Post-Transcriptional Regulation of Early T Cell Development by T Cell Receptor Signals

Yousuke Takahama and Alfred Singer

During differentiation in the thymus, immature T cells progress through an ordered sequence of developmental stages that are best characterized by variable expression of the co-receptor molecules CD4 and CD8. Crosslinking of T cell receptor (TCR) molecules on precursor thymocytes was found to block their differentiation into $CD4^+CD8^+$ cells by eliminating messenger RNA's encoding two families of developmentally important molecules: the co-receptor molecules CD4 and CD8 and the recombination activating genes 1 and 2. TCR-induced post-transcriptional regulation in early thymocytes was specific for selective messenger RNA's, required protein synthesis, and was itself developmentally regulated. These data identify a post-transcriptional mechanism that is influenced by TCR signals and that regulates early thymocyte development.

Most lymphocytes of the T cell lineage develop in the thymus and express T cell receptors (TCR's) of the αβ isotype. Differentiation of $TCR\alpha\beta^+$ T cells in the thymus proceeds through an ordered sequence of developmental stages that are best identified by the co-receptor molecules CD4 and CD8, both of which function as cell interaction molecules for developing thymocytes as well as mature T cells (Fig. 1) (*1*, *2*). Most of the cells in the thymus are $CD4^+CD8^+$ whose developmental fate is determined by the specificity of their $TCR\alpha\beta$ receptors (*3*). While TCR-specific selection events determine the fate of $CD4^+CD8^+$ thymocytes in the thymus (*3*, *4*), little is known about the role of TCR in thymocyte development prior to the $CD4^+CD8^+$ stage of differentiation.

The first cells to enter the CD4 and CD8 developmental pathway are the immediate precursors of $CD4^+CD8^+$ thymocytes and these precursors have been identified as $CD4^-CD8^{lo}$ cells (*5*–*7*). The $CD4^-CD8^{lo}$ precursor thymocytes spontaneously differentiate into $CD4^+CD8^+$ thymocytes in vitro in short-term single cell suspension culture (*5*). Whether $CD4^-CD8^{lo}$ precursor thymocytes actually express TCR molecules that might regulate their further differentiation has not been fully established (*6*–*8*). However, Hunig found that antibody to TCR inhibited the differentiation of $CD4^-CD8^{lo}$ adult rat thymocytes into $CD4^+CD8^+$ cells (*7*), and we have recently found that many $CD4^-CD8^{lo}$ thymocytes from fetal mice do express small amounts of $TCR\alpha\beta$ that are competent to transduce intracellular signals (*9*).

We have now examined the in vitro differentiation of murine $CD4^-CD8^{lo}$ thymocytes into $CD4^+CD8^+$ cells. We found that $CD4^-CD8^{lo}$ precursor thymocytes are committed to CD4 transcription and to differentiating into $CD4^+CD8^+$ cells, but that their differentiation could be inhibited by TCR crosslinking or by the drug phorbol myristate acetate (PMA), which is a potent activator of protein kinase C (PKC). We found that TCR signals did not block the differentiation of early thymocytes by inhibiting transcription but, rather, by eliminating mRNA's encoding two distinct families of molecules involved in the differentiation of early thymocytes into $CD4^+CD8^+$ cells: (i) the co-receptor molecules CD4 and CD8 that mediate critical cellular interactions in the thymus (*2*), and (ii) the recombination activating genes (RAG) 1 and 2 that are required for rearrangement of TCR gene loci (*10*). TCR induced elimination of these mRNA's required protein synthesis and was itself developmentally regulated. These results identify a post-transcriptional mechanism regulating early thymocyte development that is influenced by TCR signals, that limits further rearrangements of TCR genes, and that would function as a rapid mechanism for blocking the differentiation of early thymocytes expressing autoreactive TCR molecules.

Differentiation of $CD4^-CD8^{lo}$ precursor thymocytes into $CD4^+CD8^+$ cells. $CD4^-CD8^{lo}$ cells first appear in the murine fetal thymus on day 16 of gestation and are the immediate precursors of $CD4^+CD8^+$ thymocytes (*1*). Such $CD4^-CD8^{lo}$ precursor cells and their $CD4^+CD8^+$ progeny constitute all the $CD8^+$ cells that are present in the fetal thymus because mature TCR^{hi} $CD4^-CD8^+$ thymocytes do not appear until after birth (*1*). Therefore, fetal thymocyte populations are an excellent source of $CD4^-CD8^{lo}$ precursor cells that are not contaminated with mature cells. As a means of further increasing the frequency of precursor cells, we depleted fetal thymocyte populations of cells that had already become $CD4^+CD8^+$ in vivo by treatment with a monoclonal antibody (MAb) to CD4 (anti-CD4) plus complement (C). Placement of $CD4^-$ fetal thymocytes in short-term single-cell suspension culture resulted in the in vitro generation of $CD4^+CD8^+$ cells (Fig. 2A). Because $CD4^-$ fetal thymocyte populations actually contained two immature thymocyte subpopulations, $CD4^-CD8^-$ (70 percent) and $CD4^-CD8^{lo}$ (30 percent), either or both of these populations might have differentiated into $CD4^+CD8^+$ cells in vitro (Fig. 2A). However, physical separation by electronic cell sorting of $CD4^-$ fetal thymocytes into purified subpopulations of $CD4^-CD8^-$ cells and $CD4^-CD8^{lo}$ revealed that only the $CD4^-CD8^{lo}$ thymocyte subpopulation became $CD4^+CD8^+$ during short-term culture; $CD4^-CD8^-$ fetal thymocytes did not undergo significant phenotypic change (Fig. 2A). During their differentiation into $CD4^+CD8^+$ thymocytes, $CD4^-CD8^{lo}$ precursor cells: (i) proliferated, in that the number of $CD4^+CD8^+$ thymocytes recovered after overnight culture was 1.5 times greater than the initial number of input $CD4^-CD8^{lo}$ cells (Table 1); (ii) increased their expression of CD8, as their $CD4^+CD8^+$ progeny expressed five times more CD8 than they did (Fig. 2A); and (iii) marginally increased their low expression of TCR (Fig. 2B). Thus, $CD4^-CD8^{lo}$ fetal

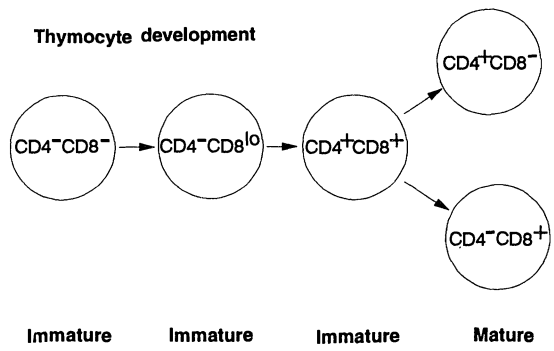


Fig. 1. Simplified scheme of intrathymic differentiation. Characterization of thymocytes as immature or mature refers to whether they have attained functional competence.

The authors are with the Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

thymocytes are the immediate precursors of $CD4^+CD8^+$ thymocytes, and essentially all $CD4^-CD8^{lo}$ cells resident in the fetal thymus are precursor cells with the capability of differentiating into $CD4^+CD8^+$ thymocytes.

To determine the molecular events involved in the differentiation of $CD4^-CD8^{lo}$ precursor thymocytes into $CD4^+CD8^+$ cells, we examined mRNA's encoding a number of molecules important in immune cell development (Table 2), focusing initially on CD4 (Fig. 3). Among unfractionated day-19 fetal thymocytes were $CD4^+CD8^+$ cells containing CD4 mRNA, most of which were removed by treatment with anti-CD4 + C (Fig. 3A, lanes 1 and 2). The amounts of CD4 mRNA within the resulting $CD4^-$ thymocyte population increased significantly in suspension culture (Fig. 3A, lane 3), and this increase was blocked by the transcription inhibitor actinomycin D (Fig. 3A, lane 4). Direct measurements of CD4 transcription demonstrated that its rate was significantly increased in suspension culture (Fig. 3B), during which $CD4^-CD8^{lo}$ fetal thymocytes differentiated into $CD4^+CD8^+$ cells. As a control, we also examined transcription of the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same samples, and we found that its rate was not increased (Fig. 3B). That these molecular changes occurred in $CD4^-CD8^{lo}$ cells within the $CD4^-$ fetal thymocyte population, and not in $CD4^-CD8^-$ cells, was confirmed by the failure to detect either CD4 or CD8 mRNA in cultured $CD4^-$ fetal thymocytes that had been further depleted of $CD8^+$ cells (Fig. 3C). These results with fetal thymocytes contrast with those reported in adult thymocytes in which some $CD4^-CD8^-$ cells expressed very small amounts of CD4 and CD8 mRNA transcripts and were able to become $CD4^+CD8^+$ in vitro (*11*).

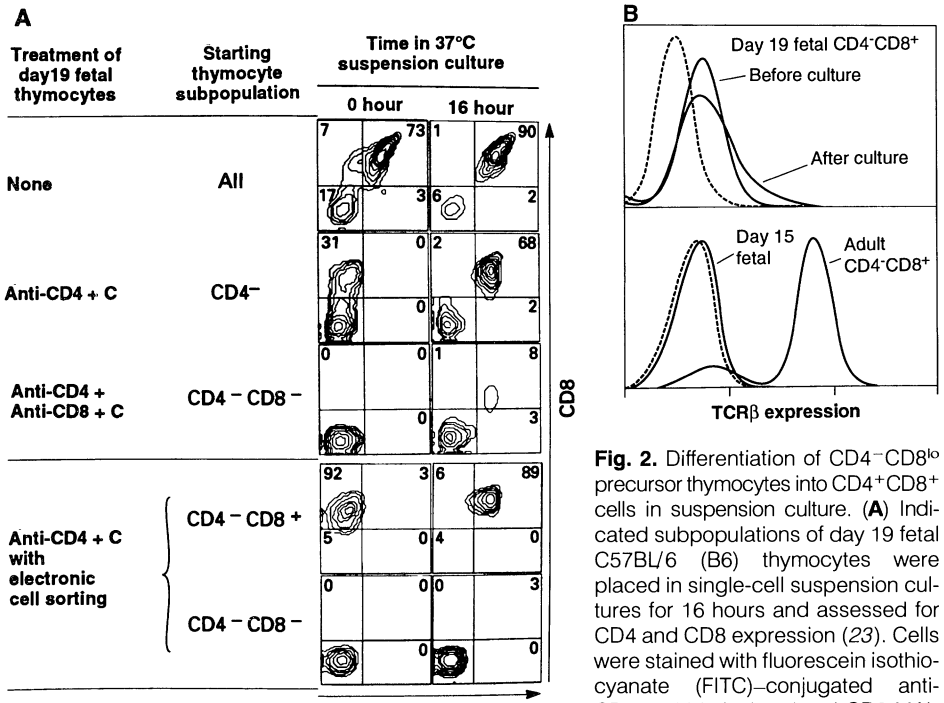


Fig. 2. Differentiation of $CD4^-CD8^{lo}$ precursor thymocytes into $CD4^+CD8^+$ cells in suspension culture. (**A**) Indicated subpopulations of day 19 fetal C57BL/6 (B6) thymocytes were placed in single-cell suspension cultures for 16 hours and assessed for CD4 and CD8 expression (*23*). Cells were stained with fluorescein isothiocyanate (FITC)–conjugated anti-CD4 and biotinylated anti-CD8 MAb and then by Texas red–linked streptavidin (TRA). Expression of CD4 and CD8 is displayed as dual parameter contour plots. Numbers within each box of contour diagrams indicate the frequency of cells within that box. The two anti-CD4 MAb's used for staining and for cytolytic depletion bound to different epitopes on CD4 and did not interfere with each other's binding. (**B**) TCR expression on $CD4^-CD8^{lo}$ thymocytes before and after 16-hour suspension cultures. Indicated subpopulations of thymocytes were stained either with FITC-conjugated anti-TCRβ (MAb H57-597) (*24*) (solid lines) or with FITC-conjugated control MAb Leu4 (dashed lines). Day-19 fetal $CD4^-CD8^+$ thymocytes expressed low but significant amounts of surface TCR both before and after culture (upper panel), as indicated after comparison of their staining with that of TCR^- day-15 fetal thymocytes and with TCR^{hi} $CD4^-CD8^+$ adult thymocytes (lower).

Table 1. Recovery of $CD4^-$ fetal thymocytes from short-term suspension culture, and the effects of TCR crosslinking and PMA. Equal numbers of electronically sorted $CD4^-CD8^+$ thymocytes (experiment 1) or $CD4^-$ thymocytes treated with anti-CD4 + C (experiment 2) from day-19 fetal B6 mice were cultured in medium, plate-bound MAb to TCRβ (×TCR), or PMA at 100 ng/ml as indicated (*23*). Numbers of viable cells recovered from each culture were measured by trypan blue dye exclusion method. Cells were also stained with MAb to CD8 to determine the frequency of $CD8^+$ cells.

Experiment	Starting population of fetal thymocytes	Time in culture (hours)	Reagent in culture	Viable cells (*N*)	$CD8^+$ cells (%)	Relative recovery of $CD8^+$ cells* (%)
1	$CD4^-CD8^+$			7.2×10^5	99	100
		12	Medium	10.7×10^5	99	149
		12	×TCR	10.0×10^5	98	138
		12	PMA	6.3×10^5	92	82
2	$CD4^-$			5.0×10^6	24	100
		6	Medium	4.7×10^6	28	110
		6	×TCR	5.0×10^6	27	113
		6	PMA	5.5×10^6	22	101

*Number of recovered $CD8^+$ cells are expressed relative to the number of input $CD8^+$ cells before culture, and were calculated by multiplying the number of recovered cells times the frequency of $CD8^+$ cells in the recovered population.

Effect of TCR crosslinking on CD4 and CD8 mRNA's. To determine whether TCR engagement influenced the in vitro differentiation of $CD4^-CD8^{lo}$ precursor thymocytes, immobilized anti-TCR MAb was included in the induction cultures. TCR crosslinking did not affect cell recovery (Table 1) but did diminish appearance of CD4 protein and accumulation of CD4 and CD8 mRNA's in most cells (Fig. 4, A and B). These inhibitory effects resulted specifically from TCR crosslinking because crosslinking of other cell surface determinants expressed on precursor thymocytes had no effect (Fig. 4A). Since TCR signals may be mediated in part by PKC, we also assessed the effect of PMA on the accumulation of CD4 and CD8 mRNA's. PMA was even more potent than TCR in blocking the accumulation of CD4 and CD8 transcripts (Fig. 4B), presumably because PMA can act on $CD4^-CD8^{lo}$ precursor thymocytes regardless of the amount of TCR they express. We then compared CD4 transcription in the absence and presence of TCR crosslinking (Fig. 5, A and B). In the absence of TCR crosslinking, CD4 transcription increased in $CD4^-CD8^{lo}$ thymocytes upon placement in single-cell suspension culture. However, CD4 transcription was not diminished by either TCR crosslinking or PMA (Fig. 5, A and B), even though both prevented the accumulation of CD4 mRNA.

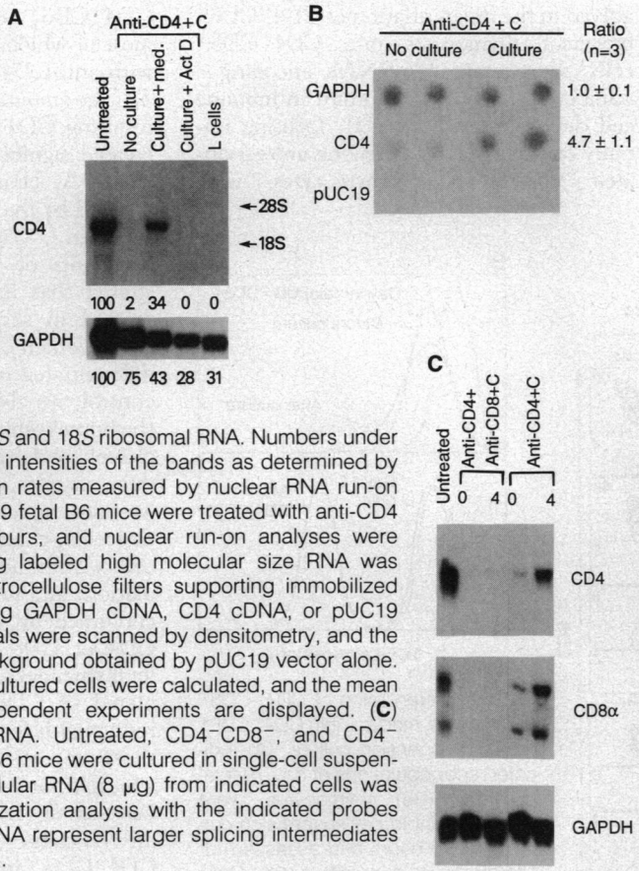


Fig. 3. Amounts of CD4 mRNA and CD4 transcription rates in cultured CD4$^-$ precursor thymocytes. (**A**) Northern blot analysis of steady-state mRNA. Thymocytes from day-19 fetal B6 mice were treated with anti-CD4 and C, and cultured in suspension for 6 hours in the absence or presence of actinomycin D at 1 μg/ml. Total cellular RNA (7 μg) from indicated cells was subjected to Northern analysis with CD4 or GAPDH cDNA probes (*25*). Arrows indicate migration points of 28*S* and 18*S* ribosomal RNA. Numbers under each lane indicate the relative intensities of the bands as determined by densitometry. (**B**) Transcription rates measured by nuclear RNA run-on assay. Thymocytes from day-19 fetal B6 mice were treated with anti-CD4 and C and cultured for 3 hours, and nuclear run-on analyses were performed (*26*). The resulting labeled high molecular size RNA was purified and hybridized to nitrocellulose filters supporting immobilized denatured plasmids containing GAPDH cDNA, CD4 cDNA, or pUC19 plasmid alone. Individual signals were scanned by densitometry, and the results were corrected for background obtained by pUC19 vector alone. Signal ratios of cultured to uncultured cells were calculated, and the mean ratio ± SE from three independent experiments are displayed. (**C**) Northern blot analysis of mRNA. Untreated, CD4$^-$CD8$^-$, and CD4$^-$ thymocytes from day-19 fetal B6 mice were cultured in single-cell suspension for 4 hours. The total cellular RNA (8 μg) from indicated cells was subjected to Northern hybridization analysis with the indicated probes (*25*). Two bands of CD8α mRNA represent larger splicing intermediates and smaller mature forms (*27*).

Since TCR- and PMA-induced signals did not inhibit CD4 transcription, we examined the possibility that they destabilized CD4 mRNA. Direct measurements of CD4 mRNA degradation rates in precursor thymocytes require that the synthesis of new CD4 transcripts be blocked during the assay. However, in the presence of actinomycin D, PMA did not affect the turnover rate of CD4 mRNA (*12*), possibly because the degradation pathway stimulated by TCR and PMA signals involved newly synthesized proteins and was itself dependent on new transcription. Indeed, protein synthesis inhibitors (anisomycin, puromycin, and cycloheximide) blocked the inhibitory effects of both TCR and PMA stimulation on CD4 mRNA accumulation in CD4$^-$CD8^{lo} precursor cells (Fig. 6, A and B). In the presence of protein synthesis inhibitors, CD4 mRNA increased in precursor thymocytes whether or not TCR complexes were crosslinked or PMA was present (Fig. 6, A and B, and Table 2). These inhibitors did not increase CD4 transcription, in that they did not themselves increase CD4 mRNA compared to controls (Fig. 6A, lanes 3, 5, 7, and Table 2). Thus, TCR and PMA signals control the appearance of CD4 transcripts, by inducing a post-transcriptional regulatory mechanism that is dependent on new protein synthesis.

Since the differentiation of CD4$^-$CD8^{lo} precursor cells into CD4$^+$CD8$^+$ thymocytes resulted in increased CD8 expression as well as increased CD4 expression, we examined whether TCR- and PMA-induced signals similarly blocked increases in CD8 transcripts occurring in cultured CD4$^-$CD8^{lo} precursor cells. Not only did PMA block accumulation of CD8α and CD8β mRNA's, but it decreased CD8α and CD8β mRNA's to below the amounts present at the initiation of culture (Fig. 6C, lanes 1 to 3). That PMA was inducing elimination of CD8α and CD8β mRNA's, as it did CD4 mRNA, was indicated by the ability of cycloheximide to reverse the PMA effect and restore CD8α, CD8β, and CD4 mRNA amounts (Fig. 6C, lanes 4 and 5). The effects of TCR crosslinking on CD8α and CD8β mRNA's were similar to those of PMA, but quantitatively less dramatic (Table 2).

Since CD4 and CD8 mRNA's are expressed in mature T cells as well as in immature thymocytes, we assessed whether PMA also reduced CD4 and CD8 mRNA's in mature T cells or whether it only did so in immature thymocytes. We compared spleen T cells and thymocytes from the same adult animals. Even though PMA stimulation markedly reduced the amounts of CD4 and CD8α mRNA among thymocytes, it did not affect those in mature spleen T cells (Fig. 7), an indication that the post-transcriptional regulatory mechanism activated by TCR crosslinking and PMA in immature thymocytes was itself developmentally regulated.

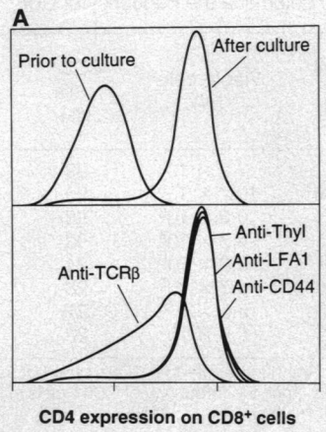
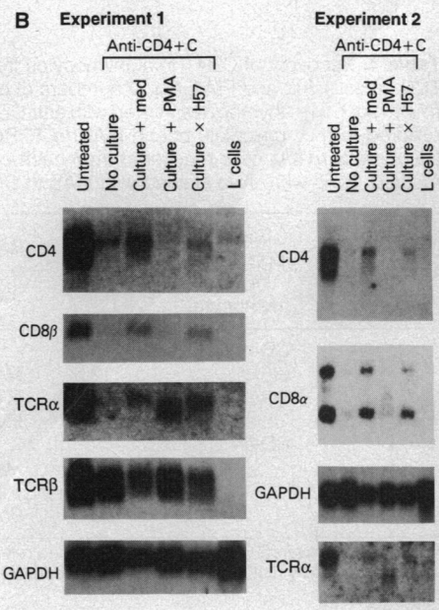


Fig. 4. Inhibitory effects of TCR-crosslinking or PMA on precursor thymocytes. (**A**) CD4$^-$ thymocytes from day-19 fetal B6 mice were cultured for 16 hours in the absence (upper) or presence (lower) of various platebound MAb (*24, 28*). After culture, the cells were stained with FITC-conjugated anti-CD4 and biotinylated anti-CD8 MAb and then by TRA and were assessed for CD4 and CD8 expression by two-color flow cytometry (*23*). CD4 expression on CD8$^+$ cells is displayed as single color histograms. (**B**) Effects of TCR-crosslinking or PMA on steady-state mRNA's in cultured CD4$^-$ fetal thymocytes. CD4$^-$ thymocytes from day 19 fetal B6 mice were cultured for 4 hours in the absence or presence of either plate-bound anti-TCRβ (MAb H57-597) or PMA at 100 ng/ml. Total cellular RNA (5 μg) from the indicated cells were analyzed by Northern blot hybridization with the indicated probes (*25*). Data from two independent experiments are displayed.



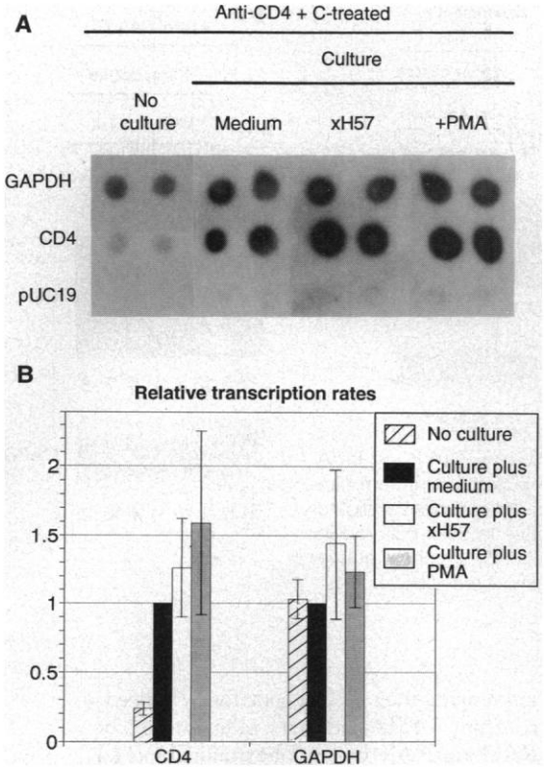


Fig. 5. Effects of TCR-crosslinking or PMA on CD4 transcription rates in $CD4^-CD8^{lo}$ precursor thymocytes. $CD4^-$ thymocytes from day-19 B6 fetal mice were cultured for 3 hours in the absence or presence of either plate-bound anti-TCRβ (MAb H57-597) or PMA at 100 ng/ml, and nuclear run-on analyses were performed (*26*). (**A**) The resulting labeled large size RNA was purified and hybridized to nitrocellulose filters supporting immobilized denatured plasmids containing GAPDH cDNA, CD4 cDNA, or pUC19 plasmid alone. (**B**) Individual signals were scanned by a densitometer with results corrected for background obtained by pUC19 vector alone. Signal ratios of cultured to uncultured cells were calculated, and the mean ratio ± SE from three to six independent experiments are displayed. Values are normalized to those of cells cultured for 3 hours in medium alone.

Effect of TCR signals on mRNA's encoding RAG, TCR, and tyrosine kinases. Having observed post-transcriptional regulation of CD4 and CD8 mRNA's in early thymocytes, we then examined the susceptibility of other immunologically relevant molecules to this same control mechanism (Table 2). We examined mRNA's encoding: (i) RAG because these molecules are necessary for productive rearrangement and expression of TCRαβ receptors, (ii) TCR α and β, and (iii) tyrosine kinases Fyn and Lck because these molecules influence thymocyte development.

1) The RAG-1 and RAG-2 mRNA's are the transcription products of genes that are required for productive rearrangements of the TCRα and TCRβ loci (*10*). Both RAG-1 and RAG-2 mRNA's were post-transcriptionally regulated in these early thymocytes in a manner indistinguishable from that regulating CD4 and CD8 mRNA's (Table 2 and Fig. 6C). RAG-1 and RAG-2 mRNA's were initially low in $CD4^-$ fetal thymocytes and both increased rapidly during culture. TCR crosslinking or PMA stimulation blocked accumulation of RAG-1 and RAG-2 mRNA's, and protein synthesis inhibitors reversed the inhibitory effect of TCR and PMA signals on accumulation of RAG-1 and RAG-2 mRNA's during culture (Table 2 and Fig. 6C). Thus, the accumulation of RAG-1 and RAG-2 mRNA transcripts in early thymocytes is post-transcriptionally regulated by TCR or PMA signals. Consequently, TCR signals would eliminate RAG transcripts and prevent further TCR gene rearrangements in early thymocytes.

2) Surface expression of TCRαβ receptors requires rearrangement and expression of both TCRα and TCRβ gene loci (*1, 13*). Since TCRβ is expressed earlier in development than TCRα (*14*), TCRα expression is the limiting factor regulating TCRαβ expression on developing thymocytes. Expression of surface TCRαβ complexes is low on $CD4^-CD8^{lo}$ fetal thymocytes, with the amount of TCRαβ expression increasing during differentiation into $CD4^+CD8^+$ cells (Fig. 2B). We found that TCRα mRNA was low in $CD4^-$ fetal thymocytes at the initiation of culture (Fig. 4B, lane 2) and rapidly increased during culture (Fig. 4B, lane 3), resembling the changes occurring in CD4, CD8, RAG-1, and RAG-2 mRNA's (Table 2 and Fig. 4B). Despite this similarity, TCRα mRNA expression was not affected by TCR engagement. TCR crosslinking had no effect on accumulation of TCRα mRNA in cultured $CD4^-$ fetal thymocytes, and TCRα mRNA accumulated to the same extent regardless of whether TCR molecules were engaged (Fig. 4B; Table 2). Thus, even though TCRα mRNA's increased during differentiation into $CD4^+CD8^+$ cells with kinetics similar to those of CD4, CD8α, CD8β, RAG-1 and RAG-2 mRNA's, TCR engagement did not affect TCRα mRNA, demonstrating that the post-transcriptional regulatory mechanism activated by TCR engagement is selective for specific mRNA's.

PMA stimulation of $CD4^-$ fetal thymocytes also failed to quantitatively interfere with accumulation of TCRα mRNA during culture (Fig. 4B and Table 2); however,

Table 2. Effects of TCR crosslinking, PMA, and cycloheximide on mRNA's encoding T cell associated molecules in $CD4^-$ fetal thymocytes. $CD4^-$ thymocytes from day-19 B6 fetal mice were cultured for 4 to 6 hours in medium or in the presence of plate-bound MAb to TCRβ (×TCR) or PMA at 100 ng/ml (+PMA). Where indicated, cultures contained cycloheximide at 10 μg/ml. Equal amounts of total cellular RNA from harvested cells were subjected to Northern blot analysis with the indicated probes (*25*). Numbers (in arbitrary densitometric units) indicate relative band densities as measured by a densitometer, and represent mean values of up to three independent experiments for each point. Densitometer values for each probe were normalized to those of cells cultured in medium alone which were arbitrarily set equal to 10.

mRNA	mRNA of $CD4^-$ fetal thymocytes*						
	Before culture	Suspension culture					
		No cycloheximide			Cycloheximide		
		Medium	×TCR	+PMA	Medium	×TCR	+PMA
CD4	1	10	4*	0*	7	8	8
CD8α	3	10	5*	1*	5	6	6
CD8β	4	10	5*	1*	5	5	5
RAG-1	1	10	3*	0*	8	9	9
RAG-2	0	10	5*	0*	11	9	10
TCRα	1	10†	11†	11‡	7†		8†
TCRβ	11	10	11	14	14		14
Fyn	11	10	12	17			
Lck	15	10	11	12			
GAPDH	11	10	10	11	10	11	10

*Significantly decreased relative to cells cultured in medium ($P < 0.05$; two-tailed Student's t-test). The mRNA values for cells cultured in the absence of cycloheximide (columns 3 and 4) were compared to cells cultured in medium alone (column 2); mRNA values of cells cultured in the presence of cycloheximide (columns 6 and 7) were compared with cells cultured in medium plus cycloheximide (column 5). †Size of transcripts hybridized with TCRα probe corresponded to mature TCRα mRNA (1.6 to 1.8 kb). ‡Size of TCRα transcripts in cells cultured with PMA were smaller (1.2 to 1.4 kb), corresponding to sterile transcripts.

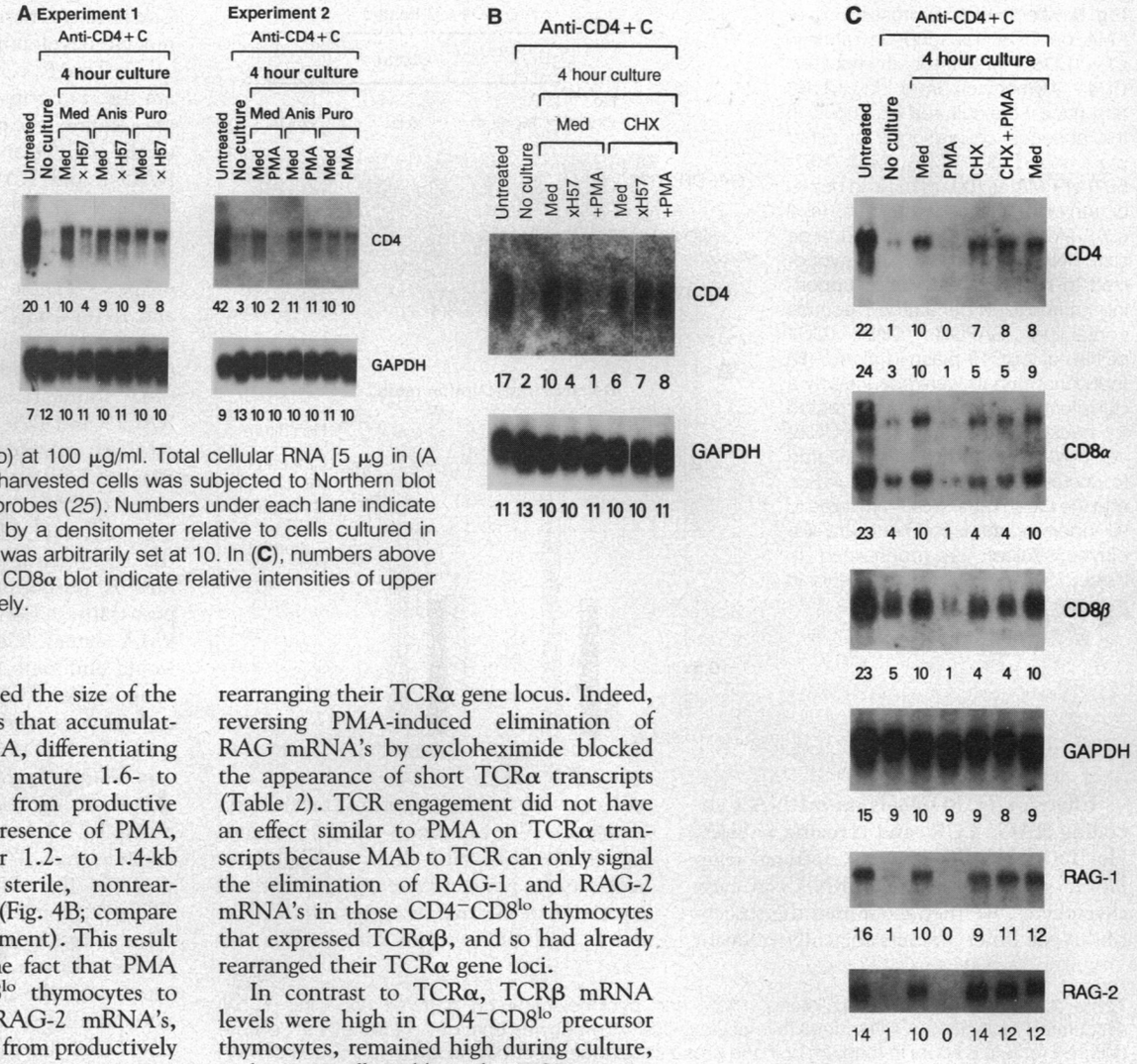


Fig. 6. Effects of protein synthesis inhibitors on steady state mRNA's in CD4$^-$ fetal thymocytes. Untreated or anti-CD4 + C–treated thymocytes from day-19 fetal B6 mice were cultured in suspension for 4 hours in the absence or presence of platebound MAb to TCRβ (H57-597) or PMA at 100 ng/ml. Where indicated, cultures also contained the protein synthesis inhibitors: cycloheximide (CHX) at 10 μg/ml; anisomycin (Anis) at 100 μg/ml; and puromycin (Puro) at 100 μg/ml. Total cellular RNA [5 μg in (A and B), 10 μg in (C)] from harvested cells was subjected to Northern blot analysis with the indicated probes (*25*). Numbers under each lane indicate band density as measured by a densitometer relative to cells cultured in medium alone whose value was arbitrarily set at 10. In (**C**), numbers above and under each lane of the CD8α blot indicate relative intensities of upper and lower bands, respectively.

PMA unexpectedly affected the size of the TCRα mRNA transcripts that accumulated. In the absence of PMA, differentiating thymocytes accumulated mature 1.6- to 1.8-kb TCRα transcripts from productive rearrangements; in the presence of PMA, they accumulated smaller 1.2- to 1.4-kb transcripts representing sterile, nonrearranged TCRα transcripts (Fig. 4B; compare lanes 3 to 5 in each experiment). This result could be explained by the fact that PMA stimulates all CD4$^-$CD8^{lo} thymocytes to eliminate RAG-1 and RAG-2 mRNA's, preventing TCRαβ$^-$ cells from productively rearranging their TCRα gene locus. Indeed, reversing PMA-induced elimination of RAG mRNA's by cycloheximide blocked the appearance of short TCRα transcripts (Table 2). TCR engagement did not have an effect similar to PMA on TCRα transcripts because MAb to TCR can only signal the elimination of RAG-1 and RAG-2 mRNA's in those CD4$^-$CD8^{lo} thymocytes that expressed TCRαβ, and so had already rearranged their TCRα gene loci.

In contrast to TCRα, TCRβ mRNA levels were high in CD4$^-$CD8^{lo} precursor thymocytes, remained high during culture, and were unaffected by either TCR engagement or PMA (Fig. 4B and Table 2).

3) The tyrosine kinases Fyn and Lck are associated with CD4, CD8, and TCR molecules in thymocytes and mature T cells. Both tyrosine kinases affect early thymocyte development, with aberrant development resulting from either their over- or underexpression (*15*). However, Fyn and Lck mRNA's were present in CD4$^-$CD8^{lo} precursor thymocytes prior to culture, did not change during culture, and were not diminished by either TCR engagement or PMA (Table 2).

Implications for early thymocyte development of a TCR-induced post-transcriptional regulatory mechanism. CD4$^-$CD8^{lo} fetal thymocytes are cells at the first step of the CD4-CD8 developmental pathway and may be the earliest cells to express surface TCRαβ molecules. The TCR-induced post-transcriptional regulatory mechanism identified in our study represents an immediate molecular mechanism for preventing early autoreactive thymocytes from differentiating any further along the CD4-CD8 developmental pathway. During development, TCR signals would be generated in early thymocytes that express autoreactive TCR with high affinity for intrathymic self-antigens. The resultant elimination of CD4 and CD8 mRNA's would rapidly inhibit the ability of autoreactive cells to express CD4 and CD8 co-receptor molecules, preventing their further differentiating along the CD4-CD8 developmental pathway and avoiding the contributions made by CD4 and CD8 molecules to the avidity with which TCR can engage antigen. Concurrently, the resultant elimination of RAG-1 and RAG-2 mRNA's would eradicate the cell's potential for further TCR gene rearrangements, fixing the specificity of the TCR molecules these cells express.

The ultimate developmental fate of CD4$^-$CD8^{lo} precursor thymocytes that have been stimulated by TCR signals is not yet certain. The data do not support the possibility that TCR stimulation of CD4$^-$CD8^{lo} precursor thymocytes induces them to undergo programmed cell death: TCR stimulation of cultured precursor thymocytes does not diminish viable cell re-

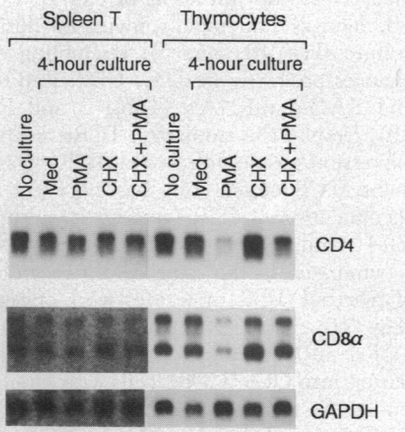


Fig. 7. The ability of PMA-stimulated signals to eliminate CD4 and CD8 mRNA is developmentally regulated. Untreated thymocytes or nylon-enriched spleen T cells from adult B6 mice were cultured in suspension for 4 hours in the absence or presence of PMA at 100 ng/ml. Where indicated, cultures also contained cycloheximide (CHX) at 10 μg/ml. Total cellular RNA (5 μg) was subject to Northern blot analysis with the indicated probes (*25*).

covery; TCR stimulation of $CD4^-CD8^{lo}$ precursor thymocytes does not induce rapid DNA fragmentation, an indicator of apoptosis (*12*); the selectivity of the mRNA's that are eliminated is inconsistent with apoptosis; and PMA, which has similar effects to those of TCR engagement in early thymocytes, protects immature thymocytes from the induction of apoptosis rather than inducing these cells to undergo apoptosis (*16*). It seems more likely that TCR stimulation of $CD4^-CD8^{lo}$ precursor thymocytes results in their becoming $CD4^-CD8^-TCR\alpha\beta^+$ thymocytes because TCR signals eliminate CD4 and CD8 mRNA's, but not TCRα or TCRβ mRNA's. In fact, a $CD4^-CD8^-$ T lymphoma cell has been described that transcribes both CD4 and CD8 mRNA's but maintains its $CD4^-CD8^-$ phenotype by actively degrading newly synthesized CD4 and CD8 transcripts (*17*). Consistent with our view, $CD4^-CD8^-TCR\alpha\beta^+$ thymocytes are increased in transgenic mice expressing autoreactive TCRαβ molecules (*3*). Moreover, we have found in such TCRαβ transgenic mice that in vivo antigen engagement prevents $CD4^-CD8^{lo}$ fetal thymocytes from differentiating into $CD4^+CD8^+$ cells (*9*).

Our study demonstrates that, in precursor thymocytes, TCR or PMA terminates RAG expression by post-transcriptionally eliminating RAG mRNA's, and therefore it may provide a molecular mechanism for the original observations of Turka *et al.* (*18*) that TCR- or PMA-induced signals terminated RAG expression in human $CD4^+CD8^+$ thymocytes. Our current observation that RAG-1 and RAG-2 mRNA's are initially low in precursor thymocytes is surprising given their initially high level of TCRβ mRNA expression. One possibility is that RAG-1 and RAG-2 mRNA's may be present in high amounts early in development, declining subsequent to TCRβ expression, and may increase again during differentiation of precursor thymocytes into $CD4^+CD8^+$ cells during which TCRα gene rearrangements occur. We think that the appearance of small, sterile TCRα transcripts induced in early thymocytes by PMA stimulation is actually a secondary consequence of rapidly terminating RAG expression, preventing productive TCRα gene rearrangements in cells that have not yet rearranged their TCRα gene loci. Whether PMA also activates a cryptic TCRα promoter that stimulates transcription of unrearranged TCRα genes in early thymocytes requires further examination. It has recently been observed that RAG expression is also terminated during positive selection of $CD4^+CD8^+$ thymocytes into mature T cells (*19*). At present, positive selection of $CD4^+CD8^+$ thymocytes is thought to result in the selective shutoff of either CD4 or CD8 transcription. However, our data suggest that post-transcriptional elimination of both CD4 and CD8 mRNA's, as well as RAG mRNA's, might be the initial consequence of intrathymic positive selection signals.

The actual mechanism by which selective mRNA's are eliminated in early thymocytes has not been determined, but presumably alters the processing, transport, or stability of target mRNA's. Because PMA has similar effects to those of TCR in early thymocytes, we think that PKC may be a necessary signal regulating the activity of this post-transcriptional mechanism. Our current perspective is that signals activating PKC induce the synthesis in immature thymocytes of sequence-specific protein or proteins that degrade specific mRNA's. The induced trans-acting proteins may be related to adenosine-uridine–binding factor that has been implicated in mature lymphocytes in destabilizing mRNA's encoding proteins controlling cell growth such as lymphokines and proto-oncogenes (*20, 21*). However, the sequence-specific proteins induced by TCR signals in precursor thymocytes might occur only in early thymocytes as TCR signals in mature lymphocytes destabilize TCRα and TCRβ mRNA's (*22*), whereas those in precursor thymocytes did not. Alternatively, it might be argued that TCR or PMA signals in precursor thymocytes do not activate a post-transcriptional regulatory mechanism at all, but rather inactivate a preexisting post-transcriptional regulatory mechanism that is constitutively active in precursor thymocytes. In this alternative view, certain mRNA's are unstable in early thymocytes and become increasingly stable during differentiation into $CD4^+CD8^+$ cells; PKC activation would prevent mRNA stabilization by blocking differentiation. However, this perspective predicts: (i) that increases in CD4 mRNA during differentiation of precursor thymocytes in $CD4^+CD8^+$ cells would result from increased CD4 mRNA stability, not increased transcription, whereas we observed increased transcription; and (ii) that PKC activation would not affect the stability of mRNA transcripts in thymocytes that have already become $CD4^+CD8^+$, whereas we observed that PMA reduced both CD4 and CD8 mRNA's in unfractionated adult thymocyte populations that consisted predominantly of $CD4^+CD8^+$ cells. Thus, taken together, our results indicate that TCR and PMA actively induce a post-transcriptional regulatory mechanism that is functional in early thymocytes and that regulates their further differentiation.

In conclusion, post-transcriptional regulation has not previously been appreciated in immune cell development having only been observed in mature lymphocytes upon activation (*22*). Nevertheless, we think that post-transcriptional regulatory mechanisms may be found to influence other important aspects of thymocyte development and selection as well.

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23. For isolation of $CD4^-$ thymocytes, single-cell suspensions of fetal thymocytes were treated with RL172, an MAb to CD4 from R. Ceredig, J. W. Lowenthal, M. Nabholz, and H. R. MacDonald [*Nature* **314**, 98 (1985)], plus C. For isolation of $CD4^-CD8^-$ thymocytes, $CD4^-$ fetal thymocytes were also treated with 3.155, an MAb to CD8 from M. Sarmiento, A. L. Glasebrook, and F. W. Fitch [*J. Immunol.* **125**, 2665 (1980)], plus C. For isolation of purified populations of $CD4^-CD8^+$ and $CD4^-CD8^-$ fetal thymocytes, $CD4^-$ thymocytes were stained with MAb to CD8 and sorted into $CD8^+$ ($CD4^-CD8^+$) and $CD8^-$ ($CD4^-CD8^-$) cells (Fluorescence Activated Cell Sorter, Becton-Dickinson Immunocytometry Systems; BDIS). Cells were cultured in Costar 24 well plates at 37°C in RPMI-1640 medium supplemented with 10 percent fetal bovine serum, 50 μM 2-mercaptoethanol, 10 mM Hepes, 2 mM L-glutamine, 1× nonessential amino acids, 1 mM sodium pyruvate, 50 units of penicillin, and streptomycin at 50 μg/ml. Where indicated, MAb's were immobilized on plastic at 50 μg/ml in PBS, pH 7.2 at 4°C for overnight. Cultured cells (1×10^6) were washed in Hanks' balanced salt solution containing 0.2 percent bovine serum albumin (BSA) and 0.1 percent NaN_3. For staining, cells were first incubated with MAb 2.4G2 [J. C. Unkeless, *J. Exp. Med.* **150**, 580 (1979)] to block binding by Fcγ receptors and then sequentially incubated with FITC-labeled anti-CD4 (MAb Rm4-5; Pharmingen) and biotinylated anti-CD8 (MAb 53-6-72, BDIS), followed by TRA (Life Technologies). Flow cytometry analyses were performed as described [Y. Takahama, S. O. Sharrow, A. Singer, *J. Immunol.* **147**, 2883 (1991)].
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