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Induction of T-cell receptor-α and -β mRNA in SL12 cells can occur by transcriptional and post-transcriptional mechanisms

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Genes encoding the α and β subunits of the T-cell receptor (TCR) for antigen require rearrangement events for functional expression. In the case of the immunoglobin genes, rearrangement events have been shown to be necessary, but they are not sufficient for full gene expression. The regulation of TCR genes, apart from the requirement for rearrangement, remains to be elucidated. The T-lymphoma cell clone SL12.4 actively transcribes both TCR-α and -β genes and the cells contain nuclear TCR precursor transcripts. However, the cells fail to accumulate appreciable quantities of mature TCR-α and -β mRNAs in either the nucleus or the cytoplasm. The protein synthesis inhibitor cycloheximide (CHX) induces a 20-fold increase in mature TCR-α transcript accumulation without a concomitant increase in TCR-α gene transcription suggesting that CHX reverses the nuclear post-transcriptional events which prevent mature TCR-α mRNA accumulation. CHX also induces full length TCR-β transcripts >90-fold while TCR-β gene transcription increases only 2- to 4-fold. The calcium ionophore A23187 induces the accumulation of TCR-α but not -β transcripts; and in contrast to CHX, it increases the rate of TCR-α gene transcription and the expression of large nuclear TCR-α precursor transcripts. Since CHX and A23187 mediated induction of TCR mRNA is both rapid and reversible, it is unlikely that new DNA rearrangements are responsible for the induction. Collectively, the data show that the accumulation of mature TCR-α and -β transcripts in SL12.4 cells can be coordinately or independently induced by nuclear events involving both transcriptional and post-transcriptional mechanisms.

Key words: post-transcriptional regulation/transcriptional regulation/T-cell receptor/protein synthesis inhibitors/calcium ionophore

Introduction

The T-cell receptor (TCR) for antigen is a heterodimer composed of variable α and β subunits in tight non-covalent association with the invariant T3 polypeptides. The TCR/T3 complex recognizes antigen in the context of major histocompatibility complex (MHC) molecules (reviewed, Kronenberg *et al.*, 1986). The TCR is first expressed on immature T-cells (thymocytes) during their journey through the thymus (reviewed, Rothenberg and Lugo, 1985). The TCR-α and -β polypeptides are encoded by members of the immunoglobulin (Ig) supergene family. Each member of the TCR and Ig gene family is composed of variable (V), joining (J), constant (C) and, in some cases, diversity (D) segments which are juxtaposed by specific DNA rearrangements during lymphocyte development.

TCR gene expression is regulated in a stage specific manner during thymocyte maturation. In the majority of fetal murine thymocytes TCR-β mRNA accumulation precedes TCR-α mRNA accumulation. TCR-β gene rearrangements can be found in the most immature thymocytes (Pgp-1^+ Thy-1^+ $CD4^-$ $CD8^-$ $Ly1^{dull}$ cell surface phenotype) recently emigrated from the bone marrow (Raulet *et al.*, 1985; Snodgrass *et al.*, 1985a; Trowbridge *et al.*, 1985). The first TCR-β gene rearrangement observed in thymocytes juxtaposes D and J, leading to the expression of 1.0 kb TCR-β transcripts which lack the V region (Clark *et al.*, 1984; Siu *et al.*, 1984). 1.0 kb TCR-β transcripts are abundant in immature (day 15) fetal thymocytes (which are predominantly $CD4^-$ $CD8^-$) as well as in adult immature $CD4^-$ $CD8^-$ thymocytes (Raulet *et al.*, 1985; Samelson *et al.*, 1985; Snodgrass *et al.*, 1985a). As thymocyte differentiation proceeds, the V and D segments are juxtaposed resulting in the transcription of full length 1.3 kb TCR-β mRNAs which contain V-D-J-C segments. 1.3 kb TCR-β transcripts are more abundant than 1.0 kb TCR-β messages in day 17 fetal thymocytes and in mature peripheral T cells.

Due to the extremely large size of the TCR-α gene (Winoto *et al.*, 1985) it has not been determined when TCR-α gene rearrangements first take place in developing T-cells. However, significant accumulation of TCR-α mRNA in fetal thymocytes does not occur until day 17 of murine development (Raulet *et al.*, 1985; Snodgrass *et al.*, 1985b) two days after TCR-β mRNA is expressed. At about the time TCR-α mRNA can be detected, the TCR/T3 complex appears on the cell surface (Snodgrass *et al.*, 1985a; Furley *et al.*, 1986). Thus, TCR-α may be the limiting factor controlling the expression of cell surface TCR/T3 during thymocyte development. However, it is not clear whether TCR-α mRNA expression is regulated by TCR-α gene rearrangements or by other regulatory mechanisms.

Here the regulation of TCR-α and -β gene expression is described in a cloned SL12.4 murine T-lymphoma cell line (MacLeod *et al.*, 1984). Lymphoma cells were chosen for this analysis since such cell lines have been valuable in elucidating the molecular events which control immunoglobulin gene expression and other events which take place during lymphocyte development (see Discussion). Furthermore, an analysis of cloned T-lymphoma cell lines circumvents the ambiguity associated with an analysis of heterogeneous thymocyte populations which differ in their maturation status and their viability *in vitro*. The SL12.4 cell clone was derived from a spontaneous thymic lymphoma from an AKR mouse (MacLeod *et al.*, 1984). SL12.4 cells lack CD4 and CD8 surface expression suggesting that this line exhibits a relatively 'immature' phenotype (MacLeod

et al., 1985; Hays *et al.*, 1986). The clone expresses truncated 1.0 kb TCR-β mRNAs but lacks full length 1.3 kb TCR-β mRNAs and contains nearly undetectable levels of TCR-α transcripts (MacLeod *et al.*, 1986). In previous studies using somatic cell hybrids formed with the SL12.4 cell line and other related T-lymphoma cell lines, evidence was presented suggesting that labile protein repressors were involved in the regulation of TCR-β gene expression (MacLeod *et al.*, 1986).

Experiments are described here which demonstrate that SL12.4 cells can be induced to express mature TCR-α and -β mRNA. The use of different inducers indicates that both transcriptional and post-transcriptional mechanisms can regulate TCR gene expression. Transcriptional regulation is a common mode of controlling the steady state levels of mRNAs. However, several reports have indicated that post-transcriptional events can also modulate the expression of mature transcripts in eukaryotic cells (Harpold *et al.*, 1979; Carneiro and Schibler, 1984; Kao and Nevins, 1986; Shaw and Kamen, 1986). Additional evidence suggests that post-transcriptional regulation can occur via changes in nuclear RNA stability and/or the rate of RNA processing (Alterman *et al.*, 1984; Vaessen *et al.*, 1987) nuclear-cytoplasmic transport (Fulton and Birnie, 1985; Young *et al.*, 1986) and cytoplasmic RNA stability (reviewed, Brawerman, 1987). Post-transcriptional events modulate immunoglobulin transcript expression during early B-cell maturation (Gerster *et al.*, 1986).

The results described here indicate that mature TCR-α and -β mRNA expression can be regulated by novel mechanisms which permit the accumulation of precursor transcripts, but which prevent the accumulation of mature transcripts. In some specific experimental conditions, increases in TCR gene transcription rates can also contribute to the regulation of these genes.

Results

Rapid and reversible induction of TCR-α in response to protein synthesis inhibitors

The SL12.4 cell clone was chosen to examine TCR-α and -β mRNA induction since it possesses a phenotype typical of thymocytes at a maturation point just prior to the expression of these transcripts (MacLeod *et al.*, 1984; 1985; 1986). SL12.4 cells lack the expression of full length TCR-α and -β mRNAs, but the cells do express T3-δ and -ϵ mRNA (MacLeod *et al.*, 1986), which encode subunits found in tight non-covalent association with the TCR. Since cycloheximide (CHX) is known to induce the expression of specific transcripts in lymphoid cells, including immunoglobulin mRNA (Ishihara *et al.*, 1984; Wall *et al.*, 1986) the effect of CHX on TCR-α mRNA expression in SL12.4 cells was examined. Figure 1 shows that CHX induces the accumulation of TCR-α mRNA in the SL12.4 cell clone (lane 2) compared to RNA from untreated control cells shown in lane 1, loaded with twice as much RNA as the other lanes. TCR-α mRNA was induced at concentrations of CHX (10 μg/ml) which inhibit [^{35}S]methionine incorporation into protein by >95%. To test whether the inductive effects of CHX on TCR-α mRNA expression can be reversed, CHX treated cells were washed free of the drug and re-cultured. Removal of CHX results in a decrease in the amount of TCR-α mRNA 2 h later; by 4 h after drug removal the amount of TCR-α mRNA returns to levels found in untreated cells (Figure 1, lanes 3-5). In contrast, actin mRNA expression remains relatively constant in untreated and in CHX treated cells (Figure 1).

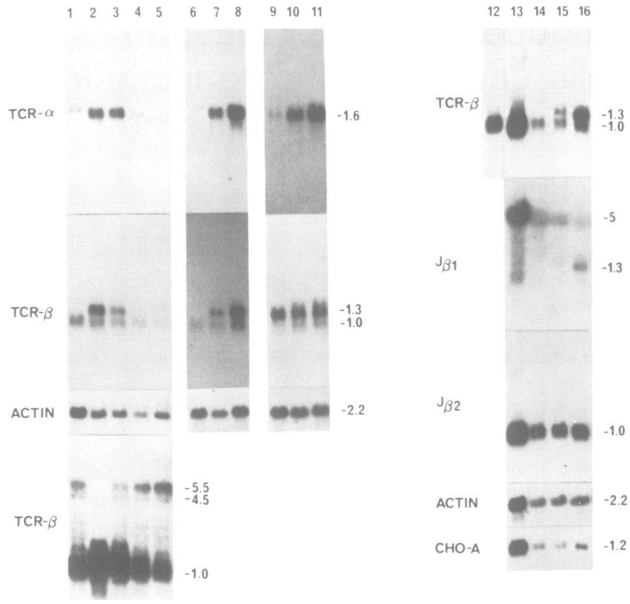

Fig. 1. Northern analysis showing reversible induction of TCR-α and -β mRNA in response to protein synthesis inhibitors. Lane 1 contains 20 μg of total cellular RNA and lanes 12 and 13 contain 5 μg of poly(A)$^+$ RNA; the remaining lanes contain 10 μg of total cellular RNA. RNA was prepared from SL12.4 cells (lanes 1–8 and 12–16) or SAK8 cells (lanes 9–11) treated as follows: untreated, lane 1; 10 μg/ml CHX for 12 h, lane 2; 10 μg/ml CHX for 12 h, washed three times in PBS, followed by resuspension in fresh media for 2 h, lane 3, 4 h, lane 4 or 8 h, lane 5. Untreated, lane 6; 6 h incubation with 10 μg/ml CHX, lane 7; 6 h with 1 μg/ml pactamycin, lane 8. Untreated SAK8, lane 9; 6 h incubation with 10 μg/ml CHX, lane 10; 1 μg/ml pactamycin, lane 11. Two autographic exposures of poly(A)$^+$ RNA from untreated SL12.4 cells, lanes 12 and 13; SL12.4 cells incubated with 10 μg/ml CHX for 0 h, lane 14, 1 h lane 15 or 5 h, lane 16. Equal loading and blotting of RNA in lanes 2–5, 6–8, 9–11 and 14–16 was demonstrated by acridine orange staining as described in Materials and methods (not shown, see Figure 4 for example).

Pactamycin, another protein synthesis inhibitor, also induces TCR-α transcripts in SL12.4 cells (Figure 1, lane 8) to a slightly greater extent than CHX (lane 7). Both CHX and pactamycin increase TCR-α mRNA accumulation in another T-lymphoma cell line SAK8 (Figure 1, lanes 10 and 11) which constitutively expresses low amounts of TCR-α mRNA (lane 9).

Coordinant induction of TCR-α and -β mRNA

SL12.4 cells constitutively express immature 1.0 kb TCR-β transcripts (Figure 1, lanes 1, 6, 12 and 13). TCR-β 1.0 kb mRNAs are expressed by T-cells which have undergone D-J jointing; these transcripts lack V_β sequences (Clark *et al.*, 1984; Siu *et al.*, 1984). Most immature fetal and adult thymocytes express these truncated TCR-β mRNAs of 1.0 kb (Raulet *et al.*, 1985; Snodgrass *et al.*, 1985b). T-cells which contain fully rearranged TCR-β genes (V-D-J joining) express transcripts with an average size of 1.3 kb (Clark *et al.*, 1985; Siu *et al.*, 1985). 1.3 kb TCR-β transcripts are not detectable in SL12.4 cells even when poly(A)$^+$ RNA is examined by Northern blot analysis (Figure 1, lane 12).

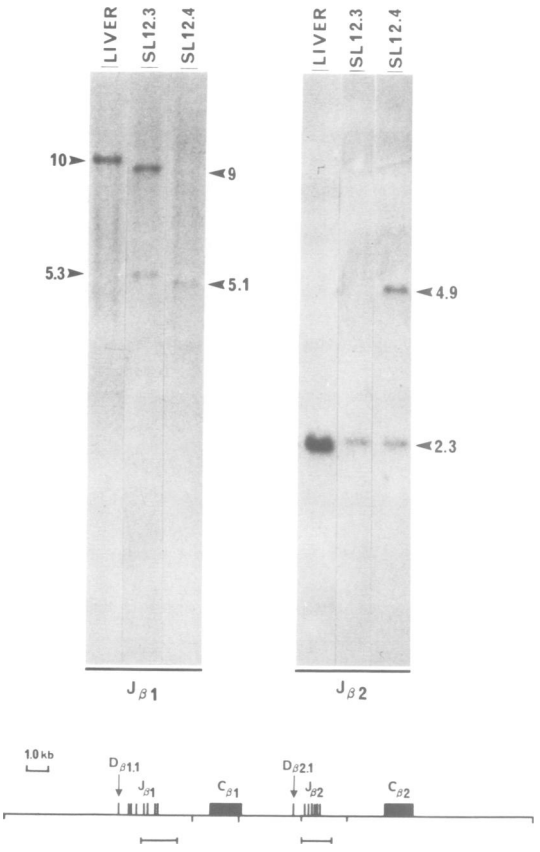

Fig. 2. TCR-β gene rearrangements in the SL12.3 and SL12.4 clones. Southern blots of total DNA from SL12.3 and SL12.4 cells digested with *Eco*RI were hybridized with murine genomic J_β probes extending over the regions indicated in the lower Panel. The *Eco*RI sites in the unrearranged (germline) TCR-β gene are shown in the diagram by the downward tick marks. The sizes of the bands hybridizing to the J_β probes were determined by comparing their migration relative to *Hin*dIII-digested λ DNA.

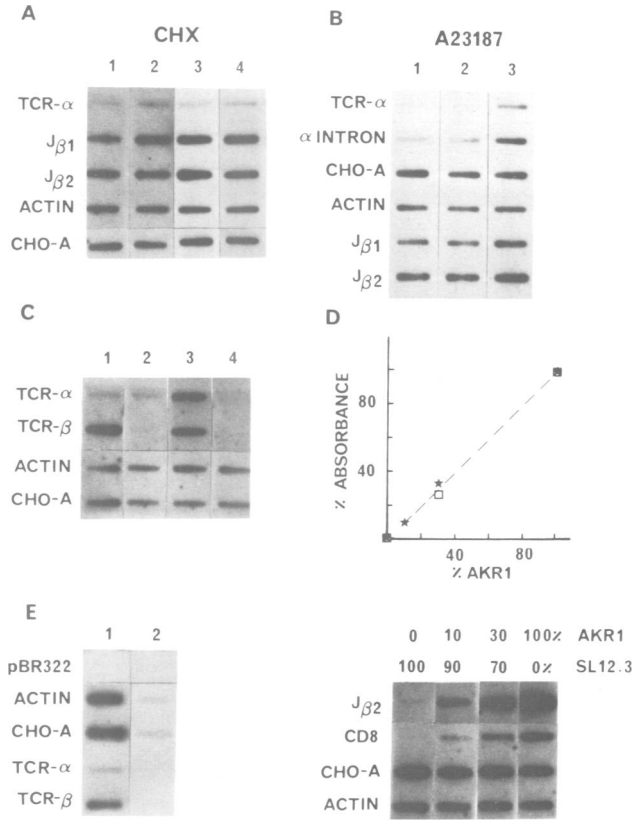

Fig. 3. Transcription of TCR-α and -β genes. Transcription rates of the indicated genes were assessed by nuclear run-off experiments described in Materials and methods using equal amounts of labeled RNA hybridized to slot blots of purified insert DNA. **Panel A**: SL12.4 cells were incubated with 10 μg/ml CHX for 0 h (lane 1), 0.5 h (lane 2), 1 h (lane 3) or 3 h (lane 4). **Panel B**: SL12.4 cells were incubated with 0.5 μg/ml A23187 for 0 h, lane 1, 1 h, lane 2, or 2 h, lane 3. **Panel C**: RNA from untreated SL12.4, lane 1; Sl12.3, lane 2, AKR1, lane 3 and RAW253, lane 4. **Panel D**: ^{32}P-labeled RNA generated from AKR1 and SL12.3 nuclei was mixed in different ratios for hybridization (equal amounts of radioactively labeled RNA were hybridized in each case), the line drawing shows the signal obtained for $J_{\beta 2}$ (★) and Lyt2 (□) gene transcription (assessed by optical densitometry) as a function of the percentage of AKR1 ^{32}P-labeled RNA; **Panel E**: SL12.4 nuclei were incubated in the presence, lane 2; or absence of 1 μg/ml α-amanatin, lane 1 during the run-off reaction (plasmid, rather than purified insert DNA was used for this hybridization).

To ascertain whether the lack of 1.3 kb TCR-β mRNA accumulation results from the absence of fully rearranged TCR-β genes in SL12.4 cells, or from other regulatory constraints, SL12.4 cells were treated with CHX. Figure 1 shows that 1.3 kb TCR-β transcripts are coordinately induced with TCR-α mRNA after a 1 h CHX treatment (lane 15) and become more pronounced 6 h after treatment (lane 16). TCR-β 1.3 kb transcripts first become detectable 15 min after CHX treatment (not shown). Pactamycin also induces 1.3 kb TCR-β transcripts (Figure 1, lane 8). The induction of 1.3 kb TCR-β transcripts by CHX was reversible with the same kinetics as TCR-α mRNA (Figure 1, lanes 3–5).

The murine TCR-β gene loci consist of two sets of D, J and C segments, each of which can be rearranged and expressed in T-cells (see Figure 2 for diagrams). J_β genomic probes can be used to distinguish between $J_{\beta 1}$ and $J_{\beta 2}$ transcripts. Northern blot analysis shown in Figure 1 demonstrates that 1.0 kb TCR-β transcripts constitutively expressed in SL12.4 cells are encoded by $J_{\beta 2}$ sequences whereas the CHX induced 1.3 kb TCR-β mRNA is encoded by $J_{\beta 1}$ sequences. Figure 1, lanes 15 and 16 clearly shows that CHX specifically enhances 1.3 kb but not the 1.0 kb TCR-β transcript expression. Larger transcripts (~5kb) are also detected by the $J_{\beta 1}$ probe. Since poly(A)$^+$ enriched RNA from SL12.4 cells has less rRNA, yet it has more of these putative precursor transcripts (Figure 1, lane 13), it is not likely to represent probe hybridization with 28S rRNA sequences. The 5 kb transcripts are found exclusively in the nucleus and are likely to be TCR-β precursor transcripts (see later). The $J_{\beta 1}$ genomic probe gives a much weaker signal with the mature 1.3 kb TCR-β mRNA than with the precursor 5 kb TCR-β transcripts. This is not surprising since the mature TCR-β mRNA contains only a single J sequence of 40–50 nucleotides and thus has a shorter region of homology with the $J_{\beta 1}$ probe than the precursor mRNAs which contain the intervening non-coding regions (see map in Figure 2).

Figure 2 shows Southern blot analysis of TCR-β gene rearrangements in SL12.4 cells, using $J_{\beta 1}$ and $J_{\beta 2}$ specific genomic probes. The data indicate that one allele of both $J_{\beta 1}$ and $J_{\beta 2}$ gene segments in SL12.4 cells is rearranged, one $J_{\beta 1}$ segment is deleted and one $J_{\beta 2}$ segment is in the germline configuration. The size of the $J_{\beta 2}$ rearranged seg-

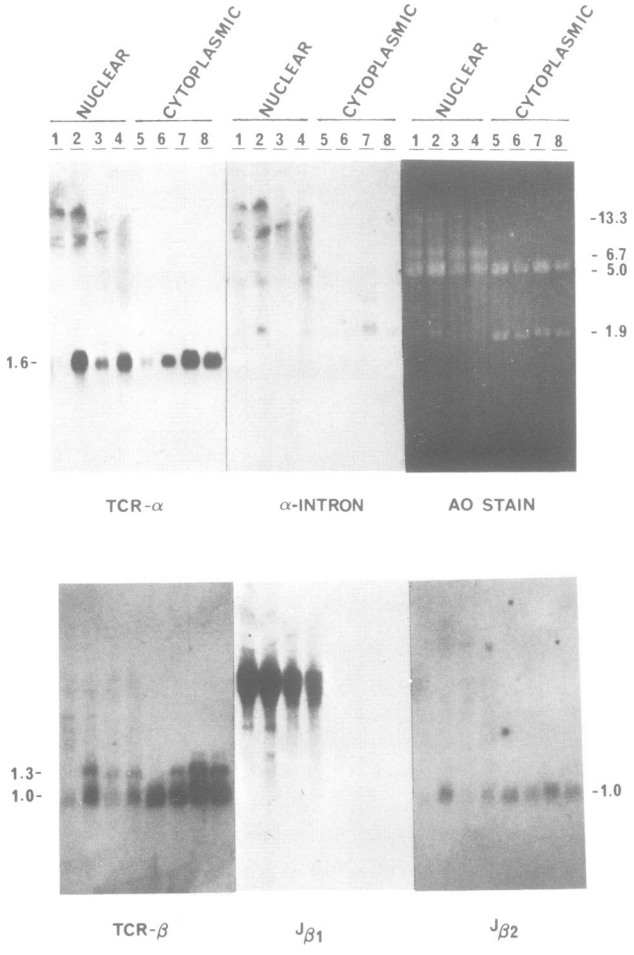

Fig. 4. Induction of TCR-α and -β mRNA in nuclear and cytoplasmic compartments. 10 μg of nuclear or cytoplasmic RNA from SL12.4 cells which were previously treated with 10 μg/ml CHX for 0 h, lanes 1 and 5; 1 h, lanes 2 and 6; 2 h, lanes 3 and 7; or 3 h, lanes 4 and 8 was analyzed on Northern blots. Acridine orange staining (AO stain) of the gel shows that the RNA samples were somewhat unequally loaded. The cytoplasmic RNA contains 18S and 28S rRNA (1.9 and 5.0 kb in length, respectively) and the nuclear RNA is enriched for 32S and 45S rRNA precursors which are 6.7 and 12.3 kb in length, respectively (Gurney, Jr, 1985). The same blot was probed successively with ^{32}P random primed DNA of purified insert from TCR-α cDNA, α TCR intron, TCR-β cDNA, TCR-$J_{\beta 1}$, TCR-$J_{\beta 2}$. The mol. wt, in kilobases (kb) is indicated on the sides of the blots and was estimated relative to the migration of rRNA and BRL standards (see Materials and methods).

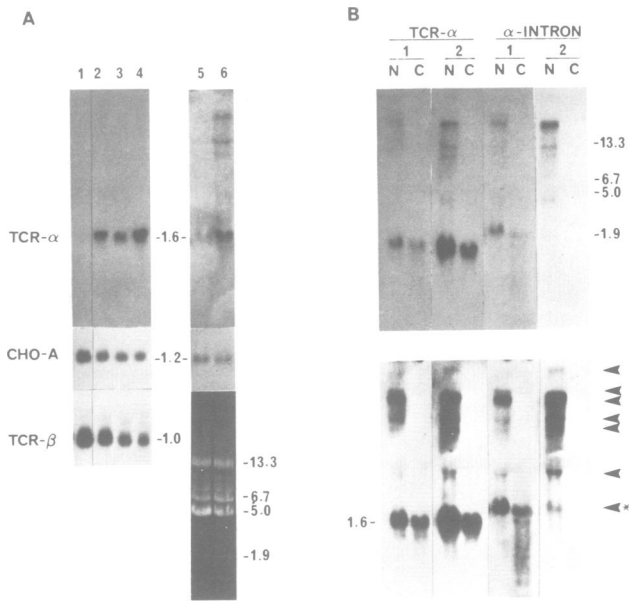

Fig. 5. Induction of TCR-α, but not 1.3 kb TCR-β mRNA in response to calcium ionophore. **Panel A**: nuclear and cytoplasmic RNA was prepared from SL12.4 cells treated with 0.5 μg/ml A23187 or 0 h, lanes 1 and 4; 1 h, lanes 2 and 6; 2 h, lane 3; or 3 h, lane 4 followed by Northern blot analysis on 1% (cytoplasmic RNA) or 0.7% (nuclear RNA) agarose gel. The lower photograph under lanes 5 and 6 shows the 0.7% gel stained with acridine orange. **Panel B**: nuclear (n) and cytoplasmic (c) RNA was obtained from SL12.4 × thymocyte somatic cell hybrid cells (1) or SL12.4 cells treated with 0.5 μg/ml A23187 for 3 h (2). The RNA was electrophoresed in a 0.7% agarose gel. Equal loading of the lanes and enrichment for nuclear RNA was assessed by acridine orange staining (not shown). The upper and lower sectors show autoradiographic film exposed to identical blots for 1 and 5 days, respectively. The arrows indicate TCR-α precursor transcripts. The 2.0 kb transcript (marked with an *) hybridizes to α-intron probe but not the TCR-α cDNA probe.

ment in SL12.4 cells is consistent with a V-D-J joining event (see map in Figure 2). The unrearranged $J_{\beta 2}$ segment is likely to encode the constitutively expressed 1.0 kb $J_{\beta 2}$ transcript (Figure 1, lanes 13−16) since the other one appears to be fully rearranged and would give rise to a full length 1.3 kb transcript. No 1.3 kb transcripts with $J_{\beta 2}$ sequences are detectable. The 5.1 kb size of the rearranged $J_{\beta 1}$ gene segment is consistent with V-D-J joining (assuming that the rearrangement occurred by deletion) and thus is likely to transcribe the 1.3 kb TCR-β mRNA induced by CHX in SL12.4 cells (Figure 1, lane 16).

Not all SL12 cell clones can be induced to express TCR-β mRNA. SL12.3 cells lack detectable 1.0 and 1.3 kb TCR-β transcripts, yet they contain TCR-β gene rearrangements of $J_{\beta 1}$ segments (Figure 2 and MacLeod *et al.*, 1986). CHX failed to induce TCR-β mRNA in SL12.3 cells under conditions which induce its expression in SL12.4 cells (data not shown). The lack of inducibility of TCR-β genes in SL12.3 cells could result from unproductive TCR-β gene rearrangements or because the cell clone is not yet 'competent' to respond to the stimulus.

TCR-α and -β gene transcription

The observation that CHX induces TCR-α and -β mRNA suggests that a labile inhibitor protein may repress the accumulation of these transcripts. To determine whether the putative inhibitor polypeptide(s) acts at the transcriptional or post-transcriptional level, TCR-α and -β gene transcription rates were measured using the nuclear run-off assay. Figure 3, panel A shows that SL12.4 cells constitutively transcribe the TCR-α gene. The rate of TCR-α gene transcription in SL12.4 cells is similar to the rate in SL12.3 cells, yet SL12.4 cells accumulate ~50-fold less mature TCR-α mRNA than SL12.3 cells (MacLeod *et al.*, 1986). CHX mediated induction of TCR-α transcripts in SL12.4 cells occurs in the absence of measurable increases in TCR-α gene transcription rates during the period when the majority of the CHX induced TCR-α messages accumulate (Figure 3, panel A). Thus, CHX appears to induce the accumulation

of TCR-α transcripts by acting on post-transcriptional events.

SL12.4 cells constitutively transcribe both TCR $J_{\beta 1}$ and $J_{\beta 2}$ genes (Figure 3, Panel A) although untreated cells accumulate only $J_{\beta 2}$ transcripts (Figure 1, lane 12). CHX induces an increase in $J_{\beta 1}$ gene transcription about 2−4-fold in SL12.4 cells suggesting that the increased transcription rate accounts, at least in part, for the induction of 1.3 kb $J_{\beta 1}$ mRNAs. However, since the cells transcribe the $J_{\beta 1}$ gene at a high rate in the absence of CHX and yet lack mature $J_{\beta 1}$ mRNA, we conclude that the increase in $J_{\beta 1}$ transcription rate is unlikely to account fully for the induction of 1.3 kb TCR-β transcripts in response to CHX and that post-transcriptional events also regulate TCR-$J_{\beta 1}$ mRNA expression.

In contrast to SL12.4 T-lymphoma cells, non T-cells display an extremely low rate of TCR-α and -β gene transcription. TCR-α and -β gene transcription was almost undetectable in the B-cell line RAW253 and in murine embryo fibroblasts (Figure 3, Panel C, 4 and data not shown) showing that their transcription is T-lymphocyte specific, as expected.

The nuclear run-off assay was validated by the following tests: the inhibitor of polymerase II dependent transcription, α-amanatin, decreased the transcriptional signal of all genes tested by >10-fold when it was included during the nuclear run-off reaction (Figure 3, Panel E). In order to determine whether the transcriptional signal obtained in the nuclear run-off assay was 'linear' with respect to the rate of gene transcription, two cell lines (AKR1 and SL12.3) which differ in their rates of $J_{\beta 2}$ and CD8 gene transcription were used as a source of ^{32}P-labeled RNA. The labeled RNA was then mixed in different ratios for hybridization. The signal obtained for the $J_{\beta 2}$ and CD8 genes titrate in a linear fashion, while actin and CHO-A genes which are transcribed at nearly equal rates by both cell lines show an equal signal in all the ratios tested (Figure 3, Panel D).

Expression of TCR-α and -β transcripts in nuclear and cytoplasmic compartments

Post-transcriptional events appear to be at least partly responsible for the CHX induction of TCR-α and 1.3 kb TCR-β transcripts in SL12.4 cells. To localize the post-transcriptional regulation to the nuclear or cytoplasmic compartments, RNA was prepared from each compartment before and after CHX treatment. The enrichment for nuclear RNA was verified by staining the unblotted gel with acridine orange (Figure 4; note that precursor rRNA molecules are enriched in the nuclear RNA fraction). Figure 4 shows the detection of nuclear precursor TCR-α mRNAs (>10 kb in size) prior to CHX treatment which is consistent with the finding that TCR-α transcription occurs in the absence of CHX treatment. The large TCR-α mRNAs are likely to represent precursor transcripts since they are highly enriched in nuclear RNA preparations and they are recognized by a probe to the J-C α-intron, which contains sequences spliced out in mature TCR-α mRNAs. In contrast, SL12.4 cells lack mature 1.6 kb TCR-α transcripts in either the nucleus or the cytoplasm (Figure 4, lanes 1 and 5). The absence of 1.6 kb TCR-α mRNA accumulation in the nucleus makes it unlikely that a block in nuclear-cytoplasmic transport is responsible for the low constitutive levels of cytoplasmic TCR-α transcripts in SL12.4 cells.

Upon induction with CHX, mature 1.6 kb TCR-α transcripts appear in the nucleus of SL12.4 cells (Figure 4, lanes 2−4) implying that CHX alters post-transcriptional events in the nucleus. CHX also increases TCR-α mRNA accumulation in the cytoplasm (Figure 4, lanes 6−8). It is reasonable to suppose that the increase in cytoplasmic TCR-α mRNA accumulation results from an increase in the amount of TCR-α mRNA transported from the nucleus. However, we cannot rule out that CHX also directly influences cytoplasmic events, such as cytoplasmic mRNA stability.

Figure 4, lane 1 shows that full length 1.3 kb TCR-β transcripts are not detectably present in the nucleus or cytoplasm of untreated SL12.4 cells; this transcript only appears after treatment with CHX (lanes 2−4 and 6−8). Since the 1.3 kb TCR-β mRNA contains $J_{\beta 1}$ sequences which are actively transcribed in the nucleus, yet no mature RNA is detectable, CHX appears to act on post-transcriptional events localized to the nucleus. TCR-β precursor RNAs of 4.5 and 5.5 kb (Figure 1, lane 1) were also enriched in the nuclear compartment of SL12.4 cells prior to CHX treatment (Figure 4, lane 1). As discussed earlier, these are likely to be precursors to the 1.3 kb mature transcript based on analysis with the $J_{\beta 1}$ probe (Figure 1). Although the signal is low, large RNA precursors are detectable with the full length TCR-β probe (see Figure 1, lane 13, and Figure 4, lane 1).

The accumulation of the $J_{\beta 1}$ precursor transcripts is decreased after 2−5 h of CHX treatment (Figure 1, lane 16; Figure 4, lanes 2−4); precursor transcripts are almost undetectable by 12 h after treatment (Figure 1, lane 2). Since the decrease in TCR-β precursor transcripts occurs concomitantly with the dramatic increase in mature TCR-β accumulation, it is possible that CHX acts by relieving a block in processing of the precursor RNAs to the mature form. It is noteworthy that no precursor transcripts to the constitutively expressed 1.0 kb $J_{\beta 2}$ mRNA were detected (Figure 4). However, we cannot exclude the possibility that instead, or in addition to its effect on RNA processing, CHX acts by altering TCR-β RNA stability.

TCR-α and -β genes are constitutively transcribed, their precursor RNAs accumulate and yet mature 1.6 kb TCR-α RNAs and 1.3 kb TCR-β mRNAs accumulate only after CHX treatment. We conclude that CHX acts by increasing nuclear RNA stability and/or by reversing a specific block to RNA processing.

Calcium ionophore induces TCR-α but not -β gene transcription

The calcium ionophore A23187 induces biological responses and alterations in gene expression in several cell types, including T-cells (Albert *et al.*, 1985; Truneh *et al.*, 1985; Wiskocil *et al.*, 1985). A23187 rapidly induces TCR-α mRNA expression in SL12.4 cells (Figure 5, Panel A, lanes 2−4). The induction of TCR-α mRNA by A23187 is reversible; by 8 h after removal of A23187, TCR-α mRNA expression is undetectable (not shown). A23187 and CHX appear to induce TCR-α mRNA by different mechanisms. Unlike CHX, A23187 increases the rate of TCR-α gene transcription as determined by the nuclear run-off assay (Figure 3, Panel B). In addition, A23187 rapidly increases nuclear TCR-α precursor transcripts (Figure 5, Panel A, lane

6). Discrete nuclear transcripts of 4, 10, 12 and > 15 kb which hybridize to TCR-α cDNA and α-intron probes are clearly present after A23187 treatment (Figure 5, Panel B). Very large precursor TCR-α transcripts are expected since fully rearranged TCR-α genes can contain J-C introns of >50 kb, depending on the particular V-J rearrangement which takes place (Winoto *et al.*, 1985). As expected, these large TCR-α transcripts are not found in the cytoplasm of A23187 treated SL12.4 cells (Figure 5, panels A and B). Nuclear RNA from the S194/5 B-lymphoma cell line lacks detectable expression of TCR-α precursor or mature transcripts (data not shown).

The J-C intron probe also hybridizes to a 2 kb transcript, distinct in size from the 1.6 kb mature TCR-α transcript, which is unlikely to be a precursor TCR-α transcript because it is found in both the nuclear and cytoplasmic compartments and it does not hybridize to the TCR-α cDNA probe (Figure 4). Furthermore, the 2 kb mRNA is found in S194/5 B-lymphoma cells which lack detectable TCR-α mRNA and measurable TCR-α transcription (data not shown). The identity of the 2 kb transcript is not known.

A somatic cell hybrid formed by the fusion of SL12.4 with a normal murine thymocyte (Hays *et al.*, 1986) constitutively expresses TCR-α precursor and mature RNAs (Figure 5B). The sizes of the transcripts detected in the hybrid cells are identical to those found in A23187 treated SL12.4 cells. The hybrid cells contain an additional ~20 kb transcript not present in SL12.4 cells. This 20 kb transcript could derive from a rearranged TCR-α gene contributed by the normal thymocyte parental cell. Taken together, our results show that while both A23187 and CHX induce mature TCR-α transcripts by altering nuclear events A23187 acts, at least in part, by stimulating transcription, whereas CHX effects nuclear post-transcriptional events. The inducers also differ in that, unlike CHX, A23187 fails to increase the amount of 1.3 kb TCR-β mRNA (Figure 5, Panel A, lanes 2–4).

Discussion

The studies presented here show that SL12.4 cells can respond to specific induction signals, resulting in increased expression of TCR transcripts encoding molecules which participate in T-cell recognition. Investigation into the mechanisms responsible for the induction of TCR-α and -β gene expression indicate that labile repressor proteins might be involved since protein synthesis inhibitors induce their mRNAs. The notion that *trans*-negative protein(s) regulate TCR-β mRNA expression was first indicated by experiments showing that TCR-β mRNA expression is suppressed in somatic cell hybrids formed between TCR-β^+ and -β^- SL12 cell clones, and that the repression is relieved by treatment of the cells with CHX (MacLeod *et al.*, 1986). The existence of labile inhibitor proteins which regulate gene expression has been postulated on the basis of several studies examining the effects of protein synthesis inhibitors. The accumulation of transcripts encoding c-*myc*, c-*fos*, histones and immunoglobulin are increased in appropriate cell types following treatment with protein synthesis inhibitors (Ishihara *et al.*, 1984; Greenberg *et al.*, 1986; Schumperli, 1986). In mature T-cells repressor proteins may regulate T-cell activation events, including the expression of interferon-γ (Wilkinson and Morris, 1984; Young *et al.*, 1986). Here, evidence is provided that labile repressors can also regulate the expression of molecules that are important for the differentiation of immature T-cells.

A number of studies using protein synthesis inhibitors suggest that labile repressor proteins can regulate transcription (Graves and Marzluff, 1984; Ringold *et al.*, 1984; Greenberg *et al.*, 1986; Wall *et al.*, 1986) and/or post-transcriptional events (Graves and Marzluff, 1984; Schumperli, 1986; Young *et al.*, 1986). The data described in this report indicate that the induction of TCR-α and -β mRNA is, at least in part, mediated by nuclear post-transcriptional events. Only a few examples of post-transcriptional regulation localized to the nucleus have been reported. Nuclear post-transcriptional mechanisms appear to regulate dihydrofolate reductase (DHFR) and histone H3 mRNA accumulation; both of which are tightly coupled to the cell cycle and the rate of cell proliferation. Although no direct evidence for nuclear regulation of DHFR and histone transcripts was obtained, it was inferred from the lack of changes in transcription rate and cytoplasmic RNA stability (Alterman *et al.*, 1984; Leys *et al.*, 1984). Nuclear post-transcriptional mechanisms may also be responsible for the glucocorticoid induction of α_1-acid glycoprotein mRNA (Vannice *et al.*, 1984). Nuclear post-transcriptional mechanisms could regulate the reduction of class I MHC mRNAs in adenovirus transformed cells (Vaessen *et al.*, 1987). The transcription rates of the class I MHC genes is similar in transformed and non-transformed cells, but both precursor and mature MHC RNAs are decreased in virally transformed cells. The TCR genes appear to be the first examples of developmentally regulated genes controlled by nuclear post-transcriptional mechanisms.

SL12.4 cells constitutively transcribe TCR-α and -β genes, and they accumulate precursor TCR mRNAs in the nucleus. However, the cells fail to accumulate significant quantities of mature 1.6 kb TCR-α or 1.3 kb TCR-β transcripts in either the nucleus or the cytoplasm unless the cells are treated with the protein synthesis inhibitors CHX or pactamycin. We interpret this to indicate that either: (i) mature and/or partially processed TCR transcripts are inherently unstable in the nucleus of SL12.4 cells due to the presence of a labile protein(s); or (ii) a labile protein(s) specifically inhibits the processing of TCR-α and -β transcripts in the nucleus. In the case of 1.3 kb TCR-β transcripts, we favour the hypothesis that there is a specific block in RNA processing due to two observations: first, precursor $J_{\beta 1}$ transcripts dramatically decline in accumulation when the cells are induced to express $J_{\beta 1}$ 1.3 kb TCR-β mRNA; and secondly, there is no detectable accumulation of precursor $J_{\beta 2}$ transcripts corresponding to the $J_{\beta 2}$ 1.0 kb TCR-β mRNA constitutively expressed in SL12.4 cells. Direct measurement of mRNA stability and precursor–product analysis will be required to definitively determine the mechanism by which mature TCR-β transcripts fail to accumulate in SL12.4 cells. The sequences responsible for the differential accumulation of 1.0 and 1.3 kb TCR-β mRNA in SL12.4 cells is likely to reside in the highly variable V, D and J sequences. For example, the V_β region (not present in 1.0 kb transcripts) could confer a specific susceptibility to nucleases or it may be involved in regulating RNA splicing or other RNA processing steps.

CHX could increase the accumulation of TCR-α and -β mRNA by inhibiting the translocation of ribosomes thereby protecting the message from degradation. This possibility

seems unlikely since TCR-α and -β mRNA is also induced in SL12.4 cells by pactamycin which inhibits translation initiation, not ribosome translocation at the concentrations used in our experiments (Sive *et al.*, 1984). Further, the post-transcriptional events responsible for the regulation of TCR-α and -β mRNAs appear to be localized in the nucleus, although cytoplasmic events such as stabilization of TCR RNAs have not been excluded by our experiments.

The coordinant induction of TCR-α and -β mRNA in response to CHX is consistent with results obtained by Ohashi *et al.* (1985) in which the transfection of TCR-β cDNA into variant human T-lymphoma cells lacking TCR-β 1.3 kb mRNA results in expression of the transfected gene product and, suprisingly, an elevation of TCR-α mRNA levels. The authors concluded that TCR-β 1.3 kb mRNA, or the encoded protein, may positively regulate TCR-α gene expression. This hypothesis is in accord with the fact that the majority population of developing thymocytes expresses TCR-β mRNA prior to TCR-α mRNA (for review, see Rothenberg and Lugo, 1985) and the finding that most cell lines and clones express either TCR-β mRNA alone or both TCR-α and -β (Davey *et al.*, 1986; Furley *et al.*, 1986; Sangster *et al.*, 1986). However, some T-cell lines are capable of expressing TCR-α mRNA in the absence of TCR-β mRNA (Davey *et al.*, 1986; MacLeod *et al.*, 1986; Sangster *et al.*, 1986). Moreover, we demonstrate here that A23187 treatment induces substantial quantities of TCR-α mRNA in the absence of any full length 1.3 kb TCR-β mRNA. The clear conclusion from this data is that 1.3 kb TCR-β mRNA expression is not an obligate requirement for mature TCR-α mRNA accumulation.

TCR-β gene expression may also be regulated by a labile protein which represses gene transcription. CHX induces a 2- to 4-fold increase in the rate of TCR-β gene transcription. This putative inhibitor could act by binding directly to TCR-β gene regulatory sequences. We have previously demonstrated that *trans*-acting factor(s) repress TCR-β mRNA accumulation in somatic cell hybrids (MacLeod *et al.*, 1986); recently we have shown that repression results from substantial decreases in TCR-β gene transcription (M.Wilkinson and C.MacLeod, unpublished observations). However, 'inhibitor' factors could also act indirectly by altering the activity of DNA-binding factors. Recently, Sen and Baltimore (1986) demonstrated that CHX increases the activity of the DNA-binding protein NF-κB, which stimulates κ immunoglobulin gene transcription. The authors speculate that CHX may inhibit the translation of a labile repressor which normally inactivates NF-κB.

TCR-α mRNA accumulation can also be transcriptionally regulated. The calcium ionophore A23187 induces an increase in the rate of TCR-α gene transcription, an increase in the expression of large TCR-α precursor RNAs and the accumulation of mature cytoplasmic TCR-α transcripts. Thus, TCR-α mRNA expression can be regulated by at least two pathways involving either transcriptional or post-transcriptional regulation.

Since the induction of TCR mRNA expression in response to either CHX or A23187 is rapid and reversible, it is likely to result from regulatory events rather than the induction of new TCR gene rearrangements. Support for the hypothesis that regulatory events control the expression of TCR-β genes is provided by the finding that TCR-β gene rearrangements (consistent with a V-D-J joining event) are present in SL12.4 cells before CHX induction. Other studies have indicated that the presence of gene rearrangements are not sufficient for TCR-α and -β mRNA expression (Davey *et al.*, 1986; Owen *et al.*, 1986; Sangster *et al.*, 1986). However, these studies did not examine whether the lack of TCR-α and -β expression was due to non-functional rearrangements or regulatory constraints. In the present report we show that rearranged TCR-α and -β genes are transcribed but mature TCR-α and -β transcripts do not accumulate unless the cells are appropriately induced.

It is possible that TCR gene expression in normal thymocytes is regulated by complex transcriptional and post-transcriptional mechanisms which govern the appropriate amount of expression during thymocyte ontogeny and T-cell activation. Although one must be cautious before extrapolating molecular events observed in lymphoma cells to normal thymocyte differentiation, it is clear that T- and B-lymphoma cell lines have been instrumental in revealing key mechanisms governing lymphocyte differentiation (for reviews, see Calame, 1985; Hanley-Hyde and Lynch, 1986; Greaves, 1986). The collection of inducible and non-inducible SL12 T-lymphoma cell clones may comprise a useful model system in which to identify thymic factors, cellular receptors, putative labile inhibitor molecules, secondary messengers and regulatory genetic elements which are involved in the induction and modulation of TCR gene expression during T-cell maturation.

Materials and methods

Cell culture

The SL12 cell lines used in this study and their culturing requirements have been described (MacLeod *et al.*, 1984; 1985). The RAW253 (Hyman and Stallings, 1978) and AKR1 (Hyman *et al.*, 1980) cell lines were kindly provided by Dr Hyman, Salk Institute. The cells were seeded at 5×10^5 cells/ml in Dulbecco's Modified Eagles Medium and 10% fetal calf serum before treatment with pactamycin (gift of Dr Baglioni, State University of New York, Albany), CHX or A23187 (Sigma, St Louis, MO). Murine embryo fibroblasts (MEF) were prepared from a Balb/c embryo as described by Freshley (1983).

Protein synthesis determination

To assess the effectiveness of the protein synthesis inhibitors, SL12.4 cells were incubated with several concentrations of CHX or pactamycin for 1 h, followed by incubation with 25 μCi/ml [^{35}S]methionine (Amersham) for 3 h. The incorporation of [^{35}S]methionine into protein was determined by precipitation of whole cell lysate proteins in 10% trichloroacetic acid (in the presence of 0.5% casamino acids) on glass-fiber filters; the precipitated material was counted in a β-scintillation counter.

Northern blot analysis

Total cellular RNA was isolated from cells as described (Maniatis *et al.*, 1982) except that the cells were lysed in 4 M guanidinium isothiocyanate, 1 M 2-mercaptoethanol, and 25 mM sodium acetate (pH 5.2). Nuclear and cytoplasmic RNA were prepared from 5×10^7 cells which were washed in Tris−saline, resuspended in 0.5 ml Tris−saline (pH 7.5), followed by addition of 4.5 ml of 1% NP-40, 0.5% sodium deoxycholate and 0.005% dextran sulfate in Tris−saline. The suspension was centrifuged at 2000 r.p.m. for 5 min. The supernatant (cytosolic fraction) was phenol:chloroform extracted in the presence of 1% SDS and 0.1 M NaCl, followed by ethanol precipitation. The nuclear pellet was treated like the cells used to prepare total cellular RNA as described above. In general, 10 μg of RNA per lane was electrophoresed in 1% formaldehyde agarose gels and transferred to nitrocellulose paper (when 0.7% gels were used it is noted in the figure legends). Poly(A)$^+$ RNA was made from total RNA as described (Maniatis *et al.*, 1982). Equal loading and transfer of RNA per lane was assessed by acridine orange staining: after electrophoresis, the gel was stained for 3 min in 30 μg/ml acridine orange, 10 mM sodium phosphate (pH 6.5) and 1.1 M formaldehyde, followed by de-staining for 20 min in the same buffer without acridine orange; the gel was examined by UV and photograph-

ed. Transcript sizes were determined by comparing their migration relative to rRNA or an 'RNA ladder' (BRL, Bethesda, MD). The blots were hybridized with ^{32}P-labeled random-primed (Amersham) insert probes in the presence of 10% dextran sulphate and 50% formamide for 12–18 h at 42°C (Meinkoth and Wahl, 1984). To remove the label so that the filters could be reprobed, the RNA blots were washed with 0.1 × SSPE and 0.1% SDS at 98°C, allowed to cool to room temperature, air-dried, and stored under vacuum until hybridized with the next probe.

Southern blot analysis

DNA was isolated from cells as described (Maniatis *et al.*, 1982) and digested with *Eco*RI according to the supplier's conditions. Twenty micrograms of digested DNA was applied to each lane of a large (250 ml) agarose gel and electrophoresed for at least 48 h. Southern blots were prepared as described (Meinkoth and Wahl, 1984) hybridized and washed as described for Northern blot analysis.

Nuclear run-off assay

The experiments were performed as described (Wang and Calame, 1985) with the following modifications: nuclei were prepared by lysing 10^8 cells (per conditions) in STKM buffer [30% sucrose (w/v), 40 mM Tris (pH 7.5), 37 mM KCl, 12 mM $MgCl_2$] in the presence of 0.5% Triton X-100. The transcription reaction was carried out in isolated nuclei in the presence of 0.8 mCi/ml [^{32}P]GTP (3000 Ci/mmol, Amersham), 0.5 mM ATP, and 0.25 mM UTP and CTP. The labeled RNA was purified by a series of steps to remove DNA, protein and free [^{32}P]GTP. The reaction was incubated for 10 min at 37°C with 30 μg/ml DNase I (BRL) in the presence of 1 mM $CaCl_2$, followed by 30 min treatment at 45°C with 100 μg/ml proteinase K in the presence of 5 mM EDTA and 1% SDS. NaCl was added to 0.12 M, one phenol/chloroform extraction was performed, followed by precipitation in 10% trichloroacetic acid at 4°C for 30 min. The labeled RNA was recovered on type HA nitrocellulose filters (0.45 μm pore size) by suction filtration. The filters were incubated with 50 μg/ml DNase I in the presence of 50 mM Tris (pH 7.5), 5 mM $MgCl_2$ and 1 mM $CaCl_2$ for 30 min at 37°C. The labeled RNA was eluted from the filters by adjusting the buffer to 5 mM EDTA and 1% SDS and raising the temperature to 68°C. Elution was performed three times, 10 min each. The eluate was incubated with 25 μg/ml proteinase K at 21°C for 30 min followed by the addition of NaCl to 0.12 M, phenol/chloroform extraction, two steps of ethanol precipitation (the second precipitation in the presence of 1 M ammonium acetate), 80% ethanol wash and resuspension in 20 μl of hybridization buffer (Wang and Calame, 1985). Typically, 3×10^7–10^8 c.p.m. of ^{32}P-labeled RNA was recovered per reaction. For the hybridization, 1 μg of purified insert DNA was denatured, slot blotted in duplicate onto nitrocellulose, and vacuum baked for 1 h at 80°C. After hybridization for 3 days at 42°C, the filters were washed at 45°C; two times (30 min each) in buffer A [300 mM NaCl, 2 mM EDTA and 10 mM Tris (pH 7.5)] containing 0.1% SDS followed by 30 min wash in buffer B [10 mM NaCl, 2 mM EDTA, 5 mM Tris (pH 7.5), 0.4% SDS]. The filters were dried and exposed to X-ray film overnight. The results shown are filters which have been further incubated in buffer A containing 10 μg/ml RNase A and 1 μg/ml RNase T1 for 20 min at 37°C, followed by autoradiography for 3–10 days. The signal is 3–5-fold weaker in RNase treated filters compared to untreated filters, but the background signal is substantially reduced. RNase treated and untreated filters display similar patterns of hybridization (unpublished results).

DNA probes

The murine TCR-α cDNA (pαDO) obtained from Dr E.Palmer is a full length sequence containing V, J and C sequences (Yague *et al.*, 1985). The murine JC intron$_\alpha$ genomic segment obtained from Dr S.Hedrick and Dr M.Becker is a 1.2 kb *Eco*RI fragment 1.7 kb 5′ of C_α (Winoto *et al.*, 1985). The murine TCR-β cDNA and $J_{\beta1}$ and $J_{\beta2}$ genomic sequences were also obtained from S.Hedrick. The murine TCR-β cDNA (86T5) contains D, J and C sequences and part of the V region (Hedrick *et al.*, 1984). The murine $J_{\beta1.3-1.7}$ and $J_{\beta2.1-2.7}$ genomic sequences are 1.6 kb *Bam*HI/*Sac*I and 1.2 kb *Eco*RI/*Cla*I fragments, respectively (Gascoigne *et al.*, 1984). CHO-A is a chinese hamster ovary cDNA obtained from Dr M.G.Rosenfeld which recognizes transcripts highly transcribed by most mammalian cells (Harpold *et al.*, 1979). The murine muscle actin cDNA is 0.9 kb *Pst*I fragment obtained from K.Calame.

Densitometry scanning

Autoradiographic XAR-5 film was exposed to the extent that it was in the linear range of the LKB UltraScan XL 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 ^{32}P.

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