Different Early-Signaling Pathways Coupled to Transcriptional and Posttranscriptional Regulation of Gene Expression during Mitogenic Activation of T Lymphocytes

MICHAEL W. WHITE, AMY K. OBERHAUSER, CHRISTINE A. KUEPFER, AND DAVID R. MORRIS*

Department of Biochemistry, University of Washington, Seattle, Washington 98195

Received 29 December 1986/Accepted 13 May 1987

Two categories of mitogen-induced mRNAs were defined in T lymphocytes. The type 1 messages (represented by *c-myc*) were regulated transcriptionally, and their expression seemed to be calmodulin dependent. The type 2 messages (ornithine decarboxylase, actin, and α -tubulin) were regulated posttranscriptionally through activation of protein kinase C.

Shortly after exposure of resting animal cells to mitogens, the cellular levels of a variety of mRNAs are elevated (for a review, see reference 8). The corresponding growth-associated genes have been termed the competence gene family, and their products are thought to be critical for induction of a state of competence for entry into the cell cycle (for a review, see reference 25). Regulatory cascades initiated by interaction of mitogens with specific receptors presumably modulate the expression of the competence genes. A variety of putative signaling processes have been observed (for a review, see reference 22). In this study we investigated the relationship between these early-signaling pathways and the expression of several competence genes in T lymphocytes.

Bovine T lymphocytes begin to enter the S phase of the cell cycle 24 h after exposure to the mitogen concanavalin A (ConA) (10). Similar to what occurs in other mitogen-activated cells (for a review see reference 8), the cellular levels of a variety of mRNAs are elevated in T lymphocytes before entry into the S phase (19, 20). This is illustrated for four of these mRNAs by Northern blots (Fig. 1) and quantitative results (see Fig. 3).

Run-on transcription of the corresponding genes was examined in nuclei isolated at various times after activation of lymphocytes with ConA. Total incorporation as a function of time after mitogenic activation is shown in Fig. 2. On the basis of sensitivity to α -amanitin, 80 to 90% of the incorporation was due to RNA polymerase II over the time shown. The proportion of incorporated radioactivity hybridizing to a bovine actin cDNA clone (expressed in parts per million) is also shown in Fig. 2. It is clear that, although the incorporation per cell increased ca. 2.5-fold in the first 24 h, the proportion of transcription product hybridizing to the actin clone actually decreased by a similar amount. The hybridization efficiency was monitored by the use of an internal [^3H]cRNA standard (18) and found to vary between 25 and 40% from experiment to experiment but to be constant for the time points in a single experiment (data not shown, but the data shown in Fig. 2 are corrected for hybridization efficiency). Thus, there was no significant elevation of the rate of actin gene transcription per cell in the first 24 h after mitogenic activation of lymphocytes, even though the cellular level of actin mRNA increased about sixfold over the same period (7; Fig. 3). The rates of in vitro

transcription were also compared with the mRNA levels for *c-myc*, ornithine decarboxylase (ODC), and α -tubulin (Fig. 3). Consistent with the results of others with other cell types (13), *c-myc* transcription was elevated. On the other hand, as we found with actin mRNA, major increases in the cellular levels of the mRNAs coding for ODC and α -tubulin occurred with no consistent elevation of the rates of transcription of the genes in vitro. Densitometric scanning of the autoradiograms demonstrated that the signals for ODC, tubulin, and actin transcription were a function of radioactivity added to the hybridization reaction mixtures (see the legend to Fig. 3), and therefore increases in labeled transcripts commensurate with the elevated mRNA contents of the cells could have been detected. The slight increase in tubulin gene transcription at 10 h was not seen in other experiments. The ODC gene transcription data in Fig. 3 were obtained by using single-stranded antisense DNA on the hybridization filters. Identical results were observed with the double-stranded 850-base-pair ODC plasmid (17) and subcloned fragments from the 5' and 3' ends of this ODC cDNA.

The influence of activating protein kinase C with phorbol esters on the levels of the mRNAs coding for *c-myc* and ODC was examined as a function of time (Fig. 4). Although ODC mRNA was strongly induced by tetradecanoyl acetate (TPA), there was no influence on the level of *c-myc* mRNA over the 8-h period examined. The first 2 h were examined in greater detail, with the first data taken at 15 min; again, no significant induction of *c-myc* was observed. The *c-myc* mRNA was elevated normally by ConA in the presence of TPA (Fig. 4). Phorbol dibutyrate behaved similarly to TPA, whereas the biologically inactive 4- α -phorbol didecanoate was ineffective in inducing ODC at concentrations up to 300 nM. TPA also elevated the levels of the actin and α -tubulin mRNAs in resting lymphocytes (data not shown).

Cytosolic Ca^{2+} is elevated within seconds after activation of lymphocytes with mitogenic lectins (26). The elevation of both *c-myc* and ODC messages by ConA, observed at 1 and 2 h, was inhibited by either 2 or 10 mM EGTA [ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] concentrations which were sufficient to block 80% of the increase in cytosolic Ca^{2+} , as measured by flow cytometry (P. Rabinowitch and D. Morris, unpublished results). The induction of ODC mRNA by TPA, measured at 2 h, was unaffected by EGTA, excluding the possibility of generalized cytotoxicity due to the presence of the chelating agent. The influence of the intracellular Ca^{2+} antagonist Cd^{2+} on

* Corresponding author.

FIG. 1. Northern blot analysis of T-lymphocyte RNA isolated during early mitogenesis. Total RNA from bovine T lymphocytes stimulated with ConA (24) was extracted (4) at various times, separated on formaldehyde-agarose gels, and blotted to nitrocellulose (16). The blots were hybridized to the following nick-translated probes: actin, bovine cDNA clone pBA1 (7); tubulin, bovine α -tubulin cDNA clone containing 1,080 base pairs of coding sequence (unpublished data); ODC, mouse cDNA clone pOD48 (17); *c-myc*, mouse *Pst*I exon 2 fragment (3).

FIG. 2. Actin gene transcription in isolated lymphocyte nuclei. Nuclei were isolated at the indicated times after ConA activation, and run-on transcription assays were performed (14, 18). The total amount of incorporation of [α - 32 P]UTP was determined at the various time points (top graph). The labeled transcription products were hybridized to filter-bound plasmids containing a bovine actin cDNA insert (7), and the proportion of the total radioactivity which hybridized was measured. The results are expressed as the proportion of radioactivity associated with actin transcripts in parts per million (bottom graph).

the induction of the *c-myc* and ODC genes was particularly interesting; a dose-response curve for CdCl_2 is shown in Fig. 5. The induction of *c-myc* was strongly inhibited, whereas there was relatively little effect on the ODC gene over the same range of concentrations. The induction of the ODC gene by TPA was also unaffected by 0.6 mM CdCl_2 (data not shown).

We suggest that there are at least two regulatory classes of growth-associated genes in mitogen-activated lymphocytes, defined on the basis of both transcriptional activity and the early-signaling pathways to which they are responsive. As expected based on previous studies with other cell types (13), transcription of the *c-myc* gene was elevated after mitogenic activation. On the other hand, regulation of the ODC, α -tubulin, and actin genes seemed to be mediated by posttranscriptional stabilization of either mature message or intranuclear precursors. Posttranscriptional regulation of mRNA levels is common in viral systems (e.g., see reference 6), but in addition, examples of such regulation are now rapidly appearing for uninfected cells (for example, see references 11 and 12).

FIG. 3. Comparison of mRNA levels and nuclear run-on transcription of several competence genes. The relative changes in mRNA levels were determined by densitometric scanning of autoradiograms produced from Northern blot analysis of T-lymphocyte total RNA isolated at various times after ConA activation. The autoradiograms from in vitro run-on transcription assays are shown on the right. In the top right panel, a filter containing *c-myc* DNA (MYC) and pBR322 DNA (pBR) was hybridized to 2×10^6 cpm of [32 P]RNA transcripts. For ODC, 1×10^6 (1X) and 2×10^6 (2X) cpm were hybridized to the antisense strand of ODC cDNA cloned into bacteriophage M13mp11. The average ratio of the densitometric scans for the five time points was 1:1.7 between the two autoradiograms. For α -tubulin (α -T), 2×10^6 (1X) and 4×10^6 (2X) cpm were hybridized to plasmid DNA; the average ratio of the densities on the two filters was 1:2.1. For actin, 1×10^6 (1X) and 2×10^6 (2X) cpm were hybridized to plasmid DNA; the average ratio was 1:1.9.

FIG. 4. Effect of TPA on levels of ODC and *c-myc* mRNAs in resting and ConA-activated T lymphocytes. (A) Northern blot probed for ODC mRNA; (B) Northern blot probed for *c-myc* mRNA. Total RNA from lymphocytes was isolated at 2 h (lanes b and f), 4 h (lanes c and g), and 8 h (lanes d and h) after TPA addition. TPA at a final concentration of 30 nM was added to resting cells (lanes b to d) or to cells at the time of activation with ConA (lanes f to h). Resting mRNA levels are shown in lanes a and e. (C) Quantitation of the autoradiogram from panel A by densitometric scanning; (D) quantitation of the autoradiogram from panel B. Resting (▲) and ConA-activated (●) lymphocytes treated with TPA.

FIG. 5. Dose-response curve for effect of CdCl₂ on ODC and *c-myc* mRNA levels. Activated lymphocytes were treated with increasing concentrations of CdCl₂ (CdCl₂ was added at the time of addition of the mitogen ConA), and total RNA was prepared after 2 h of treatment. The concentrations of CdCl₂ used were 0 (lane b), 0.1

Elevation of both ODC and *c-myc* messages require increased cytosolic Ca²⁺, but the two genes seem to respond to two different intracellular signaling pathways, one sensitive to Cd²⁺ and the other not sensitive. Cd²⁺ is thought to be a calmodulin antagonist which competes with Ca²⁺ for binding (2) but seems to form an inactive complex with this regulatory protein (1, 5). The results of the present study suggest the involvement of calmodulin in the transcriptional activation of the *c-myc* gene but not in expression of the ODC gene. In this context, it is intriguing that DNA topoisomerase II is activated when phosphorylated by calmodulin-dependent protein kinase II (23); it has been suggested that this enzyme, a major component of the chromosomal scaffolding (9), is involved in the transcriptional activation of genes (21).

Our results suggest a model for the regulation of the competence gene family in T lymphocytes. ConA is thought to bind to the T3 component of the T-cell antigen receptor complex (27) and to trigger the uptake of Ca²⁺ (26) and the breakdown of phosphatidylinositol bisphosphate to diacylglycerol and inositol triphosphate (15). We propose that the

(lane c), 0.2 (lane d), 0.4 (lane e), and 0.6 (lane f) mM. Lane a, RNA from resting cells. Autoradiograms of the Northern blots (top panels), initially probed for ODC mRNA and then reprobated for *c-myc* mRNA, were quantitated by densitometric scanning, and the data (bottom panel) is expressed as the relative amount of mRNA in ConA-activated (2 h) lymphocytes (lane b).

simultaneous elevation of cytosolic Ca^{2+} and diacylglycerol synergistically activates protein kinase C, as has been shown in a reconstituted system (28). The activation of protein kinase C would in turn lead to expression of the posttranscriptionally controlled type 2 genes. The elevation of cytosolic Ca^{2+} also results in enhanced transcriptional expression of the type 1 genes, probably requiring at a minimum a calmodulin-linked step, as discussed above.

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