berg expression vector (31) pCDV-Leu-2-1 (courtesy of P. Kavathas, R. Margolski, and P. Berg)]. pLyt-2-α was constructed by replacing α' cDNA of pLyt-2-α' with a portion of Lyt-2-α cDNA containing exon IV. Direction of transcription is as indicated in the figure.

is poorly labeled by external labeling with ^{125}I. Nevertheless, its presence is clearly seen by the 68- and 64-kDa molecular mass of nonreduced Lyt-2 immunoprecipitates from thymocytes (Fig. 3 *Left*, lane 1) or the 68-kDa immunoprecipitate from lymph node (Fig. 3 *Left*, lane 6). Even on long exposure of lymph node immunoprecipitates, no 34-kDa (reduced) or 64-kDa (nonreduced) bands were seen (Fig. 3 *Left*, lane 7).

We observed an approximately 30-kDa band in immunoprecipitates of reduced [^{35}S]methionine-labeled lysates from the α' cDNA and genomic transfectants. This band was not found in reduced α cDNA transfectants or in control L cells (Fig. 3 *Center*). By immunoprecipitation of lysates that were first reduced, alkylated, and then immunoprecipitated, the 30-kDa band was shown to have Lyt-2 determinants (Fig. 3 *Right*). In contrast, the broad band of Lyt-3 around 30 kDa is not immunoprecipitated from thymus lysate by anti-Lyt-2 antibody after reduction. Thus, the 30-kDa band seems to be an incompletely glycosylated Lyt-2 polypeptide that has a similar molecular weight but is clearly distinct from the Lyt-3 polypeptide found in thymocyte lysates.

Presence and Frequencies of α and α' Lyt-2 mRNA Species in Various Cells. These results were obtained by using S1 nuclease protection experiments (Fig. 4 *Left*). We used a 690-bp probe containing the 613-bp *Eco*RI–*Eco*RI α cDNA fragment, shown in Fig. 4 *Right* connected to the priming sequence of the M13 vector.

The labeled probe hybridized with mRNA from thymocytes and genomic DNA transfectants and, digested with S1 nuclease, contained three bands of 613 bp, 374 bp, and 208 bp besides the undigested probe. The largest band corresponds to the probe protected with α mRNA, while the two smaller bands correspond to what is expected by protection with α' mRNA. Therefore, it is clear that there are two kinds of mRNA, one of which contains all five exons and the other of which lacks exon IV (cytoplasmic exon I).

Because the cDNA expression vectors were constructed by digesting the 3' untranslated region of the cDNA at the *Bgl* II site, they lack the *Bgl* II–*Eco*RI fragment (115 bp). Therefore, cDNA transfectants show the expected fully protected band near 498 bp and digested bands at 93 bp and 374 bp. This is diagrammed in Fig. 4 *Right*.

Although lymph nodes do not express detectable amounts of Lyt-2-α' polypeptide, they do have α' mRNA (Fig. 4 *Left*, lanes 8 and 9). Similarly, mRNA from an allospecific cytotoxic T-cell line CTL-108 (17) contains α' mRNA (Fig. 4 *Left*, lane 10). This shows that mature and functional T cells

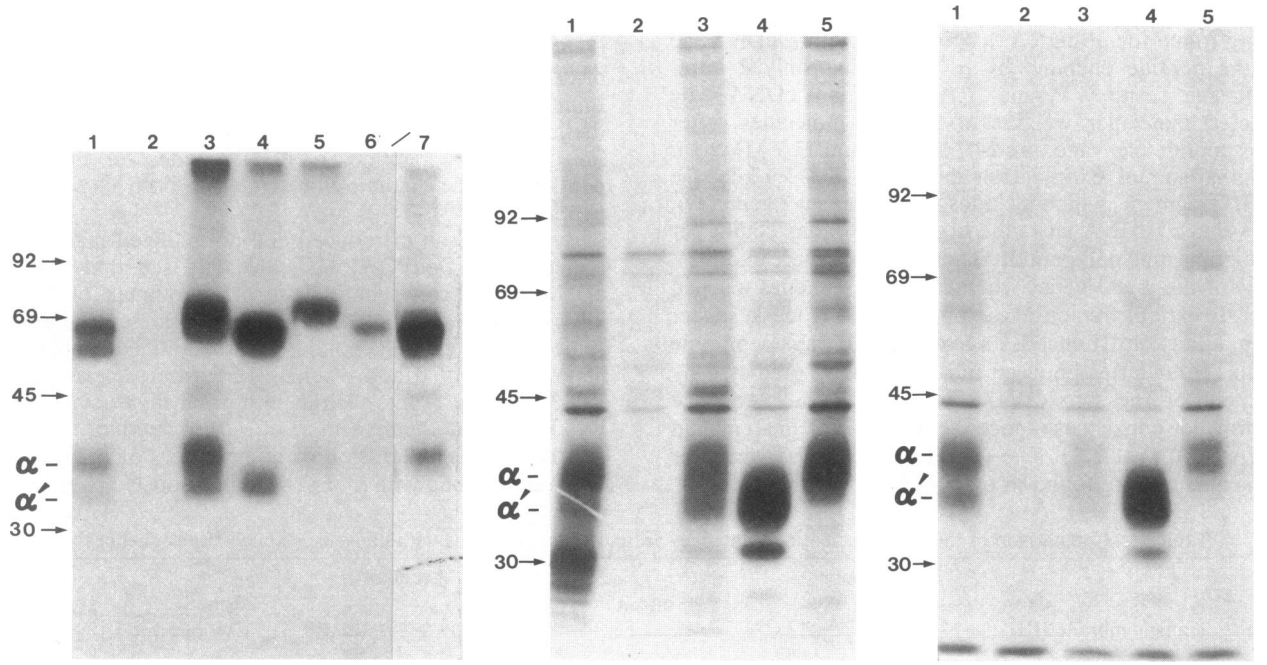

FIG. 3. Autoradiograms of Lyt-2 immunoprecipitations in NaDodSO$_4$/PAGE. Thymocytes from BALB/c (lane 1), L cells (lane 2), L cells transfected with 5.4-kilobase genomic DNA (lane 3), Lyt-2-α' cDNA (lane 4), Lyt-2-α cDNA (lane 5), and lymph nodes from SJL/J (lane 6) were labeled externally with ^{125}I (*Left*) or biosynthetically with [^{35}S]methionine (*Center* and *Right*). Lane 7 is a 3 times longer exposure of the gel in lane 6. (*Left*) A 12% acrylamide gel was used under partial reducing conditions. (*Center* and *Right*) A 10% acrylamide gel was used under reducing conditions; after the cell lysates were reduced and alkylated, immunoprecipitation was performed (*Right*). Positions of standard size (kDa) markers as well as the α (38 kDa) and α' (34 kDa) polypeptides are shown.

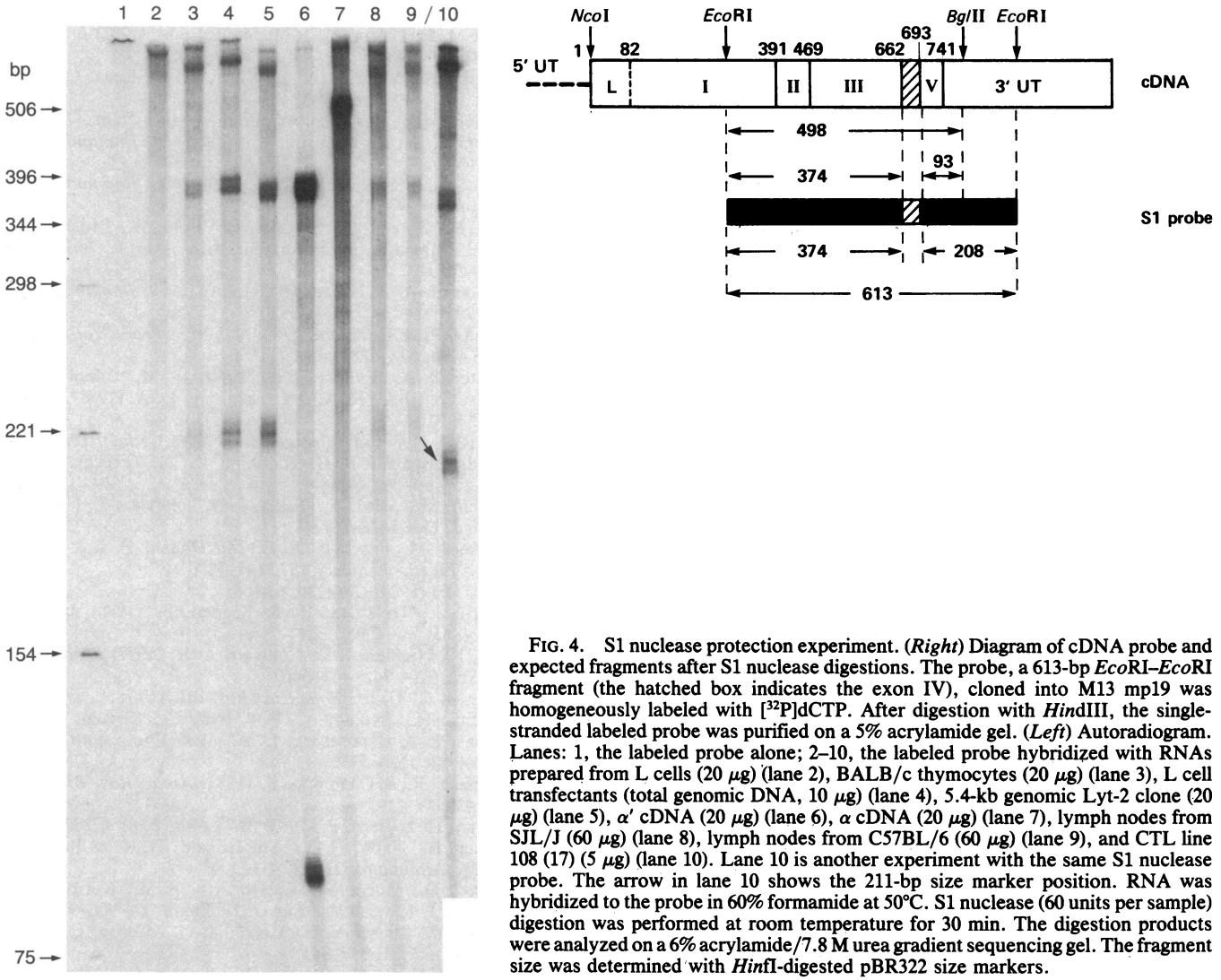

FIG. 4. S1 nuclease protection experiment. (*Right*) Diagram of cDNA probe and expected fragments after S1 nuclease digestions. The probe, a 613-bp *Eco*RI–*Eco*RI fragment (the hatched box indicates the exon IV), cloned into M13 mp19 was homogeneously labeled with [^{32}P]dCTP. After digestion with *Hin*dIII, the single-stranded labeled probe was purified on a 5% acrylamide gel. (*Left*) Autoradiogram. Lanes: 1, the labeled probe alone; 2–10, the labeled probe hybridized with RNAs prepared from L cells (20 μg) (lane 2), BALB/c thymocytes (20 μg) (lane 3), L cell transfectants (total genomic DNA, 10 μg) (lane 4), 5.4-kb genomic Lyt-2 clone (20 μg) (lane 5), α′ cDNA (20 μg) (lane 6), α cDNA (20 μg) (lane 7), lymph nodes from SJL/J (60 μg) (lane 8), lymph nodes from C57BL/6 (60 μg) (lane 9), and CTL line 108 (17) (5 μg) (lane 10). Lane 10 is another experiment with the same S1 nuclease probe. The arrow in lane 10 shows the 211-bp size marker position. RNA was hybridized to the probe in 60% formamide at 50°C. S1 nuclease (60 units per sample) digestion was performed at room temperature for 30 min. The digestion products were analyzed on a 6% acrylamide/7.8 M urea gradient sequencing gel. The fragment size was determined with *Hin*fI-digested pBR322 size markers.

carry out alternate splicing in the same fashion as do thymocytes.

The density of bands in Fig. 4 *Left* was quantitated by densitometry, and the values were adjusted to reflect labeled nucleotide content. With this technique we estimate the percentage of total Lyt-2 mRNA that is α′ mRNA to be about 45% in thymocytes, 20% in lymph nodes, and 15% in the CTL line. The thymocyte frequency accords with the α′ cDNA frequency in the thymocyte cDNA library.

DISCUSSION

Here we confirm the finding of two different Lyt-2 cDNA and mRNA species. We formally show with cDNA expression vectors containing Lyt-2-α and Lyt-2-α′, the two cDNAs, that Lyt-2 polypeptide chains found in thymocytes are the result of differential splicing of RNA from a single gene, not post-translational modification of peptides (32–34). We found no other pattern of RNA splicing in the cells analyzed. In one of the splicing patterns, the deletion of the 31 bp of exon IV causes a reading frame shift to create a cytoplasmic tail of two amino acids instead of the full 27-amino-acid tail encoded by mRNA, including exon IV.

The conversion of α/α′ cDNA expression at the polypeptide level in thymocytes to only α expression in lymph nodes might be related to a T-cell maturation process and be due to a change in the RNA splicing pattern. However, S1 nuclease analysis of lymph node RNA shows that, although lymph nodes express only 38-kDa Lyt-2-α polypeptide, 20% of the Lyt-2 mRNAs are α′ form as compared with 45% in thymocytes. This implies that splicing, as well as other mechanisms such as translational control, contributes to regulation of the differential expression of the two Lyt-2 chains.

It has been reported that some CTL lines express one or more Lyt-2 chains that migrate at 40–44 kDa (5, 33, 35). Although we do not have protein data at this point, S1 nuclease analysis of CTL-108 shows that a splicing mechanism similar to that operating in lymph nodes is operating in this cell line.

Leu-2 and MRC-OX8 are CD-4 T-cell differentiation proteins found in humans and rats, respectively, that have been shown to be homologous to Lyt-2 at both the functional and sequence levels (36–41). Although there are a number of differences among the genes encoding these proteins, the short cytoplasmic tail, as in Lyt-2-α′, is found only in the mouse. The ability of the mouse system to preferentially express the α chain in more mature cell types suggests a regulatory mechanism that the rat and human systems have lost or do not use to produce the α′ form of Lyt-2.

By transfection into L cells, we show that each chain can associate with itself to form a homodimer. Such homodimers are not seen in thymocytes. Therefore, there is some mechanism operating in Lyt-2-bearing cells that facilitates the formation of Lyt-2,3 complexes as opposed to Lyt-2 homodimers. Transfection of individual cDNAs provides us with an experimental system to analyze the function of each

molecule separately. Since Lyt-2 is considered to bind MHC class I molecules, such a transfection system allows examination of the biological significance of these two chains.

An approximately 30-kDa molecule is precipitated from α' cDNA transfectants when metabolic labeling is used. This molecular weight approximates that reported for Lyt-3. However, since these transfectants could not be stained with anti-Lyt-3 antibody (53-5.1) (3) and because reduction/alkylation experiments show the 30-kDa band carries the Lyt-2 antigenic determinant, we conclude that this molecule is not Lyt-3. Additionally, transfection of another α' cDNA expression vector with the Moloney murine leukemia virus LTR promoter in place of the Lyt-2 genomic promoter also reveals the 30-kDa and 34-kDa bands (data not shown). Therefore, we conclude that the 30-kDa form is an intermediate glycosylation form of mature α' chain, possibly caused by lack of a full cytoplasmic tail, and cannot be expressed on the cell surface properly because of the lack of the specific sugar moieties. [A similarly sized molecule was identified by Luescher *et al.* (16) in their investigations of glycosylation precursors.] Because we do not observe a 98-kDa band in nonreduced gels, we rule out the possibility that this 30-kDa band is an anchor protein that associates in the cytoplasm with Lyt-2-α' homodimers.

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