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Influence of 5′ flanking sequences on TL and H-2 expression in transfected L cells

(major histocompatibility complex class I antigens/chimeric genes/transfection)

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ABSTRACT **TL (thymus leukemia) antigens are encoded by genes in the major histocompatibility complex (MHC) of the mouse. Although similar in overall structure to other class I MHC antigens (H-2, Qa), TL expression is regulated in a highly distinctive fashion. In contrast to the broad distribution of H-2 and the intermediate distribution of Qa, TL expression is restricted to cells of T-cell derivation during development in the thymus and is lost when T cells migrate to the periphery. Some mouse strains do not express TL antigens on thymocytes (TL^- strains), but leukemias occurring in these mice can have a TL^+ phenotype, indicating activation of normally silent TL genes. In transfection studies with H-2 or TL genes in L cells (mouse fibroblasts), H-2 is expressed at high levels, whereas TL is poorly expressed. To identify genetic elements that regulate expression in transfected L cells, chimeric genes were constructed by transposing the 5′ and 3′ regions of TL and H-2 genes. Antigen expression was not influenced by transposing the cytoplasmic domain and 3′ untranslated region. In contrast, interchanging the 5′ flanking sequences and exon 1 had a marked influence on antigen expression, with 5′ sequences from the H-2 gene increasing TL expression 10- to 50-fold, and 5′ sequences from the TL gene markedly decreasing H-2 expression. With both the parental TL gene (p20-TL) and the highly expressed chimeric TL gene (construct 3), levels of TL mRNA and TL antigen correlated with the number of transfected gene copies. However, in cells transfected with equal copy numbers, much higher levels of TL mRNA and TL antigen were found in construct-3 transfectants than in p20-TL transfectants. In addition, there was marked heterogeneity in TL mRNA size in L cells transfected with p20-TL, in contrast to a more homogeneous transcript size in construct-3 transfectants. These results point to regulatory sequences in the 5′ flanking region of class I genes that control proper initiation and processing of TL transcripts.**

TL antigens of the mouse are cell surface antigens restricted to thymocytes and leukemia cells (1). Three levels of regulation of antigen expression exist: (*i*) TL^+ vs. TL^- thymocytes (strain-specific expression); (*ii*) TL^+ vs. TL^- T cells (differentiation-specific expression); and (*iii*) anomalous occurrence of TL^+ leukemias in TL^- mice (leukemia-specific expression). To examine the basis for these differences in TL regulation, we and others have cloned and sequenced TL genes from normal and leukemia cells of various mouse strains (2–5). Apparently competent TL genes have been isolated from TL^- (non-expressor) strains, and no obvious structural basis has as yet been found to account for strain-specific or leukemia-specific TL expression (1, 2, 4). Both H-2 and TL genes belong to the major histocompatibility complex (MHC) class I family and show clear structural homology, with the major distinction being the number of exons coding for the 3′ cytoplasmic domain (one exon in TL genes and three exons in H-2 genes) (2, 4). Although TL expression can be detected in L cells transfected with TL-coding genes, antigen levels are low and variable, in contrast to the uniformly high expression of transfected H-2-coding genes. To investigate the basis for this differential expression in L cells, chimeric TL and H-2 genes with transposed 3′ and 5′ sequences were constructed and their antigen-coding capacities were compared to parental TL and H-2 genes.

MATERIALS AND METHODS

TL, H-2, and Chimeric Genes. p20-TL, containing the T3 TL gene, was obtained by subcloning an 8.5-kilobase (kb) *Kpn* I fragment from λ20, a genomic T3 clone isolated from the TL^+ C57BL/6 (B6) leukemia ERLD. p20-TL is identical to C25.1 (2) except that the latter lacks a part of exon 1 and the 5′ flanking sequences. pC-H-$2K^b$, containing the H-$2K^b$ gene of B6/Kh, was derived by subcloning a 10.5-kb *Eco*RI fragment of C1.4.1 (6). Four chimeric genes produced by fusion of p20-TL and pC-H-$2K^b$ genes were constructed as described in the legend to Fig. 1. Plasmids pUC18 and -19 were used as vectors.

Enzymes. Restriction endonucleases and other DNA-modifying enzymes were purchased from New England Biolabs or Boehringer Mannheim.

Transfection. TL, H-$2K^b$, or chimeric genes were transfected into thymidine kinase-negative L cells along with the herpes simplex virus thymidine kinase gene by the calcium phosphate precipitation method (8).

Serological Analysis. TL and H-$2K^b$ antigens expressed in L-cell transfectants were analyzed by erythrocyte rosetting assays and fluorescence-activated cell sorting (FACS) [with FACStar (Becton Dickinson)], using rat monoclonal TL antibodies HD168 and HD177 (2) and mouse monoclonal H-$2K^b$ antibodies K9/178 (9) and HB-11. For detailed analysis of TL specificities, cytotoxicity and absorption assays were performed with the following test systems (10): TL1, B6 anti-A strain leukemia ASL1 serum on ERLD leukemia cells; TL2, (B6 × A-*Tla*^{b}) F_1 anti-ASL1 serum on strain 129 thymocytes; TL3, (BALB/c × C3H/An)F_1 anti-ASL1 serum [preabsorbed *in vivo* in (B6 × A-*Tla*^{b})F_1 mice carrying ERLD leukemia] on B6-*Tla*^{a} thymocytes; TL4, (A × B6-*Tla*^{a})F_1 anti-ERLD serum on ERLD cells.

Preparation of DNA and RNA. Plasmid DNA was prepared by the alkali/sodium dodecyl sulfate ($NaDodSO_4$) lysate method (11). DNA and RNA of transfectants were isolated by the proteinase K/$NaDodSO_4$ procedure (12) and by the guanidine thiocyanate/CsCl procedure (13), respectively.

DNA and RNA Dot Blot and RNA Gel Blot Analysis. DNA and RNA of transfectants were dotted on nitrocellulose

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Abbreviations: FACS, fluorescence-activated cell sorting; MHC, major histocompatibility complex.

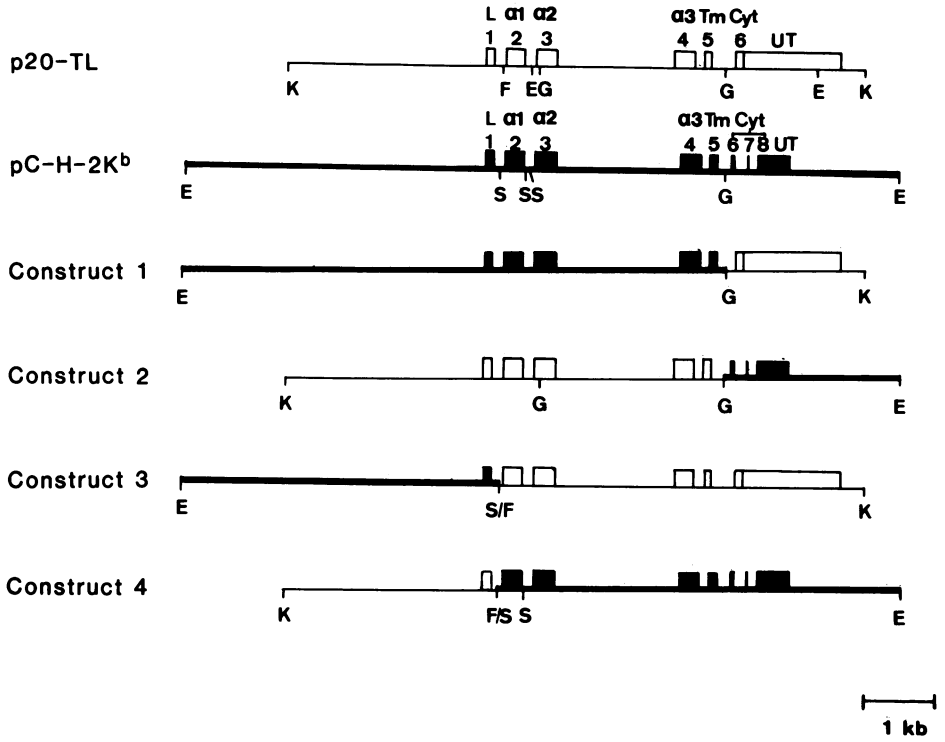

FIG. 1. Construction of chimeric genes. TL clone p20-TL is an 8.5-kb *Kpn* I–*Kpn* I fragment containing the T3 TL gene of B6 TL$^+$ leukemia ERLD and its 2.9-kb 5′ and 0.4-kb 3′ flanking regions (2). H-2K^b clone pC-H-2K^b is a 10.5-kb *Eco*RI–*Eco*RI fragment containing the H-2K^b gene of B6 mice and its 4.4-kb 5′ and 1.6-kb 3′ flanking regions (6). Construct 1 has the H-2 5′ flanking region and exons 1–5 (contained in a 7.9-kb *Eco*RI–*Bgl* II fragment) ligated to TL exon 6 and 3′ flanking region (contained in a 2.1-kb *Bgl* II–*Kpn* I fragment). Construct 2 has the TL 5′ flanking region, exons 1 and 2, and the 5′ part of exon 3 (contained in a 3.7-kb *Kpn* I–*Bgl* II fragment); TL 3′ part of exon 3 and all of exons 4 and 5 (contained in a 2.6-kb *Bgl* II–*Bgl* II fragment); and H-2 exons 6–8 and 3′ flanking region (contained in a 2.6-kb *Bgl* II–*Eco*RI fragment). Construct 2 was made in two steps; the two distal fragments were ligated and the resulting *Bgl* II site was used to insert the *Bgl* II–*Bgl* II fragment. Construct 3 has the H-2 5′ flanking region and exon 1 (contained in a 4.7-kb *Eco*RI–*Sma* I fragment) ligated to TL exons 2–6 and 3′ flanking region (contained in a 5.3-kb *Fsp* I–*Kpn* I fragment). Construct 4 has the TL 5′ flanking region and exon 1 (contained in a 3.1-kb *Kpn* I–*Fsp* I fragment); H-2 exon 2 [contained in a 300-base-pair (bp) *Sma* I–*Sma* I fragment]; and H-2 exons 3–8 and 3′ flanking region (contained in a 6.5-kb *Sma* I–*Eco*RI fragment). Construct 4 was made in two steps: the two 5′ fragments were first ligated, and then the resulting DNA was ligated to the 3′ fragment. The 51-bp *Sma* I–*Sma* I fragment of H-2 intron 2 was removed in construct 4; however, no influence of this deletion on H-2K^b expression has been reported (7). Proper structures of these chimeric clones were confirmed by restriction mapping and by sequencing. Exons represented by numbered open (TL) or filled (H-2K^b) boxes encode leader (L), extracellular (α1, α2, and α3), transmembrane (Tm), and cytoplasmic (Cyt) domains of the proteins or the 3′ untranslated (UT) region of the mRNAs. Restriction enzyme sites are identified as follows: E, *Eco*RI; F, *Fsp* I; G, *Bgl* II; K, *Kpn* I; S, *Sma* I.

filters in a Minifold apparatus (Schleicher & Schuell). For RNA gel blots, RNA was fractionated in 2.2 M formaldehyde/agarose gels and transferred to nitrocellulose filters (14). Blots were analyzed with [α-^{32}P]dCTP-labeled nick-translated hybridization probes (15). The conditions for hybridization and washing have been described (2, 4).

RESULTS

Two sets of chimeric genes were constructed with TL and H-2 sequences: one set (constructs 1 and 2) with transposition of exons coding for the cytoplasmic domain and 3′ untranslated region, and the other set (constructs 3 and 4) with transposition of the 5′ flanking region and exon 1 (Fig. 1). L cells were transfected with these four constructs and the parental TL and H-2 genes, and primary transfectants were analyzed for expression of TL and H-2 antigens. Fig. 2 shows results obtained by FACS analysis. Erythrocyte rosetting assays gave similar results. L cells transfected with the parental TL gene (p20-TL) expressed low levels of TL and no H-2K^b. L cells transfected with the parental H-2K^b gene showed strong H-2K^b expression and no TL expression. Expression of TL and H-2 in cells transfected with construct 1 or 2 (3′ transposition) was similar to that of cells transfected with parental genes. In contrast, transposition of the 5′ flanking region and exon 1 greatly augmented TL expression (construct 3) and markedly reduced H-2 expression (construct 4).

To confirm these results with cloned cell populations, a large number of clones (>150) were isolated from primary L-cell cultures transfected with parental or chimeric genes. Clones were analyzed for antigenic expression by FACS analysis and by quantitative absorption tests (Table 1), and the copy number of transfected parental or chimeric genes was estimated by DNA dot blot analysis. Results with the cloned cell populations were similar to findings with the primary transfectants, as shown in Fig. 2. The expression of individual TL specificities was assayed by quantitative absorption analysis using L cells containing similar numbers of transfected gene copies. Cells transfected with p20-TL (parental), construct 2 (3′ H-2 transposition), and construct 3 (5′ H-2 transposition) expressed TL1, TL2, and TL4 specificities, with construct-3-transfected cells expressing 10–20 times higher antigen levels. Cells transfected with λ17.3 [the T13 gene from BALB/c mice (16)] expressed somewhat less TL1 and TL2 than the p20-TL transfectants. λ17.3 transfectants expressed no TL3 or TL4, and cells transfected with p20-TL and the TL chimeric genes expressed TL4 but no TL3, in accordance with the normal/leukemic TL phenotypes of BALB/c and B6 mice (1, 10). There was a clear correlation between TL antigen level and gene copy number in clones derived from the same primary transfectants. Fig.

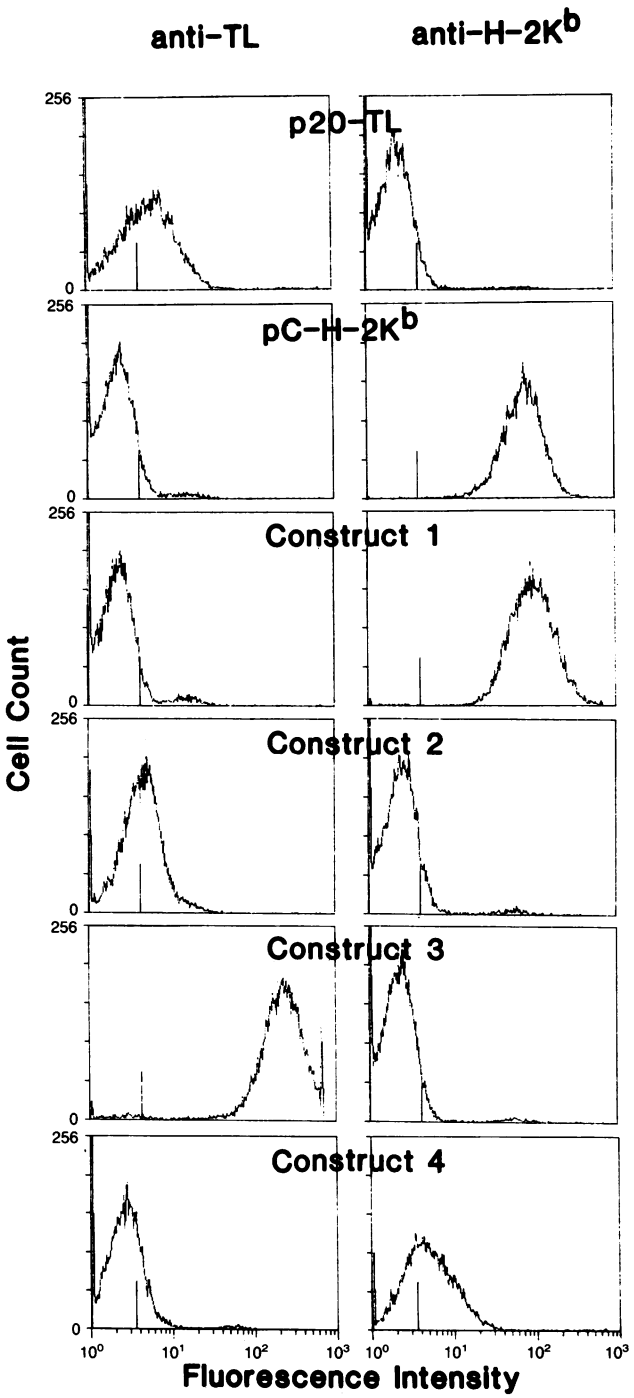

FIG. 2. Expression of TL and H-2K^b antigens in primary transfectants. Parental or chimeric genes (1 μg of DNA) and the thymidine kinase gene (20 ng of DNA) were cotransfected into 4 × 10^6 L cells. After 2–3 weeks in hypoxanthine/aminopterin/thymidine selection medium, all thymidine kinase-positive transfectants were harvested and tested for the expression of the antigens by FACS analysis with rat monoclonal TL antibodies (HD168 and HD177) and mouse monoclonal H-2K^b antibodies. Essentially identical results were obtained with HD168 and HD177, and the results with HD168 are shown. Transfectants analyzed had comparable copy numbers of transfected genes, as determined by DNA dot blot and gel blot analysis (data not shown).

3 shows results with six clones derived from L cells transfected with parental p20-TL genes and three clones transfected with construct 3 (carrying the 5′ H-2 flanking sequence). Comparing levels of TL antigen at various copy numbers, we estimate that the 5′ sequence of H-2K^b is 10–50 times more active in directing TL antigen synthesis than the corresponding 5′ p20-TL sequence.

To determine the relation between TL transcripts, gene copy numbers, and antigen expression, the amount of TL message was estimated by RNA dot blot analysis. In L cells transfected with the parental p20-TL gene, the number of RNA transcripts correlated with number of DNA copies. At comparable copy numbers, construct-3 transfectants had >10 times more TL transcripts than p20-TL transfectants. However, this difference in transcript level cannot account for differences in TL expression in p20-TL and construct-3 transfectants, since p20-TL transfectants with extremely high DNA copy numbers can have TL RNA levels comparable to those in construct-3 transfectants yet have far lower TL antigen expression.

The RNA transcripts in p20-TL and construct-3 transfectants were characterized by RNA gel blot analysis (Fig. 4). All p20-TL clones had similar hybridization patterns; there were no prominent bands, but there were several bands of intermediate intensity ranging from 9.6 kb to 1.7 kb. In contrast, construct-3 clones showed a major band at 2.1 kb. Fig. 4 also shows the pattern for ERLD, the B6 leukemia from which the p20-TL gene was derived. A prominent band was observed at 2.4 kb, quite distinct from the pattern seen with p20-TL transfectants, and larger than the transcripts in construct-3 transfectants.

DISCUSSION

Chimeric MHC class I genes have been useful in identifying coding sequences for the antigenic determinants recognized by humoral and cellular immune responses (7, 17–20), analyzing the function of the transmembrane domain (21, 22), and defining sequences that control tissue-specific expression (23, 24). In past studies, we noted that L cells transfected with TL coding genes expressed low levels of TL antigen, in contrast to high H-2 expression after transfection of H-2 coding genes. To investigate the basis for this restriction of TL expression, chimeric TL and H-2 genes with transposition of 5′ or 3′ flanking regions were constructed. In comparison with the parental H-2 or TL genes, replacement of 3′ sequences did not influence the antigen-coding capacity of the transfected genes, whereas replacement of the 5′ flanking regions and exon 1 had a marked influence, increasing TL expression and decreasing H-2 expression. Although exon 1 transposition may have contributed to these results, we think this unlikely. Putative exon 1 of p20-TL genes is 15 bases longer than H-2K^b exon 1 and has three in-frame methionine codons at the 5′ end (1). From the last methionine codon, p20-TL exon 1 clearly resembles H-2K^b exon 1 (65% sequence identity at the DNA level and 61% identity at the amino acid level) and codes for a typical hydrophobic leader peptide.

Sequence information is available on 512 bases in the immediate 5′ flanking region of exon 1 of p20-TL, and a 50% identity with the corresponding region of H-2K^b has been found. In the case of H-2K^b, the 35-bp sequence IV at −193 to −159 (25) and the 13-bp perfect dyad-symmetry sequence at −171 to −159 (26) are important for antigen expression. Homologous sequences are found in the corresponding regions of p20-TL (≈75% identity), but there is an 11-bp insert at the position corresponding to −163 in H-2K^b. In addition, the TATAA and CCAAT sequences of H-2K^b (27) are replaced by TACAA and GCAAT in p20-TL, and the α-interferon response sequence of p20-TL has diverged from the consensus sequence in H-2K^b (25). Whether these differences in the promoter regions of the TL and H-2 genes account for the differential expression in transfected L cells requires a more precise structural and functional analysis of the 5′ flanking region of p20-TL.

Table 1. Quantitative absorption tests for individual TL specificities

DNA transfected	Number of transfected genes	TL antigen expression*			
		TL1	TL2	TL3	TL4
ptk†	—	Neg	Neg	Neg	Neg
λ17.3	32	2×10^7	1.5×10^7	Neg	Neg
pC-H-2K^b	32	Neg	Neg	Neg	Neg
p20-TL	32–64	10^7	10^7	Neg	10^7
Construct 1	32	Neg	Neg	Neg	Neg
Construct 2	32–64	10^7	10^7	Neg	2×10^7
Construct 3	32–64	5×10^5	5×10^5	Neg	10^6
Construct 4	32–64	Neg	Neg	Neg	Neg

Appropriately diluted antisera (100 μl) were absorbed for 30 min at 4°C with cell pellets containing known numbers of transfected cells. After centrifugation, supernatants were tested in cytotoxicity assays with the appropriate test cells and complement (10). For each DNA preparation tested, at least two independently cloned transfected cell lines were analyzed.

*Results are expressed as number of cells required for absorption of antibody. Neg, no absorption with $>2 \times 10^7$ cells.

†Control (thymidine kinase gene alone).

There was a marked difference in the quantitative and qualitative characteristics of the TL transcripts in L cells transfected with the parental p20-TL gene or the high TL expressor, construct 3. Comparing L cells with similar copy numbers of transfected genes, construct-3 transfectants had much higher amounts of TL RNA than p20-TL transfectants. TL transcripts in p20-TL-transfected cells were heterogeneous in size, ranging from 9.6 kb to 1.7 kb, with some transcripts larger than the transfected p20-TL gene. Independently cloned lines of p20-TL transfectants showed a similar pattern of RNA transcripts, implying that transcript heterogeneity is not due to different chromosomal integration sites of the transfected gene, but is a property of the transfected gene. In contrast to the size heterogeneity of p20-TL transcripts, TL transcripts in construct-3 transfectants were more homogeneous, with the predominant species being 2.1 kb. As the H-2K^b transcript is 1.6 kb, and the 3′ untranslated region of p20-TL is 500 bp longer than that of H-2K^b, it seems likely that transcription of construct 3 involves the major initiation site of the H-2K^b gene and is controlled by H-2K^b promoter sequences.

Low TL expression in p20-TL transfectants cannot be

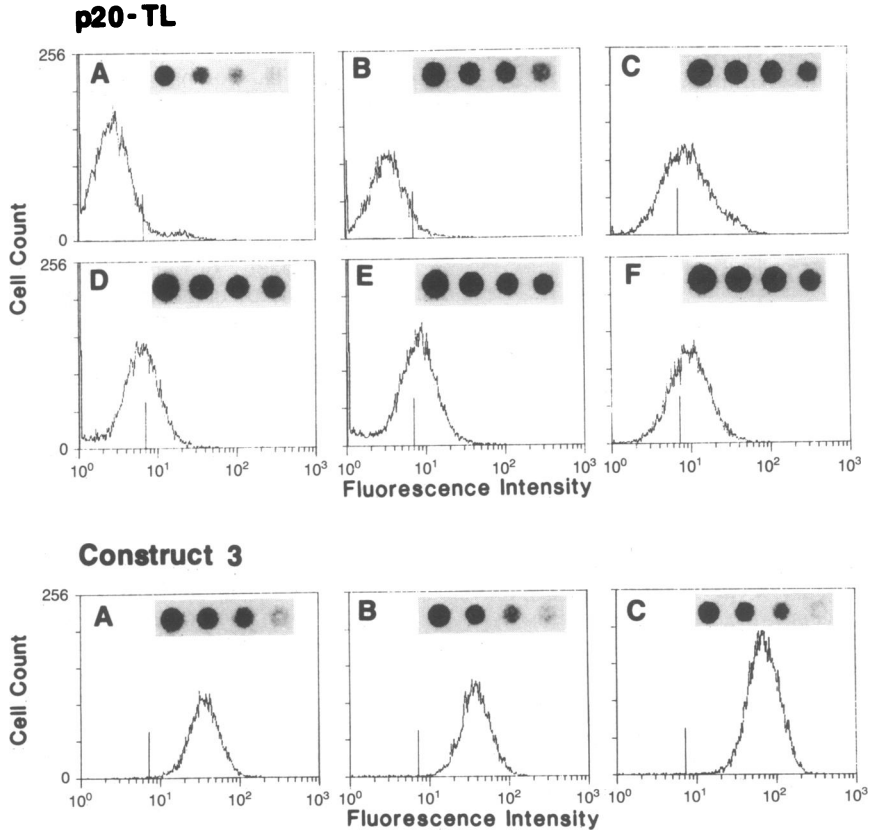

FIG. 3. Comparison of TL antigen expression and DNA copy number in cloned cell lines of p20-TL and construct-3 transfectants. Expression of TL antigen was analyzed by FACS with HD168 monoclonal antibody as described for Fig. 2. Copy number in each cell line was estimated by comparing the results of dot blots (*Insets*) hybridized with a TL-specific probe, pTL1 (2), with those of normal B6 thymocytes (>4 pTL1$^+$ hybridizing genes per cell), A-strain normal thymocytes (>8 genes), and L cells (presumably 4 genes). Estimated copy numbers in p20-TL cell lines were 4 (*A*), 4–8 (*B*), 16 (*C*), 64 (*D*), 32–64 (*E*), and 64 (*F*). The estimated copy number was 4–8 for all construct-3 lines. For dot blot analysis, 4, 2, 1, and 0.5 μg of DNA was hybridized with pTL1.

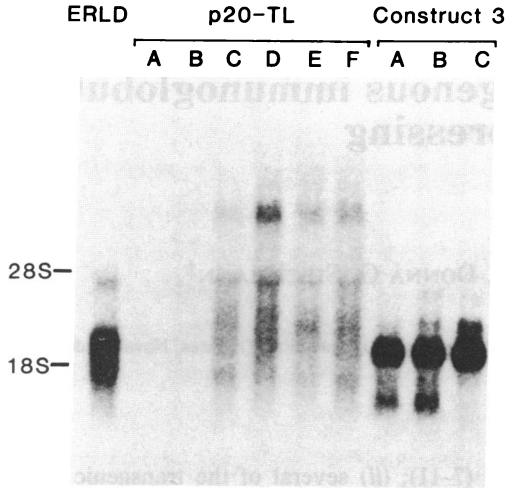

FIG. 4. RNA gel blot analysis of p20-TL and construct 3 transfectants with pTL1. Total RNA from cloned cell lines p20-TL A–F and construct 3 A–C (see Fig. 3) was analyzed with ^{32}P-labeled pTL1. Ten micrograms of total RNA was loaded per lane. Ten micrograms of poly(A)$^+$ RNA from the ERLD leukemia was included for comparison. The 28S and 18S ribosomal RNAs were used as markers and correspond to 4.7 kb and 1.9 kb, respectively.

accounted for by low levels of TL mRNA alone, since transfectants with high p20-TL copy numbers can have high amounts of TL message, despite low TL antigen expression. Nor can poor expression be accounted for by posttranslational events or by rapid turnover of TL mRNA due to its unique 3′ untranslated region, since construct 3-transfected L cells express high levels of TL message. Low TL expression and size heterogeneity of TL transcripts are not unique to p20-TL transfectants; similar findings have been made with L cells transfected with TL-coding genes from the A strains [*Tla*^{a}*-2* and *Tla*^{a}*-3* genes (4)] and T13 (λ17.3) from BALB/c mice. One possibility to account for poor TL expression and transcript heterogeneity in transfected L cells is that transcripts are initiated at multiple inappropriate sites and are not properly processed. This in turn could be due to the lack of tissue-specific factors in L cells that promote proper initiation and splicing of TL message or the presence of factors that inhibit these processes. Whatever the explanation, 5′ flanking sequences of the H-2 gene overcome these restrictions in L cells and allow for efficient synthesis of TL message and TL antigen. Since TL genes are expressed appropriately in TL$^+$ thymocytes and TL$^+$ leukemias, further understanding of tissue-specific factors controlling TL expression will require transfection systems using permissive T cells or studies with TL-transgenic mice. Methods are now available to identify promoter-specific DNA-binding proteins (28), and factors that bind to sequences in the promoter region of the H-2K^b gene have been reported (26, 29).

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