COT Kinase Proto-oncogene Expression in T Cells

IMPLICATION OF THE JNK/SAPK SIGNAL TRANSDUCTION PATHWAY IN COT PROMOTER ACTIVATION*

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COT/Tpl-2 proto-oncogene encodes a serine/threonine kinase implicated in cellular activation. In this study we have identified the human COT gene promoter region and three different human COT transcripts. These transcripts, with the same initiation site, display heterogeneity in their 5’ untranslated regions and in their subcellular localization. Activation of Jurkat T cells with either calcium ionophore A23187 or oC3d and a phorbol ester increases the levels of the different COT transcripts. Analysis of the 5′ flanking region of the human COT gene reveals a unique transcription initiation site and a TATA element 20 nucleotides upstream. Transient expression of COT promoter constructs containing a reporter gene indicates that the transcriptional activity of the 5′ flanking region of the COT gene is regulated by T cell-activating signals. Cotransfection of a dominant negative version of SEK-2 abolishes the inducible transcriptional activity of COT promoter, indicating that the inducible expression of the COT gene by T cell-activating signals is mediated by the JNK/SAPK signal transduction pathway. All these data indicate stringent regulation of COT proto-oncogene expression.

COT/Tpl-2 is homologous to members of the mitogen-activated protein kinase kinase family (MAP3K) and has been implicated in cellular activation (1–5). Overexpression of COT/Tpl-2 induces activation of MEK-1 and SEK-1 kinases, activating the ERK and JNK/SAPK signal transduction pathways, respectively (1, 6, 7). COT/Tpl-2 kinase regulates the transcription of tumor necrosis factor α and interleukin 2 genes during T cell activation (8, 9) by activating at least AP-1 and NF-κB response elements in the gene promoters (9–13).

Several COT/Tpl-2 cDNAs comprising the complete coding sequence and 3′ UTR have been reported: two different human cDNAs (GenBank™ accession numbers Z14138 and D14497) (14, 15), two rat cDNAs (GenBank™ accession numbers M94454 and L15358) (16), and one murine COT cDNA (GenBank™ accession number D13759) (17). Identities between human, rat, and murine COT cDNAs in their coding sequences and 3′ UTRs are 85 and 75%, respectively. The 5′ UTR of the human, rat, and murine COT cDNAs did not reveal any homology, with the exception of the 23 nt upstream from the first COT/Tpl-2 ATG codon.

The human COT gene is a single copy locus (14, 18) localized on the short arm of chromosome 10 at band p11.2 (14). Ohara et al. (17) proposed that the human COT gene contains nine exons, of which the last seven are coding exons. COT kinase was first identified in a truncated form in transformed foci induced in SHOK cells by transfection of the genomic DNA of a human thyroid carcinoma cell line (18, 19). This rearrangement occurs in the penultimate coding exon and provides transformation capacity (15). The rat homologue of the human COT gene (Tpl-2) was identified as an oncogene associated with the progression of Moloney murine leukemia virus-induced T cell lymphomas in rats (4, 16). As with the human and murine homologues, the disruption of the last coding exon of Tpl-2 by insertion of the Moloney murine leukemia virus enhances mRNA levels and appears to mask the oncogenic potential of the protein (4, 15, 20, 21). It has been suggested recently that an amplification of the genomic locus of the COT gene plays a role in human breast cancer (22).

In this paper we have studied the expression of the human COT gene in T cells. We have identified three different human COT mRNAs and the 5′ region flanking the transcription initiation site of the COT gene. We also provide evidence that T cell activation up-regulates the levels of the three COT kinase mRNAs and increases the transcriptional activity of the human COT gene promoter through the JNK/SAPK signal transduction pathway.

EXPERIMENTAL PROCEDURES

Primers—Primers located 3′ from the transcription initiation site of the COT gene (+1 nt) have been designated according to their location in COT-1 cDNA: −8D (−1082 to −1060 nt), 5′-CTGAGATGGGAGAGGTAACAT; −7D (−788 to −768 nt), 5′-CCTTTCTACAGGGTTGACTGATGTC; −6D (−723 to −704 nt), 5′-AGAAAGATTTCAGAGGTCAGA; −5D (−650 to −631 nt), 5′-TGTTGGAGGATTTCTACACT; −4D (−66 to −488 nt), 5′-GGATGGGAGGAATGACAGC; −3D (−340 to −318 nt), 5′-AATTGGCAGCATGTGTTT; −2D (−229 to −210 nt), 5′-CCTTTCTAGTACCAGC; −1D (−102 to −83 nt), 5′-AGGGGATCCAGAGGCTCA; 1D (122/141 nt), 5′-CCGAGATGGGAGAGGTAACAT; 2D (589/699 nt), 5′-CCCGATCCTCCCAAATGCTGG; 3D (107/109 nt), 5′-CTTCTCTCTCTCTCTCTCT; 4D (1731/1752 nt), 5′-CTTCCTTTGTGCGAC; 5D (5237/5258 nt), 5′-TAAATCGGAGGAGAAGACCT; 6D (2898/3008 nt), 5′-ATCTCTTCTACAGGGTTGACTGATGTC; 7D (3984/4003 nt), 5′-CAAGATGTGTTGTTGCTA; 8D (4643/4662 nt), 5′-CAAGATGTGTTGTTGCTA.
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5′-CCATCTTGATGATCGTT, 9D (4749/4768 nt), 5′-ATGGATGC-ATGACTGTGGA; UpTATA (-494–30 nt), 5′-CTCTTTGTCACTACAGCCAG; PR (98/115 nt), 5′-GCGTTGGAGGCGACGAG; PC (123/140 nt), 5′-AGGTTTCGCGCTGATGCCG; RT (588/607 nt), 5′-AGCATTTGG- GCAGCTATGCATGGTCAACC (307/309 nt), 5′-AGAACCGTACGCATTTTCAAGG; 4R (2762/2784 nt), 5′-ATTGAGCTCCGTTTTTACTGACG; 5R (3006/3033 nt), 5′-AAAGTGGTGCTACCTTGGCTCAGGATAAAGG; DR (5135/5152 nt), 5′-GAGAACGGTCTTCGATTTACCC; 7R (4932/4951 nt), 5′-TGGCTGACTAAGCAGCCAGG; 9R (6206/6229 nt), 5′-GGCCCTGTTGAGGAGCGACGAGAA; 5′-GCAGAAATATAGACGCTTGGG; 7D (4979/5005 nt), 5′-GCCCTGTTGAGGAGCGACGAGAA; 7R (4932/4951 nt), 5′-TGGCTGACTAAGCAGCCAGG; 9R (6206/6229 nt), 5′-GGCCCTGTTGAGGAGCGACGAGAA; 5′-GCAGAAATATAGACGCTTGGG.

DNA Isolation and COT Promoter-Luciferase Reporter Vector Constructs—Genomic DNA was obtained as described previously (23). A 6.1-kb DNA fragment containing the 5′ flanking region of the COT gene was obtained with the human genomic DNA PromoterFinder™ DNA walking kit (CLONTECH), using two specific reverse primers deduced from the first of the three COT coding exons. Different DNA fragments of the 5′ flanking region of the COT gene were generated by PCR with different direct primers (−8D, −7D, −6D, −5D, −4D, −3D, −2D, and −1D) and PR as reverse primer. The 7783 A23187 probe was performed with −7D and upTATA primers. Purified PCR products were cloned in pMOS Blue T-vector (Amer sham) and sequenced. Single clones were selected, and sequences were compared with the original template. From these constructs, the KpnI/HindIII fragments were cloned in the pGL3-BasicLuc-reporter vector (Promega). Sequencing, using specific oligonucleotides and Sequenase (U. S. Biochemical Corp.), was performed by the Sanger method (24). Gene Jockey II, DNAstrider 1.1, MacPattern Folder, and Blast programs were used to analyze the DNA sequences.

Cells, Transient Transfection Analysis, and Polysome Gradients—Human leukemia T Jurkat cells were electrophoresed as previously described (9) with 20 μg of the different pGL3-Luc constructs. After a 2-h incubation, cells were stimulated for 12 h with different stimuli: soluble cd3D (10 μg/ml, obtained as indicated in Ref. 9), calcium ionophore A23187 (0.25 μM, Sigma), and/or PDBu (50 ng/ml, Roche Molecular Biochemicals), Cyclosporin A (100 ng/ml, 8-Br-cAMP (0.5 mM, Roche Molecular Biochemicals), PD 98059 (MEK inhibitor) (20 μg/ml, Calbiochem), or SB-20580 (HO/p38 mitogen-activated protein kinase inhibitor) (20 μg/ml, SmithKline Beecham), and/or okadaic acid (100 ng/ml, Calbiochem) were added 30 min before stimulation. Luciferase activity and protein concentration were measured as described previously (9).

To perform the polysome fractionation, 8 × 10⁶ cells were lysed as described previously (25) and centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was spun in a 10–50% linear sucrose gradient buffer with 20 mM HEPES (pH 7.3), 250 mM KCl, 20 mM MgCl₂, 2 mM dithiothreitol, and 500 μg/ml heparin at 36,000 rpm for 2 h at 4 °C. Fractions of 1 ml were collected, and ethanol precipitation was performed with the RNA isolation kit (Promega). DNA was sequenced from the region flanking the translation initiation site. To establish the 5′ UTR of the three COT-1 mRNA species detected in the leukocyte poly(A)+ RNA Northern blot (Fig. 1A) should correspond to COT-1, and the 3.0-kb signal

RESULTS

Identification of Three Human COT Transcripts—Hybridization of a leukocyte poly(A)+ RNA Northern blot with a coding COT probe yielded two hybridization signals at 3.0 and 7.3 kb (Fig. 1A). To investigate whether the occurrence of these transcripts was due to the different length of the 5′ UTR, we cloned and sequenced a 6.1-kb DNA genomic fragment containing the 5′ flanking region of the COT translation initiation site. Several probes from this region were generated by PCR (Fig. 1A).

The poly(A)+ RNA Northern blot with these probes revealed a hybridization signal only at 7.3 kb (Fig. 1A), indicating that this COT mRNA species has a large 5′ UTR. To determine the sequence of the 5′ UTR of the different COT mRNAs, RT-PCR analysis was performed. The direct and reverse primers used were deduced from the sequence of the 6.1-kb genomic fragment containing the 5′ flanking region of the COT translation initiation site. PCR of overlapping fragments was performed using as a template reverse transcribed mRNA of Jurkat cells. Different PCR products were performed with the combinations of each direct primer and all the different 3′-located reverse primers. Control samples treated with DNase I and not exposed to RT were also subjected to PCR. Controls without a template, with genomic DNA as a template, and with one single primer were also performed. The different PCR products obtained (Fig. 1B) were analyzed by restriction mapping and by sequencing (data not shown). The same overlapping PCR products were obtained when human leukocyte cDNA was used as a template (data not shown). This analysis revealed that the COT gene is transcribed with three different 5′ UTRs. The transcription start site of these three 5′ UTRs was delimited to the same 30-nt region by PCR analysis, using as a template cDNA from Jurkat cells as well as human leukocyte cDNA (data not shown). (See “Determination of the Transcription Start Site of the Human COT Gene” for the location of the exact start transcription site.) To establish the 5′ UTR of COT-1 by a method other than RT-PCR, S1 nuclease analysis was performed. A DNA probe (probe D) complementary to the 589–2784 nt sequence of the 5′ UTR of COT-1 was hybridized with RNA obtained from Jurkat cells and incubated with nuclease S1 (Fig. 1C). This probe contains the entire sequence of probe B and is extended to the DNA sequence of probe A. The intron/exon boundaries of the three different 5′ UTRs of COT transcripts are shown in Table I.

We also investigated the possibility of alternative splicing in the coding sequence and 3′ UTR of COT transcripts. RT-PCR analysis revealed no alternative splicing in these regions. The coding sequence and 3′ UTR region of COT transcripts have a size of 2.5 kb (data not shown). Considering the length of the 5′ UTR of the three COT transcripts, the 7.3-kb COT mRNA species detected in the leukocyte poly(A)+ RNA Northern blot (Fig. 1A) should correspond to COT-1, and the 3.0-kb signal
should correspond to COT-2 and COT-3. The three COT transcripts were detected by RT-PCR in all human tissues tested (Fig. 1D), indicating that none of the different COT transcripts is tissue-specific, although the relative amounts seem to vary between the different tissues. When the PCRs were performed as described in the legend to Fig. 1D, the ratio of COT-2 to COT-1 oscillated between 0.5 for liver or pancreas and 1.6 for muscle. The ratio of COT-3 to COT-2 varied from 9.8 for lung to 1.6 for muscle. The ratio of COT-3 to COT-2 varied from 9.8 for lung to 1.6 for muscle.

Determination of the Transcription Start Site of the Human COT Gene—The transcription start site of the COT gene was delimited by RT-PCR analysis to a 30-nt region (data not shown). The exact transcription start site of the COT gene was determined by primer extension on poly(A)⁺ RNA from stimulated Jurkat cells with a primer complementary to primer 1D (PE primer). This sequence is complementary to the three COT transcripts. As shown in Fig. 2, a single product corresponding to a 140-base extended fragment was detected. The first transcribed base has been designated +1, to facilitate numbering of the different COT transcripts. Sequence analysis revealed a putative TATA box located at position −20 nt (Figs. 2 and 3A), which is in agreement with the preferred position occupied by this element in a typical eukaryotic promoter (26).

To confirm that the DNA region 5' flanking the defined transcription start site of the human COT gene has promoter activity, transient expression experiments with the pGL3-Luc basic vector linked to different fragments of this DNA region were carried out. Jurkat cells were transfected with pGL3, pGL3–778 (5'-3'), and pGL3–778 as well as with the pGL3–778 (3'-5') construct, and luciferase activity was measured (Fig. 3B). The pGL3–778 (3'-5') construct, containing nt −770 to +115 of the COT gene, exhibited a transcriptional activity 20-fold higher than that of the empty pGL3 vector. The −30 to +115-nt region is essential in maintaining this increase, because deletion of the −30 to +115-nt fragment, in the pGL3–778 construct, resulted in transcriptional activity similar to pGL3. No promoter activity over background levels was detected with the pGL3–778 (3'-5') construct, indicating that this region contains the transcription start site of the COT gene and not only cis-response elements.

| mRNA | Exon | Size | nt |
|------|------|------|----|
| COT-1 | 1    | 5084 | 1-5084 |
| COT-2 | la   | 192  | 1-192 |
|       | lb   | 229  | 2984-3212 |
|       | lc   | 359  | 4726-5084 |
| COT-3 | 1a   | 192  | 1-192 |
|       | lc   | 359  | 4726-5084 |

**TABLE I**

Intron-exon organization of the 5' region of the human COT gene

Exon sequences are shown in uppercase letters; intron sequences are shown in lowercase letters.

Sequences at intron-exon junction

- Splice acceptor: ATAAACATGTGTAGTTTCT
- Splice donor: AGGCCCGAGTAATCCAGG
- Splice acceptor: TTCGTTTTAGATGCAATCTTC
- Splice donor: TCCACATGAGTGAGGCTG
- Splice acceptor: TCTGTTCCTAGACTCTCCAGA
- Splice donor: TCTTAGATGTAGTTTCT
- Splice acceptor: TCTGTTCCTAGACTCTCCAGA
- Splice donor: TCTTAGATGTAGTTTCT

**FIG. 1. Identification of three COT transcripts.** A, human leukocyte poly(A)⁺ RNA Northern blot was hybridized with the coding sequence of COT kinase and with three different probes from the 5' flanking region of the COT translation initiation site. A schematic representation of the localization of these probes in the genomic DNA fragment is also shown. According to the numbering given to the sequence of the COT-1 transcript, probe A corresponds to nt 290–608, probe B to nt 2234–2784, and probe C to nt 3837–4646. These probes were generated by PCR. The coding COT probe was generated by PCR with primers 9D and 9R and reverse transcribed mRNA from Jurkat cells as a template. B, PCRs from cDNA of Jurkat cells were performed with the indicated primers. Diagrams show the positions of the primers in the different human COT cDNAs. Molecular weight markers. C, S1 nuclease protection analysis of Jurkat cells with probe D (nt 589–2784). RNAs (30 μg) from Jurkat T lymphocytes (T) or yeast (Y) were used. Undigested probe (U.P.) was also electrophoresed. D, relative levels of COT-1/COT-2 and COT-2/COT-3 transcripts were measured by RT-PCR in different human tissues. Lanes: 1, liver; 2, pancreas; 3, muscle; 4, peripheral blood leukocytes; 5, lung; and 6, kidney. Relative levels of COT-1/COT-2 were determined using the primers 6D (0.5 μM), 8D (0.5 μM), and 8R (1 μM). The 630- and 565-bp PCR products correspond to COT-2 and COT-3 transcripts, respectively. Relative levels of COT-2/COT-3 transcripts were detected with primers 1D (0.5 μM), 6D (0.5 μM), and 7R (1 μM). The 527- and 209-bp PCR products correspond to COT-2 and COT-3, respectively.
mids containing 1082 bp of the COT gene 5' flanking region (Figs. 3 and 4A) as well as 5' deletion fragments (Fig. 4A).

Comparison of the relative promoter activities of the different constructs indicated that the progressive removal of 5' sequences up to −650 did not significantly affect the COT promoter activity in unstimulated cells (Fig. 4B). Deletion of the −468 to −650 DNA fragment significantly decreased the transcriptional activity. Further deletion of nt −340 to −229 further decreased the activity of the COT promoter (Fig. 4B). However, the pGL3−229 and pGL3−102 constructs still exhibited a transcriptional activity 7-fold higher than vector pGL3 (data not shown).

To determine whether COT promoter activity is regulated by T cell-activating signals, Jurkat cells were transfected with the different COT promoter-derived constructs and stimulated with PDBu (50 ng/ml) and calcium ionophore (0.25 μM) or with αCD3 (10 μg/ml) and PDBu (50 ng/ml). Comparison of the luciferase activities produced by each different plasmid in unstimulated and stimulated cells showed that deletion of the sequences located between positions −1082 to −340 did not significantly affect the 3-fold induction relative to the unstimulated activity of each construct. Further removal of the sequences from position −340 to −229 abolished the 3-fold induction by these signals (Fig. 4B).

One AP-1 binding site (27, 28) is found at position −327 nt of the 5' flanking region of the COT gene (Fig. 3A). The AP-1 transcription factors are up-regulated in T lymphocytes activated with PDBu and calcium ionophore or with αCD3 and PDBu, and the JNK/SAPK signal transduction pathway mediates its activation. To determine whether this signal transduction...
pathway regulates, at least in part, activation of the COT promoter. Jurkat cells were cotransfected with pGL3–340 or pGL3–788 together with the dominant negative form of JNK kinase, DN-SEK-2 (MKK7-KL), that inhibits the activation of c-Jun. Cells were stimulated or not with PDBu (50 ng/ml) and calcium ionophore (0.25 μM) or with αCD3 (10 μM) and PDBu (50 ng/ml). As shown in Fig. 4C, the expression of the DN-SEK-2 abolished the increase of the promoter-driven transcription of the pGL3–340 and pGL3–778 constructs by T-cell activating signals.

To further analyze the signal transduction mechanism by which the COT promoter is activated, transient transfection experiments with the pGL3–340 construct were performed. Addition of PDBu or calcium ionophore by itself did not increase the luciferase activity, indicating that an integration of both signals has to occur to activate COT promoter-driven transcription (Fig. 4D). Transfected cells were also incubated with different inhibitors or activators of protein kinases or protein phosphatases. The transfected cells were incubated with PDBu (50 ng/ml) and calcium ionophore (0.25 μM) in the presence or absence of cyclosporin A (100 ng/ml), MEK inhibitor (20 μM), HOG inhibitor (20 μM), okadaic acid (100 ng/ml), or 8-Br-cAMP (0.5 mM) at doses that have already been reported to regulate the activation of Jurkat T cells (9). Because addition of PDBu and calcium ionophore to Jurkat T cells increases the phosphorylation state of many proteins involved in the signal transduction mechanism, the addition of okadaic acid, an inhibitor of protein phosphatases 1 and 2A, to these activated Jurkat cells could induce a further increase in the phosphorylation state of the proteins. According to the results obtained in Fig. 4D, the addition of okadaic acid did not increase the luciferase activity. Addition of cyclosporin A prior to activation of the cells reduced the luciferase activity, indicating that calcineurin (protein phosphatase 2B) is at least partially involved in the activation of the COT promoter. MEK inhibitor, which blocks the ERK signal pathway, and HOG/p38 mitogen-activated protein kinase inhibitor hardly diminished the stimulatory signal of PDBu and calcium ionophore. Activation of CAMP-dependent protein kinase by the addition of 8-Br-cAMP increased the luciferase activity by about 1.7-fold (Fig. 4D).

Up-regulation of COT mRNA Levels—An RNase protection assay was performed to determine whether the increase in the transcriptional activation of the 5′ flanking region of the human COT gene by T cell activating signals correlates with an increase in COT mRNA levels after T lymphocyte stimulation. A riboprobe from the COT coding sequence was hybridized with RNA isolated from Jurkat cells stimulated with soluble αCD3 (10 μg/ml) and PDBu (50 ng/ml) for different times. As shown in Fig. 5A, αCD3 and PDBu stimulation transiently increased COT mRNA levels (~4-fold). A similar increase in the level of COT transcripts was detected by RT-PCR analysis of mRNA of Jurkat cells stimulated with PDBu and calcium ionophore in different times, using primers that amplified a COT coding sequence fragment. As a control, a 187-bp fragment of β-actin was also amplified in each reaction (Fig. 5B).

To distinguish COT-1 from COT-2 and COT-3, a Northern blot with total RNA from Jurkat cells stimulated for different times with PDBu (50 ng/ml), calcium ionophore (0.25 μM), and okadaic acid (100 ng/ml) was hybridized with the coding COT probe (Fig. 6A). Whereas a hybridization signal of 3.0 kb, corresponding to COT-2 and COT-3, was detected 3 h after stimulation, the COT-1 message was first detected 6 h after stimulation. In agreement with the Northern blot analysis, COT-1 transcript levels determined by RT-PCR were not increased at 4 h after stimulation of Jurkat cells with the stimuli described above (Fig. 6B). At this time of stimulation, COT-2 and COT-3 levels were increased to an equal extent (Fig. 6B).

Subcellular Distribution of COT Transcripts—Next, we decided to investigate the subcellular distribution of COT-1, COT-2, and COT-3. RNA was isolated from the cytoplasmic
fraction and the nuclear fraction of intact Jurkat cells. A dot blot performed with these RNAs was hybridized with a probe specific for the 5’ UTR of COT-1 (probe C), the COT coding probe, and a probe containing the −778 to −30 nt sequence of the COT promoter (Fig. 7A). Comparison of the hybridization signals obtained with the different probes and RNA fractions indicated that the COT-1 transcript is mainly located in the nuclear fraction.

We next decided to study the distribution of the different COT messengers to polysomes. The postmitochondrial supernatant of Jurkat cells was subjected to sucrose gradient fractionation, and RNA was isolated from the different fractions. The levels of the different COT transcripts were measured by RT-PCR analysis. As shown in Fig. 7B, the fraction of COT-1 located in the cytoplasmic fraction is not associated with polysomes. In addition, only a small fraction of COT-2 was not loaded with ribosomes. The fact that both COT-2 and COT-3 were detected in fractions corresponding to small polysomes indicates a low translation efficiency of these transcripts. As a control of polyribosome-associated mRNA, we assayed the same fractions for β-actin messenger (Fig. 7B). Stimulation of Jurkat cells with PDBu and calcium ionophore did not change the distribution of these transcripts in the different fractions (data not shown).
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levels were determined with primers 8D (1 primer region (nt COT 3837–4646), the isolated from the nuclear fraction was hybridized with probe C (nt with total RNA, RNA isolated from the cytoplasmic fraction, and RNA established. Nevertheless, the possibility that physiological significance of this finding remains to be estab-

lished. The occurrence of upstream open reading frames has only been detected in about 10% of vertebrate mRNAs, and their physiological role is still unclear. Interestingly, the majority of these mRNA species code for proteins involved in signal transduction (30, 31 and references therein).

FIG. 7. Distribution of COT transcripts. A, a dot blot performed with total RNA, RNA isolated from the cytoplasmic fraction, and RNA isolated from the nuclear fraction was hybridized with probe C (nt 3837–4646), the COT coding probe, and a probe generated from the COT promoter region (nt −778 to −30). B, distribution of COT transcripts in polysomes. The figure shows the relative absorbance corre-
sponding to the different sucrose fractions. The 28 S and 18 S RNAs from 6 μl of each fraction were separated on a 2.2 M formaldehyde-
agarose denaturing gel and photographed after ethidium bromide stain.

RT-PCR analysis of the different fractions was performed. COT-1 levels were determined with primers 6D (1 μM) and 7R (1 μM), COT-2 levels were determined with primers 6D (1 μM) and 7R (1 μM), and COT-3 levels were determined with primers 1D (1 μM) and 7R (1 μM). Primers for β-actin detection were used at a concentration of 0.1 μM.

DISCUSSION

In this paper we have identified the promoter region of the human COT gene and demonstrated that its activity is inducible by T cell-activating signals. We have also identified three different human COT mRNAs, COT-1, COT-2, and COT-3, with different lengths in the 5' UTR but a common transcription initiation site. This site is located 4748 nt upstream of the translation initiation site of COT kinase. The first exon of COT-1 (denominated exon 1) comprises these 4748 nt and the first 336 nt of the coding sequence of COT kinase (see Table I and Fig. 1). The lack of splicing in the 5' UTR of COT-1 mRNA species results in a predominantly nuclear distribution. The physiological significance of this finding remains to be estab-

lished. The occurrence of upstream open reading frames has only been detected in about 10% of vertebrate mRNAs, and their physiological role is still unclear. Interestingly, the majority of these mRNA species code for proteins involved in signal transduction (30, 31 and references therein).

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requirements are needed for activation of the −340 COT promoter-driven transcription, indicating that at least the AP-1 binding site present at −327 nt could play a role in the PDBu and calcium ionophore-triggered COT promoter activation.

COT kinase activity is crucial for the transduction mechanism of activating signals in T cells during G0/G1 transition (1, COT and calcium ionophore-triggered binding site present at moter-driven transcription, indicating that at least the AP-1 providing cyclosporin A.

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