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Transcriptional and post-transcriptional regulation of TcR, CD4 and CD8 gene expression during activation of normal human T lymphocytes

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We previously showed that the turnover rates of the messengers coding for the T cell receptor (TcR) α, β and γ, CD4 and CD8 molecules composing the multireceptor complex vary in normal human mature T lymphocytes according to their state of activation. Activation by soluble anti-CD3 which does not induce proliferation, promotes a weak up-modulation of the corresponding five mRNAs. In contrast, activation signals such as anti-CD3 + PMA, which lead to lymphokine mRNA expression and T cell proliferation, promote a decrease of the TcR, CD4 and CD8 mRNA levels within 4 h post-activation, followed by their gradual re-expression. Here we show that the down-modulation of these mRNAs results from early regulation controls at transcriptional and post-transcriptional levels, i.e. through a concomitant inhibition of transcription and destabilization of the mRNA. Moreover, later re-expression of the mRNA results from recovery of transcription and marked increase of the mRNA stability. Finally, down-modulation is specific for TcR, CD4 and CD8 mRNAs, all submitted to similar regulation processes. These results strongly suggest a direct correlation between down-modulation of the multireceptor complex mRNAs, and lymphokine mRNA expression, and cellular proliferation.

Key words: mRNA expression/normal human T lymphocytes/post-transcriptional regulation/T cell receptor/transcriptional regulation

Introduction

The T cell receptor (TcR) is composed of either an $\alpha-\beta$ (Saito *et al.*, 1984) or a $\gamma-\delta$ (Borst *et al.*, 1987, Moingeon *et al.*, 1987) heterodimer and is associated with the monomorphic CD3 subunits (Brenner *et al.*, 1985). TcR $\alpha-\beta$ is responsible for the recognition of antigen presented on MHC molecules (Hood *et al.*, 1985), and CD3 plays a role in transducing the activation signal (Cantrell *et al.*, 1987). The mutually exclusive CD4 and CD8 antigens previously considered as solely accessory molecules, also seem to contribute to T cell activation by delivering intracellular signals (MacDonald *et al.*, 1982; Rogozinsky *et al.*, 1984; Blue *et al.*, 1987; Carrel *et al.*, 1988) in association with the tyrosine kinase $p56^{lck}$ (Alexander and Cantrell, 1989; Barber *et al.*, 1989; Veillette *et al.*, 1989). Moreover, the TcR-CD3 complex is physically and functionally associated to CD4 or CD8 (Emmrich, 1988), all belonging to the immunoglobulin supergene family (Hood *et al.*, 1985). Therefore, the TcR-CD3 complex associated with CD4 or CD8 constitutes a multimeric complex through which physiological T cell activation occurs, and will be referred to as the multireceptor complex (MRC) in this report. Although this MRC is the natural target for physiological stimulation with antigen, artificial ligands such as monoclonal antibodies directed against TcR or CD3 molecules can also mimic the activation signals. Thus, cross-linked anti-CD3 induces proliferation of quiescent T cells (Van Vauwe *et al.*, 1980), whereas no proliferation is observed with soluble anti-CD3. In the latter case, an additional signal provided by macrophages, interleukin-1, or phorbol myristate acetate (PMA) is required to initiate proliferation (Heckford *et al.*, 1986; Meuer and Meyer, 1986). The direct consequence of T cell activation either by antigen, anti-CD3 or PMA is the internalization of TcR-CD3 as well as the co-modulation of CD4 and CD8 molecules (Hoxie *et al.*, 1986; Minami *et al.*, 1987; Saizawa *et al.*, 1987; Takada and Engleman, 1987; Weyand *et al.*, 1987). However, if modulation of the MRC proteins at the membrane has been extensively studied, little is known about the modulation of the corresponding messengers during T cell activation (Kuziel *et al.*, 1987; Lindsten *et al.*, 1988; Paillard *et al.*, 1988; Spolski *et al.*, 1988).

Recently, we have shown (Paillard *et al.*, 1988) that a suboptimal signal of activation (soluble anti-CD3 or PMA alone) resulted in a slight up-modulation of the messengers of the TcR as well as those of CD4 and CD8, concurrently with the mRNA expression of the receptor of interleukin-2 (IL-2R) and in absence of cellular proliferation. In contrast, a strong activation signal triggered via the MRC (anti-CD3 + PMA) or via the CD2 molecule (anti-CD2) resulting in T cell proliferation led to a specific transient down-modulation of messengers of the MRC within 4-7 h, followed by their gradual re-expression. Interestingly, these phenomena have been reproducibly observed in polyclonal T cells populations, but also in a $CD8^+$ T cell line and in different $CD4^+$ clones (Paillard *et al.*, 1988 and unpublished data).

Here, we show that the down-modulation of these mRNAs is the result of transcriptional and post-transcriptional regulations, i.e. through a decrease of transcription and an early destabilization of the corresponding messengers. This is followed by a recovery of transcription and a marked post-transcriptional stabilization process which account for the re-expression of the MRC mRNAs observed 24 h after activation.

Results

Transient and specific down-modulation of the MRC mRNA after anti-CD3 + PMA activation

In order to study the kinetics of expression of MRC mRNA (i.e. TcR, CD4 and CD8 mRNA), a polyclonal human T

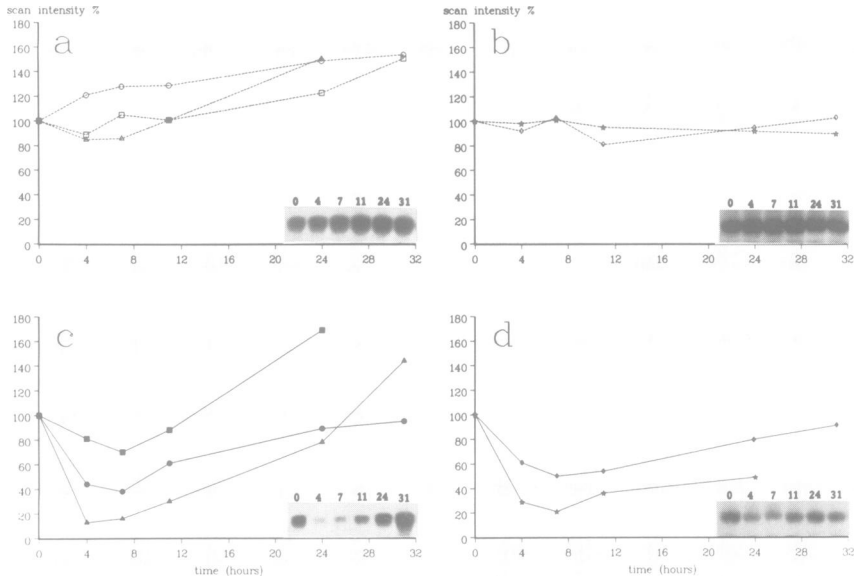

Fig. 1. Kinetics of accumulation of the MRC mRNAs. T cells were activated either with anti-CD3 (**a** and **b**), or anti-CD3 + PMA (**c** and **d**). The mRNA levels were measured at the indicated times after Northern blotting, and normalized to the 28S rRNA level of each RNA sample (see Materials and methods). Values are expressed as percentage of the mRNA level measured before activation (0 h). The experiment shown on the Figure is representative of three independent experiments. Open symbols represent anti-CD3 activation, and closed symbols anti-CD3 + PMA activation. TcRα (○,●), TcRβ (□,■), TcRγ (△,▲), CD4 (◇,◆), and CD8(☆,★) mRNA levels were analysed and plotted. On the insets are shown the Northern blots obtained for TcRγ mRNA (a and c) and CD8 mRNA (b and d).

cell population (PHA cell line) was activated either by anti-CD3 monoclonal antibodies, leading to incomplete activation with no cellular proliferation, or by anti-CD3+PMA, leading to complete activation with proliferation (800 and 34 000 c.p.m., respectively). The accumulation level of the corresponding mRNAs of the MRC genes was analysed by Northern-blot experiments of total RNA at different times after either type of activation (Figure 1). We found that anti-CD3 alone induced a slight (TcRα, β and γ; Figure 1a) if any increase (CD4 and CD8; Figure 1b) in mRNA accumulation. No change in the MRC mRNA level was observed in the absence of activation (data not shown). In contrast, a full activation leading to cellular proliferation, such as that observed after anti-CD3+PMA, induced a transient down-modulation of the MRC mRNAs. The mRNA level of the MRC genes dropped to a minimal level between 4 and 7 h and re-increased markedly thereafter (Figure 1c, d). Thus, the five mRNAs of the MRC reproducibly followed similar transient patterns of down-modulation although with different magnitudes, ranging from a 30% decrease for TcRβ, to 80% for TcRγ and CD8. In contrast, the genes known to be activated during stimulation leading to cellular proliferation, such as interferon (IFN) γ and IL-2R (Figure 2) were induced rapidly and transiently after anti-CD3+PMA activation as previously described (Vaquero *et al.*, 1984; Paillard *et al.*, 1988), whereas actin mRNA did not vary during activation (Figure 2).

Transcriptional inhibition of the MRC mRNAs after anti-CD3+PMA activation

As the MRC mRNAs present in T cells were transiently down-regulated following anti-CD3+PMA activation, transcriptional and/or post-transcriptional mechanisms could be responsible for this down-modulation.

To investigate the modulation of the steady-state mRNA at the transcriptional level, we compared the fate of the MRC

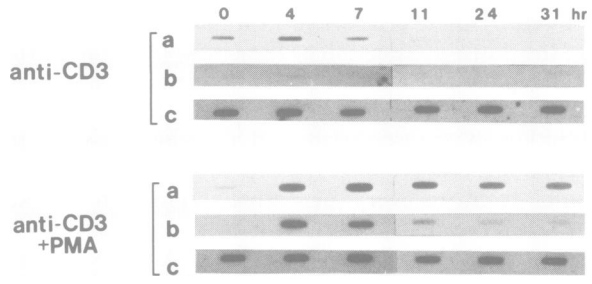

Fig. 2. Kinetics of accumulation of IL-2R, IFNγ and actin mRNAs after activation with either anti-CD3 or anti-CD3 + PMA. Slot-blots of total mRNA (5 μg) extracted at different times after activation were sequentially hybridized with IL-2R (**a**) IFNγ (**b**) and actin (**c**) riboprobes.

mRNAs early after activation with either anti-CD3 or anti-CD3+PMA, and after actinomycin D treatment. As already shown, anti-CD3 alone did not induce the down-modulation of TcRα and CD8 mRNAs (Figure 1a, b; Figure 3, lanes a) whereas anti-CD3+PMA did so (Figure 1c, d; Figure 3, lanes b). Interestingly, the decay of these mRNAs observed after anti-CD3+PMA paralleled that observed when transcription of non-treated cells was blocked with actinomycin D (Figure 3, lanes c). Similar results were also obtained with all the other genes of the MRC (data not shown). These results suggest that a strong activation signal such as anti-CD3+PMA could trigger a specific transcriptional inhibition of the MRC genes but do not exclude additional effect on mRNA stability.

The presence of a transcriptional decrease was confirmed by run-on analyses of the nuclear RNA which measure directly the transcriptional level during activation. The genes coding for the MRC molecules were transcribed constitutively in non-activated T cells (Figure 4, 0 h). Anti-CD3 either did not change, or slightly increased, the level of

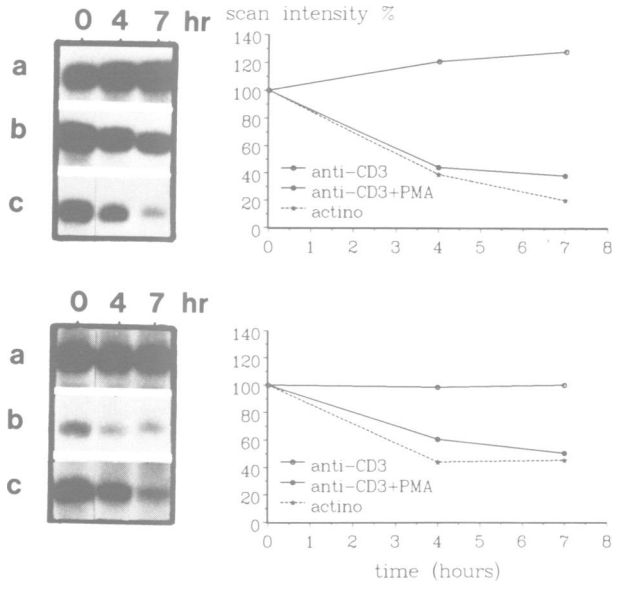

Fig. 3. TcRα and CD8 mRNAs steady-state after different treatments. T cells were treated with either anti-CD3 (**a**), or anti-CD3 + PMA (**b**) or actinomycin D (actino) (**c**). Total RNA was extracted before treatment and 4 and 7 h after treatment and was used for determination of the levels of TcRα (top) and CD8 mRNAs (bottom). The corresponding Northern-blots are shown on the left panels. The intensity of hybridization signals was normalized to 28S rRNA level in each sample by densitometric scanning and plotted (right panels) as percentage of the signal observed prior to treatment. Weaker signals observed for CD8 (lanes b) are due to lower RNA amount loaded on the membrane.

transcription of these genes (data not shown). In contrast, anti-CD3+PMA led to an early transient decrease of transcription of the various MRC genes within 2 h post-activation. This decreased level of transcription was maintained during at least 4 h post-activation (data not shown). Full recovery of MRC mRNA levels was observed at 24 h (Figure 4). These transcriptional modulations appeared to be specific for the MRC genes, since the CD2 and HLA-A3 genes, also belonging to the immunoglobulin supergene family (Sayre *et al.*, 1987 and Hood *et al.*, 1985) had no significant change of their transcriptional levels (Figure 4). In addition, and as expected, IFNγ was not transcribed at 0 h, and IL-2R only weakly, and their transcription was strongly induced after anti-CD3+PMA activation. However, although significant and specific for the MRC genes, the magnitude of these modulations of transcription depended on the MRC gene studied and were not dramatic. This suggested that the decrease of transcription was most likely not the only mechanism accounting for the down-modulation of the MRC mRNAs.

Early destabilization of the MRC mRNAs after anti-CD3 + PMA activation

Since the decrease of transcription triggered by anti-CD3 + PMA activation seemed to contribute only partially to the down-modulation observed specifically for the MRC mRNAs, this early down-modulation could be also due to an added post-transcriptional destabilization of the corresponding messengers. To address this point, we measured the half-life of these mRNAs between 0 and 4 h after anti-CD3 + PMA activation using actinomycin D treatment to block RNA transcription. Figure 5 shows as an illustration the detailed data of TcRα and CD8 mRNA. In the quiescent T lymphocytes their calculated half-lives were 4 h and 4.75 h respectively. Similar ranges of mRNA degradation rates were observed for all the MRC mRNAs analysed (between 3.15 and 4.75 h), as summarized in Table I. When T cells were activated by anti-CD3 + PMA, destabilization of the TcRα mRNA occurred within 1 h post-activation. At this time point, the half-lives of TcRα and CD8 mRNAs were equal to 2.05 h and 3 h, respectively (Table I and Figure 5). Thereafter, the half-life of the MRC mRNAs re-increased progressively to reach the initial level (0 h) approximately 3 h after activation (Table I). Moreover, this early and transient destabilization appeared to be specific for the MRC mRNAs since no variations of the actin mRNA half-life were observed (data not shown). It is then likely that both this destabilization and the inhibition at the transcriptional level accounted for the marked down-modulation of the MRC mRNAs.

Stabilization of the MRC mRNAs 3 h after anti-CD3 + PMA activation

Run-on experiments indicated that the rate of transcription re-increased at 24 h after anti-CD3 + PMA activation (Figure 4). However, it was still possible that variations in the mRNA stability could also account for the recovery of mRNA accumulation. To address this point, we compared the stability of the five MRC mRNAs at 0 h before activation, at 4 h when mRNA accumulation reached a minimal level, and at 24 h after anti-CD3 + PMA activation when full re-expression had occurred. The mRNA stability was also compared with that obtained after anti-CD3 activation at the same time points. Similar patterns were observed for all the MRC mRNAs and, as in the previous figure, the results obtained for the TcRα and CD8 mRNAs are presented as an example (Figure 6). When T cells were activated by anti-CD3 alone, the half-life of these mRNAs increased only very slightly throughout activation (Figure 6a, c). On the other hand, after anti-CD3 + PMA activation the MRC mRNAs became very stable after 4 and 24 h of activation, with an increased half-life ranging from 2-fold to more than 10-fold (Figure 6b, d; Table I). This re-increase in mRNA half-lives appeared to be specific of the MRC mRNAs since the half-life of actin mRNA, also constitutively expressed in T cells, did not vary (data not shown). These results strongly support that re-expression of the mRNAs observed 24 h after anti-CD3 + PMA activation resulted, at least in a large part, from an increased mRNA stability.

Discussion

We showed that two surface-mediated T cell activation pathways leading to proliferation or not, and both delivered, at least in part, via the CD3 molecule, were capable of differently altering the expression of the MRC genes. The level of MRC mRNAs is dynamically regulated during activation of T lymphocytes by anti-CD3 + PMA through variations of the rates of both synthesis and degradation. Thus, activation triggered an early decrease of both transcription and mRNA stability. Stability of MRC mRNAs re-increased rapidly to reach a level at 3 h post-activation similar to that observed prior to activation. However, at that time point, transcription of the genes was still inhibited (data

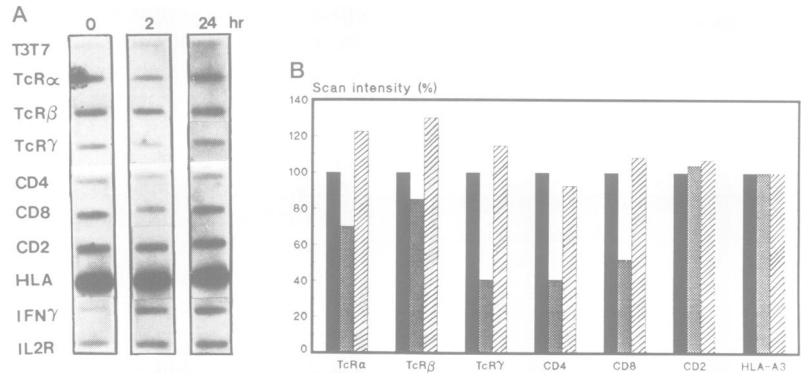

Fig. 4. Transcriptional level of MRC genes after anti-CD3 + PMA activation. Nuclear RNA run-on analyses were performed at 0, 2, and 24 h after activation as described in Materials and methods with the indicated linearized vectors (**A**). Hybridization signals were quantitated by densitometric scanning at 0 h (■), 2 h (▩) and 24 h (▨) after activation and the values were plotted as percentage of the hybridization signals obtained at 0 h (**B**). The figure is representative of 4 different experiments.

not shown) and most likely accounted for the delayed recovery of mRNA accumulation observed 7 h after activation. Down-modulation of the MRC mRNAs always paralleled the lymphokine mRNA expression induced by strong activation signals such as that triggered by either antigen, or anti-CD2, or anti-CD3 + PMA (Paillard *et al.*, 1988), as well as Con A (Kuziel *et al.*, 1987). However, it is not clear whether these two phenomena are only coincident or functionally related. Interestingly, all the activation signals mentioned above that down-modulate the MRC mRNAs, are known also to induce the early intracellular calcium increase and protein kinase C (PKC) activation, which combination leads to proliferation (Hadden, 1988). In contrast, activation signals such as anti-CD3 alone or PMA alone neither induce down-modulation of MRC mRNAs nor cellular proliferation (Paillard *et al.*, 1988). Therefore, the synergistic effects of increased intracellular calcium and activation of PKC might be involved in the down-modulation of the MRC mRNAs. Since we showed that down-modulation was the result of a concomitant early transcriptional inhibition of these genes and a destabilization of their mRNAs, these intracellular signals appeared to be synergistically required to act at two different regulatory levels: in the nucleus at the transcriptional level, and in the cytoplasm, at the level of the mRNA stability.

All the genes of the MRC are regulated similarly at both the transcriptional and post-transcriptional levels during activation, although the magnitude of these regulations could vary within the five MRC genes we studied. Therefore, the concomitant regulation of the different genes of the MRC might be due to the presence of common structural features within either their promoter and/or other non-coding and coding sequences.

No extensive study of the promoter sequences of the different MRC genes has yet been achieved. Nevertheless, a recent comparison of 14 murine TcR V β chain promoter sequences has illuminated the presence of a highly conserved decanucleotide located within a 600 bp sequence upstream from the transcription cap site (Anderson *et al.*, 1988). This sequence shares some homology with regulatory elements such as cAMP-responsiveness and AP1-binding sequences (Anderson *et al.*, 1988), and was shown to bind a protein(s) present in nuclear extracts of T cell lines and peripheral blood leukocytes, in the case of a human TcR V β promoter region (Royer and Reinherz, 1987). This decanucleotide has also been found in the TcRα gene promoter (Luria *et al.*, 1987). This sequence, and possibly other sequence elements not yet identified, present in promoters or other regions, might bind

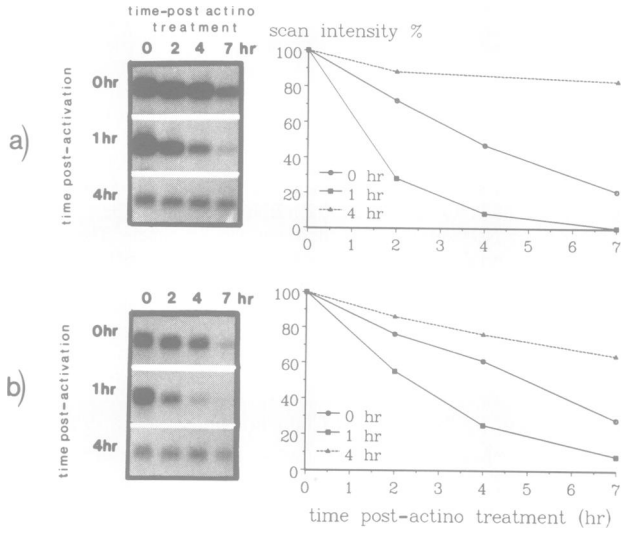

Fig. 5. Half-lives of TcRα and CD8 mRNAs early after anti-CD3 + PMA activation. Cells were treated with actinomycin D (actino) before activation (0 h), and 1 and 4 h after activation, and total RNA was extracted at the indicated times after actinomycin D treatment. The Northern blots hybridized with TcRα (**a**) and CD8 (**b**) riboprobes are shown on the left panels. Quantification analysis of each RNA time point (right panels) is performed as described in Figure 3, and values were expressed as percentage of the mRNA level observed prior to actinomycin D treatment.

Table I. Half-life of MRC mRNAs after anti-CD3 + PMA activation

Time post-activation	mRNA half-life[1]				
	TcRα	TcRβ	TcRγ	CD4	CD8
0 h	4	3.9	3.15	4.75	4.75
1 h	2.05	3	2.1	2.55	3
2 h	2.9	ND	ND	3.3	ND
3 h	4.9	ND	ND	5.5	ND
4 h	>20	>20	9.8	8.75	12.5
24 h	6.6	>20	9.5	>20	>20

[1]Half-lives expressed in hours were calculated with the least-square method (see Materials and methods) from two independent experiments (less than 10% variation)

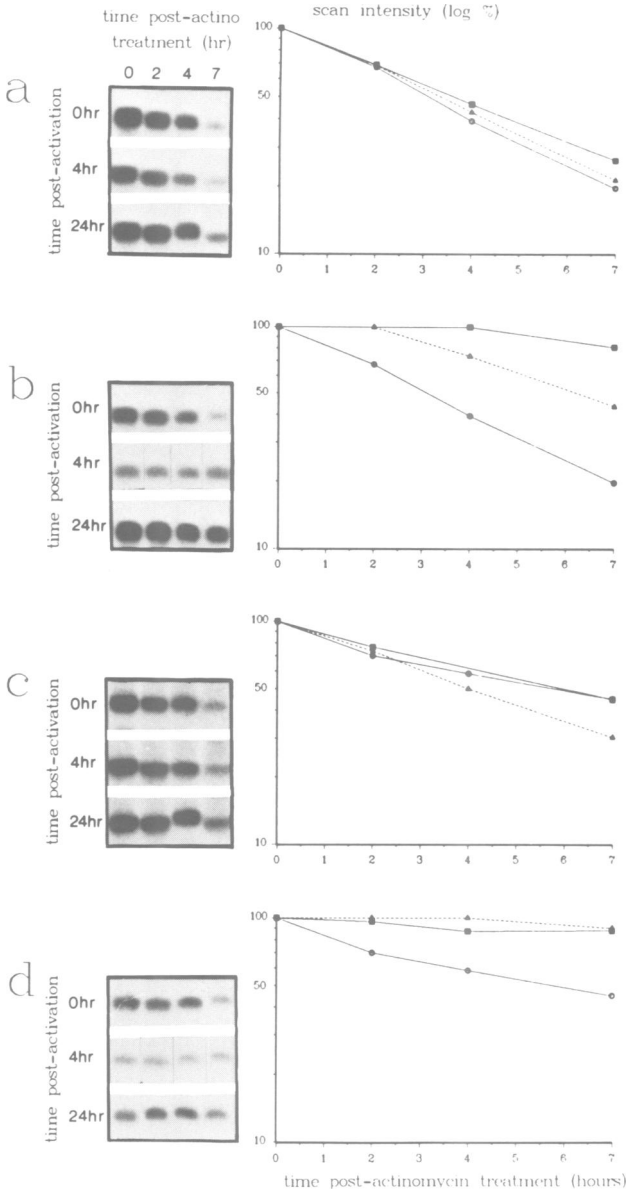

Fig. 6. Half-lives of TcRα and CD8 mRNAs after activation with either anti-CD3 or anti-CD3 + PMA. Cells were treated with actinomycin D (actino) at 0 h (⊖), 4 h (■) and 24 h (▲) after anti-CD3 activation (**a** and **c**), or anti-CD3 + PMA activation (**b** and **d**). Total RNA was extracted 0, 2, 4 and 7 h after actinomycin D treatment, and processed as described in Materials and methods. The Northern blots hybridized with TcRα probe (a and b) and CD8 probe (c and d) are shown on the left of the Figure. The mRNA levels were quantitated by densitometric scanning of the autoradiograms after normalization to 28S rRNA level of each sample, and plotted on the right as percentage of the hybridization signals obtained before actinomycin D treatment.

regulating factors induced or modified specifically during activation and therefore modulate the rate of transcription. The little differences reported in the sequences of the decanucleotide regulatory elements present in the TcRα and β, as well as differences in putative other regulatory elements, might explain the variations of magnitude we observed in the modulation of these genes.

We showed that the stability of the MRC mRNAs appeared also to be coordinately controlled. Thus, common regulatory sequences might also be present that influence mRNA degradation. The early destabilization occurring after anti-CD3 + PMA activation might involve either disappearance or inactivation of a stabilizing factor(s), or induction or activation of a destabilizing factor(s), as already described for several other mRNAs (Brewer and Ross, 1989; Weber *et al.*, 1989). The increased mRNA stability that we observed later on during activation could result from reverse modifications in the level of stabilizing and/or destabilizing factors. Such labile factors interacting with the MRC genes are yet to be identified.

Finally, no simple correlation was observed between modulation of the MRC proteins from the cellular membrane, modulation of their messengers and cellular proliferation. Indeed, either anti-CD3 alone, PMA alone, or the combination of anti-CD3 and PMA was capable to induce internalization of the MRC proteins, and a decreased expression of the proteins at the cellular membrane leading to a refractory period of activation (Reinherz *et al.*, 1982). The decreased expression of the MRC molecules at the cellular membrane might then be amplified in the case of a strong activation which induces mRNA down-modulation. However, 24 h after a strong activation, the full re-expression of the messengers we observed was not accompanied by a concomitant re-expression of the corresponding proteins, which occurs only 3 days after activation (Reinherz *et al.*, 1982). This delayed protein re-expression might be due, at least in part, to a translational regulation of the messengers. Such a complex mechanism of regulation occurring both at the mRNA and protein levels might contribute to a negative feed-back system, physiologically necessary to control T cell activation.

Materials and methods

T cell preparation and activation

Human peripheral blood mononuclear cells from healthy donors were obtained by Ficoll centrifugation. The T cells were therefore activated by phytohemagglutinin A (PHA) (1 μg/ml) and maintained in culture for 14 days in the presence of recombinant IL-2 (PHA cell lines). 14 days after PHA treatment, the cells were 100% CD3-positive as determined by immunofluorescence staining using anti-CD3 monoclonal antibodies. At that time, the T cell population expressed low levels of IL-2R, and neither IL-2 nor IFN_γ mRNAs. They were further stimulated either with anti-CD3 monoclonal antibody at 1/1000 dilution of ascitis fluid or with anti-CD3 plus PMA (1 ng/ml) (Paillard *et al.*, 1988). Cell proliferation was estimated by [^{3}H]thymidine uptake on the third day of culture. Transcription was blocked with 3 μg/ml of actinomycin D.

RNA extraction, Northern-blot and slot-blot analysis

Total cellular RNA was extracted at the indicated times by using the guanidinium isothiocyanate procedure (Vaquero *et al.*, 1982). Equal amounts of total RNA (8 μg) were fractionated by agarose gel electrophoresis after glyoxal denaturation and transferred to a PALL membrane (PALL Industries) with a vacuum-blot apparatus (LKB). For slot-blots, the RNA was directly immobilized on the membrane with a slot-blot apparatus (Minifold II, Schleicher and Schüll) after a formaldehyde denaturation step. The membranes were sequentially hybridized at high stringency with riboprobes obtained after *in vitro* transcription of T3T7 Bluescribe vectors (Vector Cloning System) containing either the 0.38 kb *Pvu*II fragment of the C region of human (hu) TcRα, the 0.4 kb *Bgl*II from the C region of hu TcRβ, the 1.4 kb *Eco*RI–*Acc*I from the DJC region of hu TcRγ (F.Triebel), the 1.4 kb *Eco*RI–*Bam*HI from hu CD4 (Maddon *et al.*, 1985), the 1.5 kb *Eco*RI from hu CD8 (Littman *et al.*, 1985), the 1.35 kb *Pst*I–*Bam*HI from IL2R3 cDNA (Leonard *et al.*, 1984), the 1 kb *Hin*cII from hu IFNγ cDNA (Paul Sondermeyer Transgene; Vaquero *et al.*, 1984), the 1.3 kb *Pst*I from HLA-A3 cDNA (D.Piatier), or the 1.2 kb *Pst*I from actin cDNA (S.Gisselbrecht). The conditions of transcription were as advised by the manufacturer. Riboprobes were used at 5×10^5 c.p.m./ml of hybridization medium (50% formamide, 5 × SSC, 10 × Denhardt's solution, 5 mM EDTA, 50 mM Tris, pH 7.5, 0.1% pyrophosphate, 1% SDS, 500 μg/ml

yeast tRNA and 5% dextran sulphate), and following high stringency washes, the radioactive signals were detected by autoradiography with intensifying screens at −80°C.

Quantitative analysis of mRNA hybridization signals was performed by scanning densitometry (Shimadzu). For all RNA preparations, MRC mRNA levels were first normalized to the corresponding 28S rRNA level measured after hybridization with the 28S rRNA-labelled oligonucleotide described by Barbu and Dautry (1989). These MRC mRNA levels were then expressed as percentage of the mRNA level observed either prior to activation or prior to actinomycin D treatment. The mRNA half-lives were determined from 'best-fit' regression lines obtained by using the least-square method from three to four time points in each experiment.

Transcription in isolated nuclei ('run-on')

10^8 cells were washed in ice-cold HB (10 mM Tris, pH 7.6, 10 mM NaCl, 10 mM $MgCl_2$) and resuspended in 0.5 ml of HB. The cells were lysed by addition of 0.2% Nonidet P-40 in HB and after 1 min on ice were diluted with 40 ml ice-cold HB. Nuclei were collected by centrifugation for 3 min at 2000 r.p.m. and resuspended in 200 μl of storage buffer (40% glycerol, 5 mM Tris-Cl, pH 8.3, 10 mM $MgCl_2$, 0.1 mM EDTA) and frozen in liquid nitrogen. Transcription reactions were performed with 200 μl of the isolated nuclei in a final volume of 400 μl with 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.05 mM S-adenosyl methionine, 0.12 mM KCl, 1 mM $MgCl_2$ and 2.5 mM DTT final concentration, incubated in the presence of 200−300 μCi of [^{32}P]UTP during 1 h at 25°C. Total RNA was extracted by the guanidinium isothiocyanate procedure (Vaquero *et al.*, 1982), treated with 0.1 N NaOH for 8 min in ice, neutralized with 0.28 M HEPES buffer and precipitated. Equivalent amounts of radioactivity were hybridized to the PALL membrane after immobilization of 5 μg of the various linearized plasmids (including the T3T7 vector as a control) using a slot-blot apparatus (Marzluff and Huang, 1984). Hybridizations were performed for three days at 52°C in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 1 mM EDTA, 10 mM Tris−HCl pH 7.4, 0.2% SDS, 500 μg/ml salmon sperm DNA, 500 μg/ml tRNA, and with 2−6 × 10^6 c.p.m./ml. Non-hybridized radioactivity was removed by washings and when necessary, by RNase A and proteinase K treatment.

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