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# PHORBOL ESTERS DOWN-REGULATE TRANSCRIPTION AND TRANSLATION OF THE CD4 GENE[1]

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**This study investigated the effects of PMA on biosynthesis and transcription of the CD4 molecule and gene in order to define mechanisms resulting in reduced cell surface expression of the CD4 molecule after treatment with PMA. Cells treated with PMA showed reduced biosynthesis of the CD4 molecule but not of class I HLA molecules. Furthermore, PMA treatment resulted in reduced steady-state levels of CD4 mRNA and inhibition of the relative rate of transcription of the CD4 gene. Cells expressing transfected CD4 cDNA gene products modulated in response to PMA, however, re-expressed CD4 earlier than cells expressing the product of the wild-type CD4 gene. These data suggest that the cell surface expression of the CD4 molecule is probably down-regulated at the level of the protein, as well as the gene, and that inhibition of transcription may affect the kinetics of CD4 expression. These observations provide further insight into the mechanisms by which HIV affects expression of CD4.**

The CD4 molecule is a 55-kDa glycoprotein expressed on a subset of T lymphocytes, monocytes, and brain, as well as on some nonlymphocytic leukemic cell lines (1). The CD4 molecule is involved in the regulation of T lymphocyte function through several mechanisms. CD4 interacts with class II molecules expressed on Ag-presenting cells, which increases the affinity of the TCR (2–9). CD4 has also been shown to initiate and regulate biochemical signals involved in T lymphocyte activation (10–14). CD4 has been identified as the receptor for HIV (15–18).

The mechanisms regulating CD4 expression are not well understood. Modulation of CD4 expression occurs after stimulation with PMA, Ag, or HIV, all of which induce phosphorylation of serine residues within the IC[3] tail of CD4 (19–27). Phosphorylation of CD4 is necessary for modulation to occur, as deletion of phosphorylation sites by site-directed mutagenesis or by truncation of the IC tail abrogates PMA-induced modulation (26–28).

Three observations suggest that PMA may also affect transcription and/or biosynthesis of CD4. First, after PMA-induced modulation, re-expression of CD4 to pretreatment levels occurs by 48 to 72 h (21, 23). Because CD4 is detected within several hours after biosynthetic labeling (1, 11), ongoing CD4 biosynthesis and transport cannot account for the relatively long time required for re-expression. Second, HIV-infected cells have reduced steady-state levels of CD4 mRNA (29). Because both PMA and HIV cause phosphorylation of CD4, these observations suggest that PMA may affect transcription, translation, and/or transport of CD4. Third, PMA treatment of murine cells results in reduced steady-state levels of CD4 mRNA (30). Unlike human cells, however, not all $CD4^+$ murine cells modulate in response to PMA, which suggests that these results cannot be extended to human CD4 (30). For these reasons, we studied the effects of PMA on CD4 biosynthesis and transcription to further define the molecular events that regulate CD4 expression.

## MATERIALS AND METHODS

*Antibodies and reagents.* The characteristics of anti-CD4 mAb, 6B10, have been previously described (11). W6/32, detecting a nonpolymorphic region of class I HLA molecules, and OKT3 (anti-CD3) were derived from hybridomas obtained from the American Type Culture Collection (Rockville, MD). Control ascites fluid containing nonbinding antibody was generously provided by Dr. James Lessard, Children's Hospital, Cincinnati, OH. PMA was purchased from Sigma Chemical Co., St. Louis, MO.

*CD4 biosynthesis.* One hundred million MOLT 4 cells ($2 \times 10^6$/ml) in RPMI 1640 containing 10% FCS were stimulated with $10^{-8}$ M PMA for 0 to 48 h at 37°C, 5% $CO_2$. Cells were washed and resuspended at $2 \times 10^6$/ml in methionine-free RPMI 1640 formula 78-0404 (GIBCO) supplemented with 10% FCS. [$^{35}$S]methionine (>1000 Ci/mM, New England Nuclear, Boston, MA) was added to a final concentration of 1000 $\mu$Ci/$15 \times 10^6$ cells. The cultures were then incubated at 37°C in 5% $CO_2$ for 4 h. Immunoprecipitation of lysate from $7.5 \times 10^6$ cells with 6B10 and control antibodies was carried out as described previously (1, 11).

*Northern blot analysis.* Total RNA was isolated from control and PMA-treated MOLT 4 cells as previously described (31). Twenty micrograms of total RNA were electrophoresed in 1% agarose gels containing formaldehyde and transferred to a nylon membrane (Gene Screen Plus, New England Nuclear) according to the manufacturer's recommendation. Verification that equal amounts of RNA were applied to each lane was also done by analysis of ethidium bromide staining of the gels (data not shown). A 3.0-kb cDNA encoding the CD4 protein and including the 1.3-kb 3′ untranslated region was generously provided by Dr. Paul Maddon, Columbia University, New York, NY (32). A cDNA probe detecting $\beta$-actin was provided by Dr. James Lessard. A cDNA probe detecting the 3′ untranslated region of the HLA-A2 gene (pHLA-2a.1) was provided by Dr. Harry Orr, University of Minnesota. Probes were labeled with [$^{32}$P]dCTP (Amersham Corp., Arlington Heights, IL), by using the random primer method (33). Membranes were hybridized to [$^{32}$P]-labeled

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[3] Abbreviations used in this paper: IC, intracytoplasmic; hnRNA, heteronuclear RNA.

probes at 42°C in buffer containing 50% formamide, 10% dextran sulfate, 1.0% SDS, 1.0 M NaCl, and denatured salmon sperm. Hybridized membranes were washed twice in 2× SSC (2× SSC = 0.3 M NaCl, 0.03 M Na citrate) for 5 min at room temperature followed by washing with 2× SSC, 1.0% SDS for 30 min at 60°C and washing with 0.1× SSC for 30 min at room temperature. Membranes were exposed to XAR film (Eastman Kodak, Rochester, NY) with two Lightning Plus intensifying screens for a period of 4 to 5 days at −70°C.

*Isolation of nuclei.* MOLT 4 cells ($5 \times 10^8$ cells at $2 \times 10^6$/ml in RPMI 1640-10% FCS) were treated with $10^{-8}$ M PMA for 16 h. Nuclei from treated and untreated cells used for in vitro chain elongation transcription studies were isolated at 4°C as described (34). Cells were pelleted by centrifugation at $50 \times g$ for 20 min and washed three times in 30 mM Tris-HCl (pH 7.4), 120 mM KCl, 5 mM Mg acetate, and 7 mM 2-ME. Pellets containing approximately $5 \times 10^8$ cells were resuspended in 5 ml of homogenization buffer, composed of 0.3 M sucrose containing 2 mM Mg acetate, 3 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.5), 0.1% Triton X-100 and 0.5 mM 2-ME. The cells were disrupted with eight strokes of a Dounce homogenizer by using a B-type pestle. The homogenate was then gently overlaied on 6 ml of 1.4 M sucrose cushion containing 5 mM Mg acetate, 10 mM Tris-HCl (pH 7.5), and 0.5 mM 2-ME in a Falcon 2059 tube. Nuclei were then pelleted by centrifugation at $2400 \times g$ for 30 min at 4°C in a Beckman JS-13 rotor. The pelleted nuclei were washed and resuspended at a concentration of $2 \times 10^8$/ml in 25% glycerol containing 5 mM Mg acetate, 0.1 mM EDTA, 5 mM 2-ME, and 50 mM HEPES/NaOH (pH 7.5). Aliquots (0.1 ml) of resuspended nuclei were quick-frozen in liquid nitrogen and stored at −70°C. Nuclei prepared by this method have been shown to retain their capacity for continued elongation of nascent primary transcripts by RNA polymerase II for more than 12 mo.

*In vitro chain elongation.* $2 \times 10^7$ nuclei were incubated for 20 min in 0.5 ml of buffer containing 7.5% (w/v) glycerol, 19 mM HEPES/NaOH (pH 7.5), 3.8 mM Mg acetate, 84 mM KCl, 30 μM EDTA, 3 mM 2-ME, 10 mM $NH_4Cl$, 130 μg/ml creatinine phosphokinase, 10 mM creatine phosphate, 1 mM ATP, 250 μM CTP and UTP, 0.5 mM S-adenosyl-L-methionine and 0.33 μM [α-$^{32}$P]GTP (35, 36). Labeling reactions were terminated by addition of RNase-free DNase I (RQ1, Promega Biotec, Madison, WI) to a final concentration of 144 U/ml. After termination of reactions, nuclei were lysed by the addition of an equal volume of a 2× lysis buffer that contained 0.6 M NaCl, 20 mM EDTA, 20 mM Tris-HCl (pH 7.5), and 0.5% SDS. The nuclear lysates were digested with 300 mg/ml of proteinase K for 1.5 h at 65°C. After extraction with phenolchloroform-isoamyl alcohol (25:24:1, v/v/v) and ethanol precipitation, [$^{32}$P]-labeled hnRNA was again subjected to RNase-free DNase I treatment for 30 min. After phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol (24:1) extractions, hnRNA was isolated by two to three 2.5 M ammonium acetate/ethanol (1:2.5) precipitations. These final high-salt ethanol precipitations essentially removed 99% of the unincorporated radionuclides.

Before the above experimentation, the optimal time course of incorporation of radiolabeled nucleotides into nascent-chain hnRNA was determined. Nuclei, labeled as above, were analyzed from time points of 0 to 70 min in 10-min intervals. Reactions were precipitated in 0.5 ml 5% TCA on ice for 45 min and filtered through Whatman 934-AH glass microfiber filters. Filters were washed with 15 ml of ice-cold 5% TCA, dried with cold isopropanol, and counted by scintillation spectrometry.

*Preparation of membranes.* Ten micrograms of the CD4 cDNA in Bluescript (Stratagene Inc., San Diego, CA), Bluescript alone as a control for nonspecific hybridization, and the cDNA coding for human β-actin were applied to 0.45 μm Nytran (Schleicher and Schuell, Inc., Keene, NH) membranes according to the procedure described by the manufacturer.

*Hybridization conditions.* Hybridization of membrane-bound dsDNA to labeled hnRNA was carried out as follows. [$^{32}$P]-labeled RNAs extracted from $2 \times 10^7$ nuclei were dried by means of a Savant Speed-Vac and dissolved in 1 ml of hybridization buffer containing 50% formamide, 6× SSPE [1× SSPE = 0.150 M NaCl, 0.010 M $NaH_2PO_4$, 0.001 M EDTA (pH 7.4)], 0.1% polyvinylpyrrolidone 10, 0.1% Ficoll 400, 100 μg/ml salmon sperm DNA, and 1.0% SDS. Three ml of the above hybridization solution containing $1.5 \times 10^7$ dpm/ml of labeled RNAs were added to prepared membranes that had been pre-hybridized in the same volume of the above buffer for a period of 24 h. Hybridizations were incubated at 42°C for 48 h. After hybridization, membranes were washed twice at room temperature for 10 min, in buffer containing 5 mM Na phosphate (pH 6.4), 1.0 mM EDTA, and 0.1% SDS. Membranes were then washed for 1 h at 42°C in 1× SSC (pH 7.0) containing 7.0 μg/ml of RNase A. The final two washes were in 0.2× SSPE, 0.1% SDS for 20 min at 48°C. Membranes were blotted dry on Whatman 3 MM paper and exposed to Kodak XAR film with two Lightning Plus intensifying screens for 2 days at −70°C. Autoradiographs were analyzed by scanning laser densitometry on a EC 910 transmission densitometer equipped with a Hewlett-Packard 3390A reporting integrator. Where applicable, integrated peak values of nonspecific, background hybridization were subtracted from specifically hybridized sequences.

*Transfection.* The 3.0-kb CD4 cDNA was subcloned into an expression vector containing the Friend spleen focus-forming virus LTR (SFFV) (37). This vector contains the neomycin resistance gene driven by the SV40 LTR and was generously provided by Dr. Dennis Loh of Washington University (St. Louis, MO). Transfection into CD4$^-$ Jurkat cells was carried out by electroporation using a Baekon 2000 electroporation apparatus. Cells were resuspended to a final concentration of $2 \times 10^8$/ml in a buffer consisting of 0.3 M sucrose and 1.0 mM $Na_2HPO_4$ (electroporation buffer). Sixty micrograms of linearized DNA in electroporation buffer was used per $1 \times 10^7$ cells. Conditions were as follows: 5 kV, 5 cycles, 12.8-s burst time, 160-μs pulse width, $2^8$ pulses. The electrode was set on a non-contact mode for a final volume of 100 μl. Cells were kept on ice for 30 min after transfection. Selection with 2.0 mg/ml of G418 was started 48 h after transfection.

## RESULTS

Time course experiments showing the effects of PMA on cell surface expression of CD4 are shown in Figure 1. MOLT 4 cells were stimulated with $10^{-8}$ M PMA for 5 to 48 h and analyzed for CD4 expression by flow cytometry. Reduced cell surface expression of CD4 was seen by 5 h after addition of PMA. The nadir of CD4 expression was between 16 and 24 h after PMA. Reexpression of CD4 was noted as early as 48 h after PMA treatment. An identical pattern of modulation and re-expression of CD4 was also seen when MOLT 4 cells were pulsed for 1 h with $10^{-8}$ M PMA (data not shown).

The delay in normalization of cell surface expression of CD4 cannot be explained by protein turnover because previous studies showed detectable CD4 protein within several hours after biosynthetic labeling (1, 11). To evaluate the possibility that PMA inhibited biosynthesis of CD4, MOLT 4 cells were treated for 16 h with and without $10^{-8}$ M PMA followed by [$^{35}$S]methionine labeling and immunoprecipitation with anti-CD4 (6B10) or anti-class I (W6/32). These results are shown in Figure 2. CD4 biosynthesis was reduced in cells treated with PMA compared with untreated controls. In contrast, biosynthesis

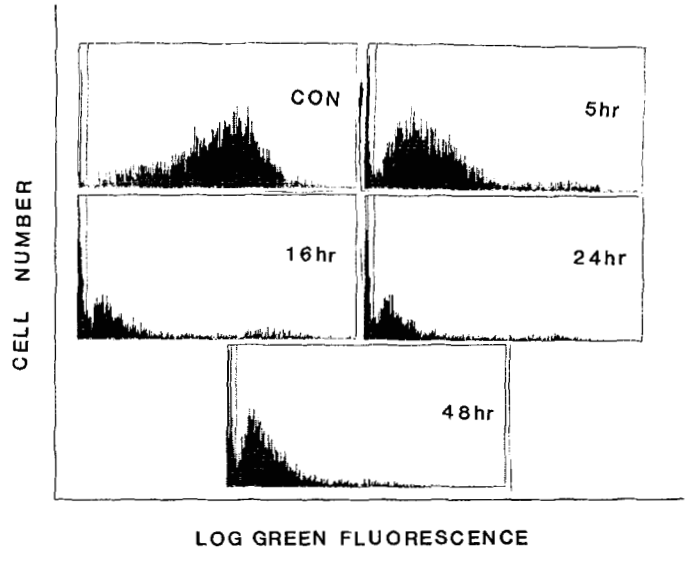


*Figure 1.* Effects of PMA on cell surface expression of CD4. MOLT 4 cells were stimulated with $10^{-8}$ M PMA for the times shown, stained with FITC-conjugated anti-CD4 (6B10), and analyzed by using a Coulter Epics C cytofluorograph. A minimum of 5000 cells were analyzed.

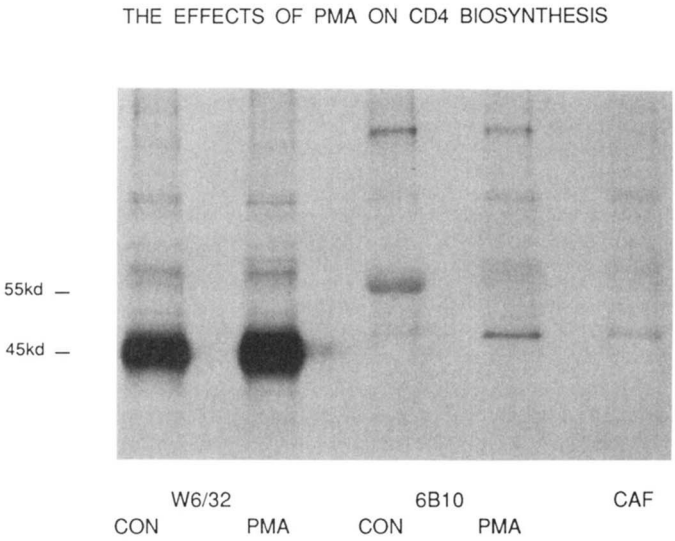


*Figure 2.* Effects of PMA on CD4 biosynthesis. MOLT 4 cells stimulated with or without $10^{-8}$ M PMA for 16 h were labeled with [$^{35}$S] methionine, lysed, and immunoprecipitated with anti-class I (W6/32), anti-CD4 (6B10), or nonbinding antibody. Immunoprecipitates were analyzed by SDS-PAGE.

of class I HLA proteins was not affected. To determine whether the reduction in CD4 biosynthesis was associated with reduced steady-state levels of CD4 mRNA, total RNA was isolated from untreated MOLT 4 cells and cells treated for 16 to 48 h with PMA and analyzed by Northern blot. Figure 3*A* shows that PMA-treated cells had reduced steady-state levels of CD4 mRNA compared with untreated controls. The nadir of expression was between 16 and 24 h and corresponded to cell surface expression of CD4. In contrast, PMA did not affect steady-state levels of actin mRNA (Fig. 3*B*) and did not affect stady-state levels of class I mRNA isolated from cells treated for 16 h with PMA (data not shown).

Reduced steady-state levels of CD4 mRNA induced by PMA may result from either the inhibition of transcription or an increase in the turnover of CD4 mRNA. To define the mechanisms by which PMA affects transcription of CD4, the effects of PMA on the relative rate of transcription was studied by using nuclei from untreated MOLT 4 cells and from cells treated for 16 h with PMA. Results of a representative experiment are shown in Figure 4. In two separate experiments, PMA treatment resulted in a reduction in the relative rate of transcription of CD4 mRNA compared with that seen from nuclei isolated from untreated cells. PMA did not affect the relative rate of transcription of actin mRNA. Densitometric analysis was performed, and integrated peak values for CD4 were normalized to values obtained for actin. Results are shown in Table 1. In two experiments, PMA treatment resulted in a 6- and 23-fold reduction in the relative rate of transcription of CD4 mRNA. Extracts from labeled nuclei did not bind to Bluescript control (data not shown).

*Cis*-acting regulatory sequences have been demonstrated to be involved in PMA-mediated effects on transcription (38, 39). To determine the impact of these sequences on PMA-induced modulation, a construct consisting of the SFFV promoter and the 3.0-kb CD4 cDNA was used. This construct, which lacks CD4-specific regulatory sequences, was transfected into CD4$^-$ Jurkat cells. CD4$^+$ transfectants were analyzed for the ability to respond to PMA. Results of a typical experiment are

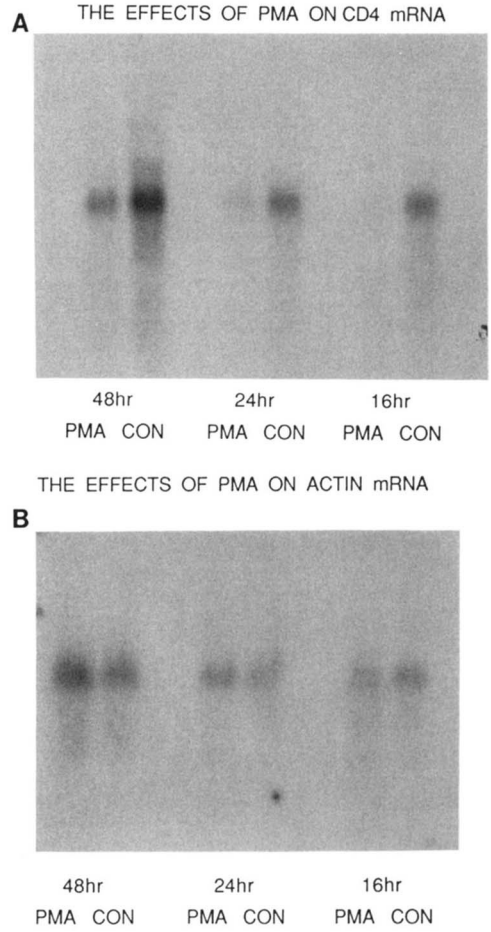


*Figure 3. A*, effects of PMA on steady-state levels of CD4 mRNA. MOLT 4 cells were treated for 16 to 48 h with $10^{-8}$ M PMA before the isolation of total RNA. Twenty μg of total RNA per lane were analyzed by Northern blot. *B*, effects of PMA on steady-state levels of β-actin mRNA.

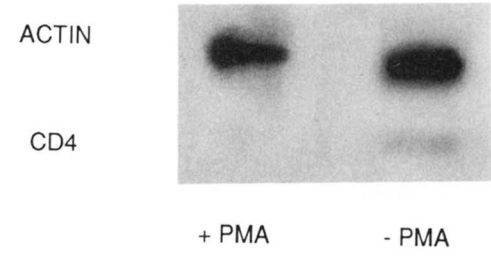


*Figure 4.* PMA inhibits the relative rate of transcription of CD4 but not actin mRNA. Nucleic acids extracted from control (−PMA) and cells treated for 16 h with $10^{-8}$ M PMA (+PMA) were hybridized to membranes containing cDNA coding for β-actin and CD4 as described in *Methods*.

TABLE I

*Densitometric analysis from nuclear run-on experiments evaluating the effects of PMA on the rate of transcription of CD4 and actin*

| Experiment | Actin | CD4 | Inhibition of Transcription[a] |
|---|---|---|---|
| 1 | | | |
| Control | 26.2[b] | 16.7 | |
| PMA | 35.2 | 3.7 | 6× |
| 2 | | | |
| Control | 37.2 | 2.6 | |
| PMA | 29.1 | 0.1 | 23× |

[a] Fold inhibition of the relative rate of transcription of CD4 after normalization to actin.

[b] Arbitrary densitometry units obtained by using a EC 910 transmission laser densitometer equipped with a Hewlett-Packard integrator.

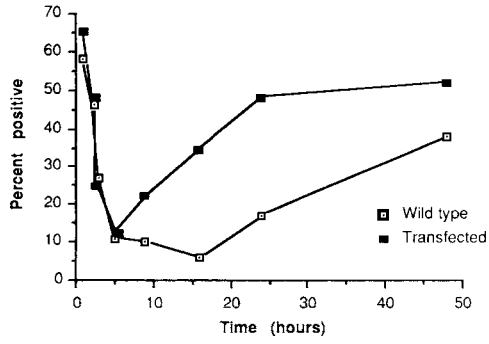


*Figure 5.* The kinetics of CD4 expression in Jurkat cells expressing transfected (cDNA) and wild-type CD4 gene products. Cells were stained with FITC-conjugated anti-CD4 (6B10) and analyzed by using a Coulter Epics C cytofluorograph. A minimum of 5000 cells were analyzed. Similar results were obtained in four additional experiments.

shown in Figure 5. $CD4^+$ transfectants showed reduced expression of CD4 within 5 h after PMA treatment. Normalization of CD4 expression was documented by 16 h after PMA treatment. Jurkat cells expressing CD4 derived from the endogenous gene also showed reduced cell surface expression of CD4 by 5 h after PMA stimulation. In contrast to cells that expressed transfected CD4, the nadir of CD4 expression in wild-type cells was between 16 and 24 h, and normalization of CD4 expression was documented by 48 h. Jurkat cells that expressed transfected and wild-type CD4 gene products showed similar kinetics of PMA-induced modulation of CD3 expression, which suggests that the differences in CD4 expression were not the result of intrinsic differences in the cells used in this study. The results shown in Figure 5 were confirmed in four separate experiments. PMA treatment did not result in reduced steady-state levels of CD4 mRNA in transfected cells, whereas PMA treatment of cells expressing the endogenous gene resulted in reduced steady-state levels of CD4 mRNA (data not shown), which suggests that the SFFV LTR does not respond to PMA.

## DISCUSSION

Stimulation of $CD4^+$ cells with PMA, HIV, or Ag results in transient modulation of CD4 expression with normalization of CD4 expression occurring by 48 to 72 h (19–27). The relatively long time for normalization of CD4 expression suggests that PMA also affects transcription, translation, or transport of CD4 to the membrane. To investigate these possibilities, we studied the effects of PMA on biosynthesis and transcription of CD4. PMA stimulation inhibited the biosynthesis of CD4, but not of class I proteins. PMA stimulation also resulted in reduced steady-state levels of CD4 mRNA. Reduced steady-state levels of CD4 mRNA may be the result of a reduced rate of transcription or an increased rate of mRNA turnover (40). Experiments were carried out to define the mechanisms regulating transcription of CD4. These experiments used isolated nuclei from cells treated with and without PMA, wherein nucleoside triphosphates were incorporated into nascent RNA chains. Previously initiated RNA polymerase II molecules elongated nascent hnRNA chains by 70 to 150 additional nucleotides. The RNAs for β-actin and CD4 were extracted and hybridized to respective cDNA, and the abundance of each RNA was quantitated by autoradiography. Relative rates of transcription, differences of which may be visually determined autoradiographically, are reflected by the density of RNA polymerase molecules present in the nucleotide region against which the labeled RNA is hybridized, which presumably represents relative rates of transcription initiation. Our studies showed that PMA treatment inhibited the relative rate of transcription of the CD4 gene but not that of β-actin.

PMA-induced inhibition of transcription of CD4 affects the kinetics of re-expression of CD4. The time to reach the nadir of CD4 expression was similar in cells expressing CD4 cDNA gene products and in cells expressing endogenously derived CD4. In contrast, re-expression of CD4 in transfected cells was quicker than in cells containing endogenous CD4. Since the cDNA used in these studies lacked non-coding regions of the CD4 gene (putative targets for the action of PMA), these observations suggest that PMA-induced inhibition of transcription and biosynthesis is mediated through non-coding regions of the CD4 gene and these regions affect the kinetics of CD4 expression after modulation.

Our studies provide further insight into the mechanisms regulating CD4 expression. Previous studies showed that cell surface modulation of CD4 is regulated by specific domains of the IC tail. PMA stimulation results in phosphorylation of serine residues within the IC domain of CD4. Deletion of the entire IC domain, or only of serine residues, prevents modulation (26–28). Our studies showed that PMA stimulation inhibits the biosynthesis and transcription of CD4. These observations suggest that PMA affects CD4 expression by at least two mechanisms. The immediate loss of CD4 after PMA treatment may be regulated by phosphorylation of serine residues within the IC tail. Re-expression is regulated by factors affecting transcription and biosynthesis of CD4.

These results may have implications in further defining HIV/CD4 interactions. The binding of HIV to CD4 results in phosphorylation and modulation of CD4, and these effects are mediated through activation of protein kinase C (25). Additional studies demonstrated that HIV-infected cells had reduced steady-state levels of CD4 mRNA, as compared with uninfected controls (29). Based on the data from our study, we hypothesize that the reduction of steady-state levels of CD4 mRNA may be the direct result of the activation of protein kinase C by HIV, and that the effects of PMA on transcription of CD4 may be a useful model to study HIV/CD4 interactions. Identifying the non-coding regions of the CD4 gene that are affected by PMA will provide insight into the mechanisms in which HIV affects transcription of the CD4 gene.

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