The Expression of Several T Cell-Specific and Novel Genes Is Repressed by Trans-Acting Factors in Immature T Lymphoma Clones

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Summary

Cell surface proteins encoded by members of the immunoglobulin supergene family are sequentially expressed during T cell ontogeny. The molecular mechanisms responsible for the regulation of these surface molecules are not well understood. To investigate this issue, we used a series of well characterized T lymphoma cell clones with phenotypes characteristic of distinct stages of early thymocyte maturation. Somatic cell hybrids formed from these cell lines were employed to detect the presence of negative regulatory molecules. The expression of CD4 and CD8 was strongly repressed in hybrids formed between a CD4+CD8+ lymphoma clone and "immature" CD4-CD8- lymphoma clones. Individual subunits of the T cell receptor (TCR)/CD3 complex displayed independent regulation in unique patterns in hybrid cells. Hybrids formed by fusing CD3+ and CD3- cells completely repressed CD3-δ mRNA expression while CD3-γ, -ε, and -ζ transcripts were moderately inhibited or codominantly regulated. Similar to CD3-δ, interleukin 2R-α (IL-2R-α), and TCR-β mRNA accumulation was trans-negatively regulated. Transcription rate measurements demonstrated that the inhibition of CD4, CD8, CD3-γ, CD3-ε, TCR-β, and IL-2R-α mRNA accumulation in hybrid cells was exerted, at least in part, at the transcriptional level. To test whether repressional regulation is a general feature of T cells, we examined the regulation of six novel genes which were selected solely on the basis of their differential expression between two of the cell lines used in this study. Five of the six novel gene transcripts were repressed in the somatic cell hybrids. Thus, inhibitor factors appear to play a general role in controlling T cell gene expression. The model system presented here may be useful for the identification and characterization of repressor molecules responsible for the regulation of genes expressed during T cell ontogeny.

T cell maturation in the thymus is characterized by the sequential expression of several different T cell surface proteins encoded by genes of the immunoglobulin supergene family. An early event in thymocyte maturation is the transient expression of the IL-2R. The expression of the IL-2R appears to be required for subsequent maturation events since antibodies against IL-2R inhibit thymocyte differentiation in vivo (1) and in organ culture in vitro (2). The individual members of the TCR/CD3 gene complex are also expressed in a stage-specific manner during thymocyte maturation. The CD3-γ, -δ, and -ε genes are expressed in the early phases of thymocyte maturation, before rearrangement and expression of TCR-α and -β genes in human fetal thymus and liver (3–5). Although CD3 proteins are detectable in the cytoplasm of immature thymocytes early in differentiation, the proteins are not expressed on the cell surface until all of the components of the complex, including both TCR subunits, are present (6, 7). The cell surface CD4 and CD8 proteins are expressed after IL-2R and most members of the TCR/CD3 complex during fetal ontogeny. In the adult thymus, CD4+CD8+ (double positive) cells are the predominant thymocyte population and they reside mainly in the thymic cortex (6). During the final stages of thymocyte maturation, a selective shut-off of either CD4 or CD8 gene expression occurs. The vast majority of mature thymocytes which exit the thymus express only one of these gene products, e.g., T helper cells express only CD4 while T suppressor/cytotoxic cells express only CD8.

The molecular signals which regulate the genes important in these developmental events are not well understood. Molecular analysis is hampered by the difficulty of obtaining sufficient quantities of pure thymocyte subpopulations at
distinct stages of maturation (8). However, cloned lymphoma cell lines have been useful in defining the regulation of genes expressed in both T and B lymphoid cell lines (9, 10). Since the targets of transformation are often progenitor cell types, immortalized cells are useful for studies of developmentally restricted gene regulation (10, 11). Cloned tumor cell lines have been instrumental in identifying numerically infrequent normal cells at specific stages of development which would otherwise be difficult to detect (10, 11) and for analyzing molecular events which underlie differentiation processes (12).

This study focuses on a set of well characterized T lymphoma cell clones which correspond in phenotype to thymocytes at distinct stages of maturation (13–16). The SL12 T lymphoma cell clones were derived from a single murine cell line (13, 14). The SL12.3 clone has the phenotype CD3+CD4-CD8-CD44+ (Pgp-1−) HSA− (J11d−) Thy1+ IL-2R-α− (14, 16, 17) which is similar to the phenotype of the most immature subset of double negative thymocytes (18–21). A second cell clone, SL12.1, is similar to the SL12.3 cell clone, although it expresses CD3-e mRNA and class I MHC cell surface protein (13, 16). The SL12.4 cell clone is also CD3+CD4−CD8−HSA−Thy1+, but unlike the other cell clones, SL12.4 cells lack CD44 and express II-2R-α (14, 15), a phenotype characteristic of "intermediate maturity" thymocytes within the double negative subset (21) that have a high potential to differentiate into mature T cells in organ culture or after adoptive intrathymic transfer (6, 18, 22). SL12.4 cells accumulate CD3-γ, -δ, -ε, and -ζ mRNA, and possess fully rearranged TCR-α and -β genes (15, 16), but they do not express TCR/CD3 on the cell surface since they lack or weakly express full length TCR-α and -β mRNAs, unless they are suitably induced (15, 23). The SAK8 cell clone has the most mature phenotype of the lymphoma cell lines examined in the present study; it expresses cell surface CD4 and CD8, and accumulates full length TCR-α and -β transcripts, analogous to cortical thymocytes (15, 16). Somatic cell hybrids were formed by fusing these several cell clones that represent different maturation stages to assess the expression of developmentally regulated T cell specific genes (24, 25).

These lymphoma cell clones and their hybrids were used to test the hypothesis that T cell gene expression is repressed by transacting factors during the early stages of thymocyte development. Such factors may function to repress T cell specific gene expression until the appropriate point of maturation has been reached. Two lines of evidence lead us to formulate this hypothesis. First, our previous work indicates that unstable proteins repress TCR/CD3, CD4, and CD8 gene expression, since protein synthesis inhibitors induce or augment these transcripts in T lymphoma cell clones (15, 16, 23, 26, 27). Secondly, using somatic cell hybrids formed from the fusion of parental cell clones which represent different stages of thymocyte maturation, we found that TCR-β and CD3-6 mRNA accumulation is blocked by inhibitory factors present in immature cell clones (16).

In the present study, we have extended the analysis of gene regulation in somatic cell hybrids formed between T lymphomas of common origin. These hybrids were chosen because the cells have the same lineage; thus the regulation observed is likely to be relevant to T cell biology and could involve a small set of regulatory factors. Our analysis of TCR-β gene expression was extended to determine whether the mechanism of repression is transcriptional or posttranscriptional. To determine the generality of represional regulation, we have broadened the analysis to include other T cell specific transcripts, including additional CD3 subunits, CD4, CD8, and IL-2R. The regulation of these known genes was compared with the regulation of six novel transcripts which are differentially expressed between two of the T lymphoma cell clones used in this study. The novel transcripts were detected with specific cDNA clones isolated by subtraction hybridization (28). Five of the six CDNA clones correspond to genes that show no significant homology to sequences present in current databases (28). Four of the genes have been mapped to murine chromosomes and have been given a three letter name based upon their expression patterns. The Tia (20.5) cDNA sequence has substantial homology with the murine ecotropic retroviral receptor (29). Both Tea and Lro (19.5) genes encode cell surface multiple membrane spanning proteins and both are expressed in lymphoid tissue (28, 29). The Tia (19.4) gene is expressed exclusively in lymphoid tissue; both Tea and Tia gene expression are strongly induced when splenic T cells are activated by the T cell mitogen ConA but not by the B cell mitogen LPS. The two unnamed genes, 19.1 and 19.2, are also expressed in lymphoid tissues. The Pem (20.2) gene encodes a highly hydrophilic protein, likely to be intracellular (30). Unlike the other five genes, the Pem gene is not detectably expressed in normal lymphoid tissue, but it is expressed in a variety of immortalized and malignant cells, including T lymphomas (30). The results of our analysis of this constellation of known and novel genes indicate that repressional control is a common mechanism which regulates mRNA accumulation, and that control is exerted at both the transcriptional and posttranscriptional level.

Materials and Methods

Cell Culture. The T lymphoma parental and hybrid cell clones used in this study and their culture requirements have been described (13, 14, 16). The cells were grown in DMEM containing 10% FCS and supplemented with glutamine and penicillin/streptomycin. The cells were seeded so that at the time of harvest their cell density was between 5 x 10^6 and 10^6 cells/ml.

Immunofluorescence Staining and Flow Cytometry Analysis. Hybridoma supernatants from rat fusions for the following antibodies were provided by Dr. Robert Hyman, Salk Institute for Biological Studies: CD8 (Lyt-2, reference 31), CD4 (3T4, reference 32), and Thy-1.2 (33). Cells (10^6) were suspended in 25 μl of buffer A (PBS containing 10% fetal bovine serum and 0.02% sodium azide) and the cell suspension was incubated with 2.8 μl of heat treated mouse serum for 20 min at 4°C to block potential Fc-receptors. Subsequently, 50 μl of rat hybridoma supernatant (diluted 1:5) was incubated with the cells for 30 min at 4°C. Following three washes in buffer A, the cells were incubated for 30 min at 4°C with diluted rabbit or goat anti-rat IgG conjugated with FITC (anti-mouse IgG activity was removed by the supplier; Zymed Laboratories Inc., San Francisco, CA). The negative control in all the experiments was anti Thy 1.2 rat anti-mouse mAb. It is of the same isotype.
as the anti CD4 and anti CD8 used in the study. The cells were analyzed on a cytofluorograph 50 H fluorescence activated cell sorter (Ortho Diagnostics Inc., Westwood, MA) operating with a laser emitting at 488 nm. Histograms were generated from 5,000-10,000 cells analyzed using a three decade logarithmic amplification scale of fluorescence emitted by single viable cells. Propidium iodide was used to gate out dead cells which were in all cases less than 30% of the cells at the time of analysis. Cell viability at the beginning of the staining procedure was >95% as assessed by trypan blue exclusion.

**DNA Probes.** The murine cDNA probes used in this study for Northern analysis were a 0.6 kb CD8 (pLY2C-1) cDNA (34), a 2.1-kb CD4 (p2C) CD4 cDNA (35), a 0.75-kb CD3-γ (pBIO.AT3y-1) cDNA (36), a 0.6-kb CD3-β cDNA (37), a 1.5-kb CD3-e cDNA (38), a 1.2-kb CD3-ζ cDNA (39), a 0.6-kb TCR-β (86T5) cDNA (40), a 1.3-kb IL-2R-α (mIL2R8) cDNA (41), and an epidermal growth factor receptor (EGFR) cDNA (42). A 1.5-kb Chinese hamster cDNA clone, CHO-A (43), which recognizes transcripts ubiquitously expressed by all known mammalian cell types, was used as a positive control for Northern blots. The following novel cDNA clones were obtained by subtraction hybridization: 19.1, 19.2, 19.4 (Tia), 19.5 (Lov), 20.2 (Pem) (28, 30), and 20.5 (Tia) (29). For the nuclear run-off analysis, single stranded probes were prepared by subcloning the following fragments into M13mp18 and M13mp19: the entire 0.6 kb CD8 cDNA clone, a 0.5 kb 5′ PstI/EcoR1 CD4 cDNA fragment, a 1.6 kb BamH1/SacI β3-1-7 genomic fragment, a 1.2 kb EcoR1/Clal β3-1-2 genomic fragment (23), a 0.5-kb 5′ EcoR1/PvuII CD3-γ cDNA fragment, a 0.4 kb 5′ EcoR1/XhoI CD3-ε cDNA fragment, 0.12-kb, and 0.5-kb PstI/PstI internal CD3-γ cDNA fragments, and a 0.5-kb 5′ PstI/BamH1 muscle actin fragment. Several CD3-β fragments were subcloned: 81, 0.2-kb 5′ EcoR1/PvuII cDNA fragment; 82, 0.3-kb EcoR1/BamH1 IVS1 genomic fragment; 83, 0.3-kb XbaI/AccI EX-IVS2 genomic fragment; 84, 0.6-kb BglII/PstI IVS2-3′ genomic fragment; 85, 0.3-kb PstI/TaqI IVS3-3′ genomic fragment; and 86, 0.8-kb TaqI/EcoR1 IVS4-3′ untranslated genomic fragment (27).

**Northern Blot Analysis.** Total cellular RNA was isolated from cells as described (44). 10 μg of RNA was electrophoresed (unless otherwise noted) in 1% agarose (formaldehyde) gels and transferred to S & S Nytran membranes (45). The integrity and equivalence of RNA loaded was assessed by acridine orange staining: the gel was stained for 3 min in 15 μg/ml acridine orange, 10 mM sodium phosphate (pH 6.5) and 1.1 M formaldehyde. After destaining 20 min in the same buffer, the gel was examined on an ultraviolet transilluminator and photographed. After transfer and UV cross-linking, the efficiency of transfer was assessed by staining the blot for 45 s with 0.03% methylene blue in 0.3 M sodium acetate (pH 5.2), followed by destaining in water for 2 min. The blot was photographed before complete removal of the stain by a 5 min wash in 1× SSPE, 1% SDS. Northern blots were hybridized with 32P-labeled random primed cDNA fragments in the presence of 10% dextran sulphate and 50% formamide for 12–18 h at 42°C, and washed with 0.1× SSPE, 0.1% SDS at 50–65°C (45). To remove the labeled probe, the RNA blots were washed at 98°C with 0.1× SSPE and 0.1% SDS and stored at room temperature until hybridized with the next probe. Autoradiograms were analyzed for signal intensity by laser densitometry. XAR film was exposed to the extent that it was in the linear range of the Helena Laboratories (Beaumont, TX) “Quick Scan R & D” densitometer. The XAR-5 film was linear over a 50-fold range as determined by measuring the optical absorbance of film exposed to known quantities of 32P.

**Southern Blot Analysis.** Genomic DNA was obtained by removing the DNA Band from cellular lysates ultracentrifuged over a 5.7 M cesium chloride cushion (44) followed by dialysis against 10 mM Tris (pH 8), 1 mM EDTA. The genomic DNA was digested with EcoR1, followed by phenol/chloroform (1:1) extraction. The DNA was electrophoresed in a TBE agarose gel and transferred by vacuum blotting in alkaline as described (46). The amount of DNA loaded and transferred per lane was assessed by ethidium bromide staining of the gel, and methylene blue staining of the blot (see Northern blot analysis). Southern blots were hybridized and washed as described for Northern blots.

**Measurement of Transcription Rates.** Nuclear run-off experiments were performed to measure the rate of nascent RNA synthesized in isolated nuclei. The nuclei from each cell line were labeled with [α-32P]UTP as described (23). 3 μg of single stranded M13 DNA containing sense and antisense sequences or 0.5 μg of double stranded insert DNA (vectorless) were used to detect the transcription signal. The incorporation ranged from 0.5 to 1 cpm/nuclei.

The number of cpm hybridized was kept constant within each experiment so that the results are comparable. The blots were washed under stringent conditions (23); including 20-min treatment at 37°C with 10 μg/ml ribonuclease A and 1 μg/ml ribonuclease T1, followed by 65°C wash in 5 mM Tris (pH 7.5), 2 mM EDTA, 10 mM NaCl and 0.4% SDS for 30 min.

**Results**

To investigate whether T cell gene expression is regulated by inhibitory factors, somatic cell hybrids were formed between parental cells which differ in the expression of specific T cell gene products. The authenticity of the somatic cell hybrids was determined by several means. The hybrids contained a near-tetraploid chromosome content, ranging from 77 to 81, with an average of 79–80 for each hybrid clone (24, 25). Since the somatic cell hybrids are an intraspecies and intralineage combination, they are unusually stable; they have maintained their tumorigenic and karyotypic phenotype for several years in culture (24, 25). Southern blot analysis indicates that the unique pattern of TCR-β gene rearrangements characteristic of each T lymphoma parent was also present in the hybrid cells (16).

**Represion of CD4 and CD8 Gene Expression in Somatic Cell Hybrids.** Table 1 provides a summary of the surface phenotype of the SL12 and SAK8 T lymphoma clones used in the present study (also described in the Introduction). Fig. 1 shows that the SAK8 T cell lymphoma clone expressed cell surface CD4 and CD8, while the SL12.3 cell clone lacked detectable CD4 and CD8 expression. Somatic cell hybrids formed from the fusion of SL12.3 and SAK8 cells (SL12.3 × SAK8) did not detectably express cell surface CD8 and expressed only negligible amounts of CD4 (Fig. 1).

The relative amount of CD4 and CD8 mRNA was assessed by Northern analysis to determine whether the repression of cell surface protein resulted from a decrease in the accumulation of the respective mRNAs. Fig. 2 A illustrates that SAK8 cells expressed CD4 and CD8 mRNA, whereas neither SL12.3 cells nor the fusion product, SL12.3 × SAK8, detectably expressed these mRNAs. Thus, the repression of

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1 Abbreviation used in this paper: EGFR, epidermal growth factor receptor.
Flowcytometry was used to determine cellsurface expressionsimilar to that shown in Fig. 1. The (+) symbol denotes that the indicated cellsurface protein was expressed in the cell line listed across the top of the table. ND = not determined. The references provide information on one or more of the cell lines; those marked with an asterisk (*) are reported for the first time in this communication.

CD4 and CD8 cell surface protein in these hybrid cells results from a striking reduction in the steady state levels of CD4 and CD8 mRNA, and not from alterations in posttranslational events such as protein transport, processing, or cell surface modulation. Table 2 summarizes a quantitative densitometric analysis of mRNA expression in parental and hybrid cells. CD4 and CD8 mRNA accumulation in SL12.3 × SAK8 hybrid cells was at least 50-fold less than in SAK8 parental cells. Similarly, the SL12.1 T lymphoma cell clone, which is nearly identical in phenotype with SL12.3 (Table 1), engendered a strong reduction of CD4 and CD8 mRNA expression when fused with SAK8 cells (Fig. 2A and Table 2). The lack of CD4 and CD8 gene expression in either the SL12.1 × SAK8 or the SL12.3 × SAK8 hybrid was not due to the loss of, or to major alterations in the CD4 or CD8 genes. Southern blot analysis of the hybrids indicated that the CD4 and CD8 genes were present and unaltered in size relative to the genes present in the parental cell lines (Fig. 2B). Although this analysis cannot rule out point mutations, small deletions or insertions which could inactivate the protein, such subtle changes are unlikely to explain the large differences in mRNA accumulation observed in this system. This conclusion is further supported by the analysis of several additional independently derived hybrid cell clones (25) which gave completely concordant results (data not shown).

The preceding data support the conclusion that double negative SL12.3 and SL12.1 cells contain transacting factors which repress the accumulation of CD4 and CD8 mRNA. Another double negative T lymphoma cell clone, SL12.4, was analyzed because it has a more mature phenotype than either SL12.1 or SL12.3 (Table 1 and introduction) and thus can be used to determine whether transacting inhibitory factors are a general feature of all double negative cells, regardless of their precise maturation status. Hybrid cells formed from the fusion of SAK8 and SL12.4 cells expressed CD4 and CD8 on the cell surface, albeit at reduced levels relative to SAK8 parental cells (Fig. 1). Likewise, CD4 and CD8 mRNA expression in SL12.4 × SAK8 cells was only slightly less than parental SAK8 cells (data not shown). Thus, unlike immature SL12.1 and SL12.3 cells, SL12.4 cells do not strongly repress CD4 and CD8 gene expression in somatic cell hybrids formed with SAK8 cells.

The transcription rate of the CD4 and CD8 genes was analyzed using the nuclear run-off assay to determine whether the strong repression of CD4 and CD8 mRNA accumulation in SL12.1 × SAK8 and SL12.3 × SAK8 hybrid cells was due to transcriptional or posttranscriptional mechanisms.

Table 1. Cell Surface Phenotype of T Lymphoma Cell Clones

|            | SL12.1 | SL12.3 | SL12.4 | SAK8 | Reference |
|------------|--------|--------|--------|------|-----------|
| CD3        | –      | –      | –      | –    | – (14)    |
| CD4        | –      | –      | –      | +    | (14, 24)  |
| CD8        | –      | –      | –      | +    | (13, 14)  |
| CD44 (Pgp1)| +      | +      | –      | –    | (13, 24)  |
| CD45 (T200)| +      | +      | +      | ND   | (13, 24)  |
| IL-2r-α    | –      | –      | +      | ND   | (15*)     |
| HSA (J11D) | +      | +      | +      | ND   | (15)      |
| Thy1       | +      | +      | +      | +    | (13, 24)  |
| TL         | +      | +      | +      | +    | (14*)     |
| H-2K³      | +      | –      | –      | –    | (13, 14)  |

Figure 1. Transacting factors inhibit cell surface CD4 and CD8 expression. The histograms show the fluorescence profile of the indicated cell lines and somatic cell hybrids assessed by flow cytometry. The solid line represents staining with antibodies against murine CD4 or CD8 as indicated on the figure. The dotted line represents the fluorescence intensity of cells stained with an antibody against Thy-1.2 (AKR mouse cells express the Thy1.2 allele).
Figure 2. Transcriptional repression of CD4 and CD8 expression in somatic cell hybrids. (A) Northern blots of total cellular RNA prepared from the indicated cell lines and hybrids were hybridized sequentially with purified inserts from CD4, CD8, and Chinese hamster (CHO-A) cDNA clones. CHO-A transcripts serve as an internal standard to assess RNA amounts in each lane. (B) Southern blots were somewhat unevenly loaded as assessed by staining with ethidium bromide, and by staining the blot with methylene blue, this accounts for the differential intensity of the hybridization in different lanes. (C) The rate of gene transcription was assessed by the nuclear run-off assay using single stranded M13 probes which recognize sense or anti-sense nascent transcripts as noted.

Table 2. Quantitative Analysis of mRNA Expression in Parental Cells and Somatic Cell Hybrids

| Gene   | SL12.3 X | SL12.4 X | SL12.4 X | SL12.1 X | SAK8 | SAK8 |
|--------|----------|----------|----------|----------|------|------|
| CD4    | <1       | <1       | <1       | <1       | 100  | <1  |
| CD8    | <2       | <2       | <2       | <2       | 100  | <2  |
| CD3-γ  | <1       | 10       | 100      | 31       | 2.7  | 1.1  |
| CD3-δ  | <1       | <1       | 100      | 1.8      | <1   | <1  |
| CD3-ε  | <1       | 6        | 100      | 16       | 10   | 45   |
| CD3-ξ  | 1.1      | 50       | 100      | <1       | <1   | 30   |
| IL2r-α | <2       | <2       | 100      | <2       | <2   | <2  |
| 19.1   | <2       | 17       | 100      | 8        | <1   | 1.2  |
| 19.2   | 4.1      | 3.0      | 100      | 10       | 1.5  | 2.7  |
| 19.4 (Tiα) | 3.9   | 4.3      | 100      | 1.1      | <1   | 3.6  |
| 19.5 (Lov) | <1 | <1       | 100      | <1       | <1   | <1  |
| 20.2 (Pem) | <1 | 32       | 100      | 42       | <1   | 1    |
| 20.5 (Tes) | <0.1 | 6.1      | 100      | 10       | <0.1 | 0.1  |

Relative values were determined by densitometric analysis of Northern blots. The cell line with highest expression was given the arbitrary value of "100". The numbers given in the table were derived by normalizing to the intensity of hybridization of a CHO-A cDNA probe as a control for the amount of RNA loaded in each lane. The data representing each gene is derived from one experiment. However, two or more (up to 10) independent experiments gave completely concordant results. In cases where more than one transcript is present only the most abundant transcript was analyzed.
We have previously shown that this assay provides a linear signal over at least a 10-fold range of transcription rates (23). Fig. 2 C shows that the CD4 gene was preferentially transcribed in the sense orientation in SAK8 cells, as expected, whereas SL12.3 cells transcribed the CD4 gene at a very low rate. The SL12.3 x SAK8 hybrid also transcribed the CD4 gene at a low rate, although higher than in SL12.3 cells (Fig. 2 C). Similarly, SL12.1 x SAK8 hybrid cells exhibited a lower rate of CD4 transcription than SAK8 parental cells. These results were confirmed using 5' and 3' double-stranded CD4 DNA fragment as probes (data not shown). Thus, a depression of the transcription rate accounts, at least in part, for the reduced CD4 mRNA content in the hybrid cells. However, since the transcription rate of the CD4 gene was higher in CD4- hybrid cells than in CD4- SL12.1 or SL12.3 parental cells (Fig. 2 C), it appears that posttranscriptional events also participate in the regulation of CD4 transcript amounts.

In SAK8 cells, the CD8 gene was detectably transcribed in the sense strand, but was not detectable in either SL12.1 x SAK8 or SL12.3 x SAK8 hybrid cells. Although this result was highly reproducible using both single stranded and double stranded probes, the low CD8 transcription rate makes it difficult to conclude that transcriptional repression completely accounts for the reduced CD8 mRNA expression; posttranscriptional events may also participate. However, lower rates of CD4 and CD8 gene transcription account for some of the repressed cell surface CD4 and CD8 expression observed in the hybrid cells.

**TCR/CD3 Genes are Differentially Repressed by Trans-Acting Factor(s).** The individual transcripts encoded by the CD3 gene complex exhibit distinct patterns of expression in cell lines (3-5, 47, 48). We examined whether CD3 transcript levels are regulated by repressional mechanisms, and if so, whether the individual CD3 molecules are regulated differently. To clarify the analysis, three distinct categories of regulation in somatic cell hybrids were defined: (a) the term "trans-negatively" regulation was used when mRNA expression was reduced in hybrid cells by greater than 20-fold, relative to the expressing parental cell line; (b) the term "inhibitory" regulation was used to describe reductions of 3-20-fold in hybrid cells; and (c) the term "codominant" regulation was used when expression was reduced less than three-fold lower than the expressing parental cell line.

Fig. 3 A and Table 2 show that SL12.4 cells expressed abundant CD3-γ, -δ, -ε, and -ζ transcripts, while SL12.3 cells expressed trace or undetectable amounts of these transcripts. CD3-δ gene expression was trans-negatively regulated in SL12.3 x SL12.4 hybrid cells (>100-fold less than parental SL12.4 cells). CD3-γ and -ε mRNA levels were inhibited 10- to 17-fold, respectively, in SL12.3 x SL12.4 hybrid cells compared to parental SL12.4 cells. In contrast, CD3-ζ transcripts were expressed in a codominant manner. SL12.1 x SL12.4 hybrid cells exhibited similar regulation, except that CD3-ζ expression was trans-negatively regulated (Fig. 3 and Table 2). Since the SAK8 clone expressed CD3-γ, -ε, and -ζ mRNAs, we also examined hybrids formed between this parent and either SL12.1 or SL12.3 cells. SL12.3 x SAK8 cells trans-negatively regulated CD3-ε and -ζ mRNA expression (>40-fold reduction in expression relative to parental SAK8 cells), but codominantly regulated CD3-γ mRNA accumulation (Fig. 3...
and Table 2). SL12.1 x SAK8 hybrid cells expressed all of the CD3 transcripts in a codominant manner (Fig. 3 A and Table 2).

The repression of CD3 mRNA expression in hybrid cells could not be explained by a lack of CD3 genes in these cells, as demonstrated by Southern blot analysis (Fig. 3 B). CD3-γ, -δ, -ε, and -ζ gene copy number (relative to a fixed amount of genomic DNA) was the same in the parental and hybrid cell lines. The small variation in the hybridization signal which is apparent between different cell lines (Fig. 3 B) results from differences in the amount of genomic DNA loaded in the lanes (see figure legend). The additional submolar bands observed in some lanes resulted from incomplete restriction digestion, not new restriction sites, as shown by independent analysis in separate experiments. We conclude that transcripts encoding the individual subunits of the CD3 complex are differentially repressed by inhibitory factors present in SL12.1 and SL12.3 cells.

CD3 gene transcription rates were measured to determine the mechanism by which CD3 mRNA accumulation was repressed in somatic cell hybrids. Several single and double stranded probes complementary to different regions of CD3-δ and CD3-ζ transcripts failed to provide a signal sufficiently above background levels to allow assessment of transcriptional regulation. CD3-γ and -ε transcriptional rates were sufficient for analysis. CD3-ε transcription rates were at least 4 fold lower in SL12.1 x SL12.4 and SL12.3 x SL12.4 hybrid cells as compared to SL12.4 parental cells (Fig. 3 C), consistent with the reduced CD3-ε mRNA accumulation found in these hybrid cells (Fig. 3 A and Table 2). Transcriptional repression also accounts for the reduction in CD3-γ mRNA accumulation in somatic cell hybrids. The CD3-γ gene was transcribed at a lower rate (at least threefold lower) in SL12.3 x SL12.4 hybrid cells than in SL12.4 parental cells (Fig. 3 C) consistent with the reduced CD3-γ mRNA levels in this hybrid cell line (Fig. 3 A and Table 2).

Since TCR-β mRNA levels are also repressed in SL12 somatic cell hybrids (16), we sought to determine whether the regulation was exerted at the transcriptional or posttranscriptional level. The SL12.4 cell clone constitutively transcribes both the Cβ1 and Cβ2 loci and accumulates high amounts of 1.0-kb Cβ2 transcripts (23). Somatic cell hybrids formed between β+ SL12.4 and β- SL12.3 cells lack detectable 1.0 kb TCR-β transcripts (16). SL12.4 cells transcribed the Cβ1 and Cβ2 genes at high rates in the sense orientation, whereas SL12.3 cells and SL12.3 x SL12.4 hybrid cells transcribed the TCR-β gene at rates >50-fold lower (Fig. 3 C). Similarly, SL12.1 x SL12.4 hybrid cells exhibited repressed accumulation of TCR-β mRNA (16) and lower transcription rates (Fig. 3 C). We conclude that the accumulation of TCR-β, CD3-γ, and CD3-ε transcripts are inhibited by transacting factors present in SL12.1 and SL12.3 cells which act, at least in part, by reducing the rate of gene transcription.

Trans-Negative Regulation of Interleukin-2 Receptor α Gene Transcription in Somatic Cell Hybrids. The IL-2R-α is transiently expressed early during thymocyte development by a population of cells destined to mature into functional T cells (18, 22). We examined IL-2R-α expression in somatic cell hybrids formed between parental cells that lack detectable expression (SL12.3 and SL12.1) and SL12.4 cells which express high levels of IL-2R-α mRNA (Fig. 4 A) and cell surface protein (15). IL-2R-α gene expression is transnegatively regulated in both SL12.3 x SL12.4 and SL12.1 x SL12.4 hybrid cells (Fig. 4 A and Table 2). Southern blot analysis revealed no detectable difference in IL-2R-α genomic structure or gene copy number in parental or hybrid cells (Fig. 4 B). Therefore, it is likely that repressor molecules present in SL12.1 and SL12.3 cells efficiently blocked the accumulation of IL-2R-α mRNA in hybrid cells formed with SL12.4. The pattern of IL-2R-α and CD3-δ gene expression in this system was highly similar (Table 2). Both IL-2R-α and CD3-δ transcripts were transnegatively regulated in hybrids formed between SL12.4 cells (IL-2R-α+ CD3-δ+) and either SL12.1 or SL12.3 cells (IL-2R-α+ CD3-δ-).

IL-2R-α transcription rates shown in Fig. 4 C demonstrate that SL12.4 cells transcribe the gene, but transcription was repressed below measurable levels in SL12.3 x SL12.4 hybrid cells. In contrast, the Thy1 and actin genes were tran-
scribed in both parental and hybrid cells, and the EGFR gene was not detectably transcribed in any of these cells, as expected (Fig. 4 C). Therefore, the repressed IL2R-α mRNA accumulation in hybrid cells must result from a relatively specific repression of IL2R-α gene transcription.

Regulation of Six Novel Genes in Somatic Cell Hybrids. The preceding analysis shows that transacting factors negatively regulate TCR, CD3, CD4, CD8, and IL2R gene expression among T cell lines which differ in maturation status. The generality of this observation was tested by assessing the regulation of novel transcripts corresponding to cDNAs selected solely on the basis of their differential expression in SL12.4 and SL12.3 cells. These six cDNA clones were isolated by subtraction-enriched differential screening of an SL12.4 cDNA library (28, 29). A brief summary of the characteristics of these six cDNA clones was presented in the introduction.

Five of the six novel gene transcripts were expressed at significantly lower levels in the SL12.3 × SL12.4 somatic cell hybrid compared with the SL12.4 parent (Fig. 5 A, Table 2, and reference 28). Three genes (19.2, Lov, and Tia) were transnegatively regulated (>20-fold repression), while two (19.1 and Tea) were inhibited 5–16-fold. Pem was the only codominately expressed novel gene in SL12.3 × SL12.4 hybrid cells (Table 2). The same general pattern of gene expression was observed in SL12.1 × SL12.4 hybrid cells (Table 2). The Tia gene is also expressed in SAK8 cells and was transnegatively regulated in SL12.1 × SAK8 and SL12.3 × SAK8 hybrid cells (Fig. 5 A and Table 2). Tea mRNA expression was inhibited at least 12-fold in the same hybrid cell lines. Since none of the parental or hybrid cells showed any detectable difference in the genes as assessed by Southern analysis (Fig. 5 B and reference 28), the repression of the genes is likely to result from transacting repressor factors derived from the less mature SL12.1 and SL12.3 cells.

Transcription rates of the novel genes were assessed to examine whether the reduced accumulation of the novel transcripts in hybrid cells was regulated at the transcriptional or posttranscriptional level. Fig. 5 C shows that five of the six novel genes were transcribed at detectable rates in SL12.4 cells (i.e., at rates greater than EGFR, which is not transcribed in T cells). The transcription rate of the Lov gene was too low to detect. The transcription rates of the 19.1, 19.2, Tia, and Tea genes were only moderately repressed (threefold or less) in hybrid cells compared with SL12.4 parental cells (Fig. 5 C). For comparison, TCR-β and CD3-ε gene transcription rates were assessed in the same experiment; the transcription rate of TCR-β gene was dramatically reduced in hybrid cells and CD3-ε gene transcription was moderately repressed in hybrid cells, while actin and Thy1 gene transcription was similar in all cell lines (Fig. 5 C). The data indicate that the depressed accumulation of 19.1, 19.2, Tia, and Tea mRNA in hybrid cells (Table 2) resulted from the combined effects of a slight decrease in transcription and a major posttranscriptional regulatory component. Pem gene transcription was similar in parental and hybrid cells (Fig. 5 C) consistent with the codominant regulation of Pem transcripts (Table 2 and reference 28).

Thus, repressional mechanisms prevent the accumulation of a wide variety of developmentally regulated transcripts in

Figure 5. Several novel T cell genes are regulated by negative transacting factors. Methods and additional information are described in the legend of Fig. 2. (A) The Northern blot was hybridized sequentially with the purified inserts Tia, Tea, and CHO-A cDNA. (B) Southern blot analysis. (C) The transcription rate was assessed using purified double stranded DNA inserts (the novel genes) or single stranded M13 DNA (TCR-β, CD3-ε, actin, and Thy1) which recognizes sense nascent transcripts. The data shown for Pem was derived from the experiment shown in Fig. 4 C. The data shown for 19.1 and 19.2 was from a shorter autoradiographic exposure, as compared with the data shown for the other genes.
immature T lymphoma cells. The cells appear to repress the accumulation of the mRNAs using different strategies, by regulating both transcriptional and posttranscriptional events.

**Discussion**

The data presented in this communication show that negative factors, present in somatic cell hybrids, regulate T cell specific gene expression. Repression was exerted on transcripts encoding cell surface molecules of known function (TCR, CD3, CD4, CD8, and IL-2R), as well as on several novel transcripts. The inhibition was mediated by factors present in immature SL12.1 and SL12.3 T lymphoma clones. Since our analysis involved fusion of cells from the same cell lineage and in many cases, from the same cell line source, it is unlikely that the results can be explained by phenotypic extinction, which occurs when somatic cell hybrids are formed between cells of different lineages. For example, immunoglobulin gene expression in myeloma cells is extinguished when these cells are fused with fibroblasts or T cells (49-51), and Thy1 expression is repressed when Thy-1+ T cells are fused with Thy-1- B cells (52). Phenotypic extinction is important since the molecules involved may participate in maintaining lineage fidelity. In contrast, since we examined hybrids formed between cells of the same lineage, the regulation exerted may be more relevant to maturational control within a lineage. The SL12.1 and SL12.3 cell clones are not generally repressive to the T cell phenotype since they express many "early" T cell gene products such as Thy1, CD44, CD45, heat shock antigen, and Thymic leukemia (Table 1).

The coregulation of CD4 and CD8 gene transcription in somatic cell hybrids may be relevant to the coexpression of these two genes in the majority of adult thymocytes. A common repressive mechanism which operates on both CD4 and CD8 expression may be relieved when double negative thymocytes undergo conversion to the double positive phenotype. Consistent with this interpretation is our observation that CD4 and CD8 transcripts are coordinately induced in SL12.4 cell clones in response to treatment with protein synthesis inhibitors or coculture with thymic epithelial cells (15). Our results from somatic cell hybrids also define two types of CD4-CD8- cells: those with an "immature" phenotype (SL12.1 and SL12.3) which strongly repress CD4 and CD8 gene transcription and cells with an "intermediate maturity" phenotype (SL12.4) that only weakly inhibit CD4 and CD8 gene expression. The apparent coordinate regulation of CD4 and CD8 gene regulation is intriguing, but further work is required to determine whether the same repressor molecule(s) control the expression of these genes. Mechanisms which independently regulate CD4 and CD8 expression must also exist since CD8 is activated before CD4 in at least some immature thymocytes, and most mature thymocytes selectively inactivate either CD4 or CD8 expression before their release from the thymus (6).

Individual members of the CD3 complex are independently regulated in our intralineage somatic cell hybrids. CD3-δ mRNA levels were strongly repressed (>50-fold) by transacting factors present in SL12.1 and SL12.3 cells. In contrast, CD3-γ, -ε, and -ζ mRNAs were inhibited to a lesser extent, and in unique patterns in different hybrid cells. Studies examining thymocytes and leukemia cells provide some evidence for selective expression of particular TCR/CD3 subunits at different stages of maturation: (a) cell clones of human origin which lack cell surface CD3 selectively express CD3-ε mRNA, but not -γ or -δ mRNA, suggesting that these latter transcripts may accumulate somewhat later in maturation (47); (b) CD3-δ transcripts are not evident in mouse fetal thymocytes until day 16 of gestation, when full length TCR-β transcripts are already expressed (53); and (c) neither CD3-δ nor -e mRNAs are detectably expressed by murine Thy1+ CD44+ thymocyte clones obtained from day 14 embryos which accumulate CD3-γ transcripts, and possess TCR-α, -β, and -γ genes in a germ line conformation (48). A common feature of these examples of asynchronous expression of CD3 subunits from human and murine T cells is that the CD3-δ gene is expressed later in ontogeny than the other CD3 genes. This is consistent with our observation that the accumulation of CD3-δ transcripts is more dramatically inhibited by transacting factors than the other CD3 transcripts. The CD3-δ protein is known to prevent the degradation of partial TCR/CD3 complexes in the endoplasmic reticulum by neutralizing the charged amino acids present in the transmembrane spanning domain of the TCR-α subunit (54). Thus, the unique regulation of CD3-δ may play a role in controlling the subcellular fate of TCR/CD3 complexes during ontogeny.

IL-2R-α gene transcription is under strong repressional control in somatic cell hybrids. The regulation of IL-2R-α and CD3-δ gene expression was identical in the cell lines and somatic cell hybrids examined. IL-2R-α and CD3-δ transcripts were not expressed by double negative cell clones (CD4+-CD8-) with an immature phenotype (SL12.1 and SL12.3) but were expressed by an intermediate maturity double negative cell clone (SL12.4). The expression of both transcripts was strongly transnegatively regulated (>50-fold) in hybrids formed between the immature and intermediate maturity cell clones. This pattern of regulation is consistent with the observation that neither of these genes is expressed in the most immature double negative thymocyte subsets, but both are expressed before CD4 and CD8 expression (11, 53). Whether the coordinate regulation of IL-2R-α and CD3-δ is coincidental or not, independent regulatory mechanisms must also exist since cell surface IL-2R is only transiently expressed during early thymocyte maturation, while CD3-δ mRNA remains constitutively expressed throughout the remaining phases of development. An interesting feature of IL-2R gene regulation is its biphasic expression during T cell maturation. IL-2R-α is first expressed early in immature thymocytes, followed by shut-off during later phases of ontogeny, and reexpression in mature antigen-activated T cells. It remains for future investigations to determine whether a common repressive mechanism operates to inhibit IL-2R expression during these distinct IL-2R- phases of maturation.

Our data support the hypothesis that repressional control is a common mode of regulating the expression of T cell transcripts which encode surface molecules of known function.
In addition, the expression of several novel transcripts was inhibited by repressor factors in hybrid cells. All five of the novel transcripts whose accumulation was repressed in somatic cell hybrids are expressed in normal lymphoid tissue. The only gene not repressed in somatic cell hybrids, Pem, is unique among the novel genes in that it is not detectably expressed in any normal adult tissues, including lymphoid organs (30). The Pem gene is expressed in a variety of immortalized and transformed cell lines from several cell lineages. Thus, the expression of Pem in T lymphoma cell clones is more likely to reflect their tumorigenic status than their lineage. Hence, it is not surprising that the Pem gene is regulated in a different manner than genes expressed by normal lymphoid cells.

The variable degree of inhibition exerted on different T cell transcripts in somatic cell hybrids could result from a single inhibitory factor which acts with variable efficiency. However, it seems more likely that multiple inhibitory factors are involved since there are both quantitative and qualitative differences in the expression of several of the transcripts examined. Many of the genes had unique patterns of expression in the panel of somatic cell hybrids. Furthermore, the mechanism by which transcript accumulation was inhibited was primarily transcriptional for some of the mRNAs, while for others, the predominant mode of regulation was posttranscriptional.

A growing body of literature supports the notion that lymphoid genes are regulated by repressor factors. Three main lines of investigation support this view. First, the induction or augmentation of specific lymphoid transcripts after treatment with protein synthesis inhibitors has suggested the important role of labile inhibitor polypeptides. Immunoglobulin \( \kappa \) transcripts are induced by cycloheximide treatment (55), perhaps due to selective removal of the inhibitor I-\( \kappa \) B, which controls the activity of the NF-\( \kappa \) B transcription factor (56). We have shown that the expression of full length TCR-\( \alpha \) and -\( \beta \) transcripts in SL12 clones is dramatically induced by transcriptional and posttranscriptional mechanisms in response to different agents, including protein synthesis inhibitors (16, 23, 26). Second, studies with somatic cell hybrids have indicated that immunoglobulin, class I MHC, TCR, Thy1, and CD44 genes are repressed by transacting factors (16, 49–52, 57–60). Third, molecular studies have identified cis- and trans-acting factors which negatively regulate the expression of several lymphoid genes, including immunoglobulin, TCR, II-2, II-2R-\( \alpha \) and c-myc (49, 50, 56, 61–65). Studies analyzing extinction of immunoglobulin expression in inter-lineage hybrids has revealed that negative regulation is exerted on the \( \kappa \) promoter and heavy chain enhancer (50), perhaps acting on the conserved octamer sequence which controls immunoglobulin gene expression (49). Silencer elements have been identified in the 3' flanking regions of the TCR-\( \alpha \) and -\( \gamma \) genes; these silencers may promote fidelity of TCR expression in \( \alpha \beta \)- and \( \gamma \delta \)-bearing T cells, respectively (64, 65). Putative repressor factors have been characterized which regulate immunoglobulin heavy chain (62) and c-myc (63) gene transcription.

The T lymphoma hybrids used in this study have several unique features useful for identifying and characterizing negative regulators which control the expression of T cell genes. The hybrids were formed from parents of common origin and lineage which could limit the number of repressor molecules under investigation. The large number of T cell transcripts regulated in the somatic cell hybrids permits a comparative analysis of regulation within a single model system. The orchestration of inhibitory factors may be analogous in some respects to positive transcriptional regulation as it is currently understood (66). An emerging model proposes that a limited number of positive transcription factors activates the expression of a wide array of genes by acting in concert in an overlapping manner (66). By analogy, negative regulation could be exerted by a small number of repressor factors which use combinatorial diversity to act on a single gene or on a set of different genes to regulate their expression. The analysis of negative regulation requires biological systems which are suitably accessible to molecular scrutiny.

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