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ARTICLES

Phorbol Ester-Induced Transcription of an Immediate-Early Response Gene by Human T Cells Is Inhibited by Co-Treatment With Calcium Ionophore

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Abstract Human T cells require two discrete signals to initiate their proliferation. In Jurkat T cells the first signal can be provided by the phorbol ester TPA and the second by the calcium ionophore A23187. We have isolated a cDNA from Jurkat T cells representing mRNA induced by TPA but inhibited by simultaneous treatment of the cells with antibody, lectin, or A23187. Sequencing revealed identity of the Jurkat clone to a cDNA, termed ETR101, recently isolated from HL60 promyelocytic leukaemia cells and shown to be an immediate early gene expressed upon TPA stimulation of these cells [Shimizu et al.: J Biol Chem 266:12157, 1991]. The gene is also induced very rapidly upon TPA treatment of Jurkat cells and is superinduced by co-treatment with cycloheximide. The predicted amino acid sequence encoded by ETR101 has weak homology to *Jun*B and *Jun*D, therefore it is of some interest that these three genes share the chromosomal localization, 19p13.2. The divergent effects of TPA treatment upon cell proliferation and differentiation in different circumstances allow some speculation about a possible role for the ETR101 gene product upon cellular differentiation.

Key words: ETR101, Jurkat cells, transcriptional regulation, chromosome localization

The process of T cell activation centers around transcriptional activation of the genes coding for interleukin 2 (IL-2) and its receptor (IL-2R) [reviewed in Crabtree, 1989]. The precise sequence of events regulating this process has not been determined but can be divided into immediate, early, and late phases. Immediately upon the interaction of the T cell antigen receptor with its ligand, several genes are activated and the products of some of these genes serve as transcriptional factors involved in the activation of the IL-2 and IL-2R genes. Later gene activations involved in the process of T cell activation appear to be dependent upon IL-2 interacting with its own receptor on the T cell membrane [Crabtree, 1989].

Physiological T cell activation depends upon the lymphocyte recognizing antigen in the context of the major histocompatibility complex (MHC) together with accessory factors such as interleukin-1 (IL-1) provided by macrophages or other antigen presenting cells. In vitro these requirements can be met with antibodies or lectins to the T cell receptor or its associated CD3 complex replacing the antigen/MHC signal, and a phorbol ester such as TPA replacing the signal provided by IL-1 [Weiss et al., 1984]. The antigen/MHC signal can also be provided by a calcium ionophore, and the ionophore A23187 by itself can initiate a degree of IL-2 independent T cell proliferation [Truneh et al., 1985]. However, IL-2 induction and the initiation of IL-2-dependent T cell proliferation is absolutely dependent on two stimuli, and neither antibody/lectin/ionophore on one hand, nor TPA/IL-1 on the other, is capable of initiating this event [Truneh et al., 1985; Weiss et al., 1984]. The molecular basis for the two signal requirement is not entirely clear. The IL-2 enhancer transmits all known physiologic regulatory information for the IL-2 gene, and in trans-

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fected T cell lines this element requires both signals in order to function [Maruyama et al., 1987; Durand et al., 1987]. Since some early response genes such as c-*jun* and c-*fos* are induced directly in response to TPA stimulation, and their products bind the AP-1 binding site of the IL-2 gene enhancer, such stimulation can be envisaged as a direct cascade of events. However, the widespread usage of common transcriptional regulatory elements such as the AP-1 and NF-κB binding sites requires feedback regulation in specific cell types and this is often accomplished by inhibitory proteins that bind and inactivate the transcriptional activators [Baeuerle and Baltimore, 1988]. One such inhibitor, IκB, has recently been identified as the product of an immediate early response gene in monocytes, thus emphasizing the complexity of signal-induced regulation. Further regulation occurs post-transcriptionally; for example association of the IκB inhibitor protein with the 65kD subunit of NF-κB is blocked by phosphorylation of IκB by protein kinase C [Haskill et al., 1991; Ghosh and Baltimore, 1990]. By contrast, the transcriptional factor, cAMP response element binding protein, will activate the AP-1 site of the c-*jun* gene promoter upon phosphorylation by cAMP-dependent protein kinase A (PKA), but in the absence of PKA activity it serves as a repressor of the c-*jun* promoter [Lamph et al., 1990].

For a number of years we have been studying the structure and function of a T cell activation antigen, termed PTA1, involved in the differentiation of T cells and T cell clones [Burns et al., 1985]. The antigen was also identified on blood platelets and shown to be involved in membrane signaling by these cells [Scott et al., 1989]. More recent studies have shown that, on Jurkat T cells, the antigen is a complex of several phosphoproteins and that the surface expression of at least some members of the complex is differentially regulated by different T cell activators: thus on these cells, surface expression of the antigen is increased 8-fold by stimulation with TPA and this effect is abrogated by co-stimulation of the cells with PHA, anti-CD3 antibodies, or the calcium ionophore A23187 [Scott et al., submitted]. To structurally characterize members of the complex, a rabbit antiserum was raised to the proteins immunopurified from platelets and used to screen a cDNA library prepared from Jurkat cells. Positive clones were further selected by their relative induction (on Northern blots) in response to cell stimulation by TPA or TPA with ionophore. The complete cDNA for one gene that is regulated in an identical fashion to the original antigen has been identified and characterized. However, during the preparation of this manuscript Shimizu et al. [1991] reported the cloning of the same gene product from HL60 promyelocytic leukemia cells, and this differed only in the length of 5′ untranslated product that was isolated. The present report therefore concentrates on presenting data showing the unusual responsiveness of this gene, termed ETR101, in response to the two discrete T cell activation signals. In addition we have mapped the ETR101 gene to chromosome 19p13.2 which may be of some significance since *Jun*B and *Jun*D—which share a region of homology with ETR101—map to the same region of the human genome [Mattei et al., 1990].

MATERIALS AND METHODS

Cloning of ETR101 cDNA From Jurkat Cell Library

The PTA1 antigen complex was immunopurified from platelet lysate using Sepharose beads coupled to a monoclonal antibody against one member of the complex as described previously [Scott et al., 1989]. The purified antigen was then used to raise a polyclonal antiserum in rabbits as described [Scott et al., 1989]. The rabbit antibodies were affinity purified over Sepharose beads coupled to protein A: the purified rabbit antibodies blocked binding of the monoclonal antibody and inhibited the differentiation of T cells [Jin et al., 1989]. A human Jurkat T cell cDNA library in λgt11 (kindly provided by Dr. Michael Crumpton, Imperial Cancer Research Fund, London) was induced and screened with the rabbit antibodies at a dilution of 1/300, and positive plaques were identified using an enzymic horse-radish peroxidase conjugate (Vector Labs, Burlingame, CA). Some 1×10^6 clones were screened to isolate 13 positive clones in the primary screen of which 10 remained positive through subsequent secondary and tertiary screens. Cross hybridization indicated that 4 of the clones were homologous. Northern blot analysis of the cloned isolates showed that the homologous clones all hybridized strongly with a 2.2 kb message isolated from Jurkat cells treated with TPA, but only very weakly from Jurkat cells treated with TPA plus ionophore (see Results). The largest of these clones was selected for sequencing but the total length of the cDNA insert was approximately

1.8 kb which did not completely account for the 2.2 kb message seen in Northern analysis. This isolate was therefore used to re-screen the Jurkat cDNA library and also a 3 day PHA blast cDNA library purchased from Clonetech (San Francisco, CA). Duplicate lifts onto Hybond N membranes (Amersham, Buckinghamshire, UK) were processed according to the protocol provided by the manufacturer and screened with the radiolabeled cDNA isolate. The cDNA probe was labeled as described [Feinberg and Vogelstein, 1983] using reagents purchased from Bresatec, Adelaide, Australia. Two positive clones, shown to possess identical inserts, were isolated from the PHA blast library and both had inserts of 0.9 kb. However, one positive clone isolated from rescreening the Jurkat library with the most 5′ PstI-EcoR1 fragment of the initial clone was found to possess an additional 84 bp in the 5′ direction. This clone, termed 11c1, was sequenced according to the method of Sanger et al. [1977] and was shown to possess a total of 1,902 bp.

Cell Culture and Stimulation

The Jurkat T cell line, LiBr melanoma, and U937 promonocytic cell lines were cultured in RPMI 1640 tissue culture medium (Cytosystems, Sydney, Australia) containing 10% FCS (Flow Labs, Washington, D.C.), 1% glutamine, 5×10^{-5} M β-ME, penicillin (100 U/ml), and streptomycin (100 μg/ml) (RPMI tissue culture medium), and maintained at 37°C in 10% CO_2/90% air. Peripheral blood lymphocytes (PBL) were prepared from peripheral blood mononuclear cells (PBMC) that were isolated from the heparinized blood of normal individuals by collecting cells from the interface after centrifugation at 350*g* for 20 min over Ficoll hypaque (Lymphoprep; Nycomed, Oslo, Norway). PBMC were resuspended in RPMI tissue culture medium containing 20% FCS and PBL prepared by monocyte depletion. PBMC were depleted of monocytes by incubating them on 150 mm polystyrene dishes (Nunc, Kamstrup, Denmark) for 2 h at 37°C. PBL were gently removed, washed, and resuspended in RPMI tissue culture medium. Macrophages were prepared by culturing freshly isolated monocytes in the continuous presence of 50 ng/ml of either GM-CSF or M-CSF (kindly provided by Genetics Institute, Boston, MA) for 9 days. Platelets were obtained as concentrates from the Red Cross Blood Center, Newcastle. The phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), calcium ionophore, A23187, and the activator of cyclic AMP, forskolin, were purchased from the Sigma Chemical Corp. (St. Louis, MO) and the lectin, phytohemaglutanin (PHA), from Wellcome (Dartford, U.K.). The phosphatase inhibitor, okadaic acid, was a gift from Prof. P. Cohen, Department of Biochemistry, University of Dundee, U.K. The hybridoma cell line producing anti-CD3 antibody, OKT3, was purchased from the American Type Culture Collection (Rockville, MD).

RNA Preparation and Polymerase Chain Reaction

Total RNA was isolated from cells by acid guanidinium thiocyanate-phenol-chloroform extraction as described [Chomczynski and Sacchi, 1987]. The integrity of the RNA was assessed on a 1% TBE-agarose slab gel (1 × TBE = 0.09 M Tris-borate/0.002 M EDTA) and single-strand cDNA synthesized for 2 h at 42°C in a 50 μl reaction mix containing 1 μg total RNA, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 500 μM each dATP, dCTP, dGTP, dTTP (dNTPs), 1 μl oligo dT (0.2 μg/μl), and 10 U AMV reverse transcriptase. Five microliters of the above reaction volume were then added to a 100 μl PCR reaction mix containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 2 μl of a 10 mM dNTP mix, 1 μl Taq DNA polymerase (Amplitaq, Cetus Corp., Emeryville, CA). Prior to the addition of the DNA polymerase, samples were heated to 98°C for 10 min and then placed on ice. DNA polymerase was added and the samples overlaid with 100 μl paraffin oil. The cycle protocol was as follows: cycles 1–3: 96°C, 3 min; 50°C, 1 min; 74°C, 1 min; cycles 4–40: 96°C, 1 min; 50°C, 1 min; 74°C, 1 min. At the completion of the PCR 10 μl of each sample was removed and digested in a 50 μl reaction with the restriction enzyme, Hae III (Pharmacia, Uppsala, Sweden). The undigested PCR products and the digested products were then analyzed on a 6.5% TBE/acrylamide gel. The primer sequences were, 5′-TAGAGAGGCGTGCAGAGC-3′ and 5′-GAGAGGTAGAGCTCCCGG-3′ and the expected size of the PCR products was 227 bp undigested, and 169 and 58 bp following digestion with Hae III.

Northern Blot Analysis

Total RNA isolated from untreated and stimulated Jurkat cells as described [Chomczynski and Sacchi, 1987] was incubated in 50% formamide, 16% formaldehyde, 1 × MOPS buffer

(20 mM 3-[N-Morpholino]-propane-sulfonic acid, 5 mM Na acetate, pH 7.0, 1 mM Na_2EDTA) at 65°C for 5 min and then analyzed (10 μg/track) on a 1.2% agarose/formaldehyde slab gel in 1 × MOPS buffer. The gel was stained with ethidium bromide, photographed, rinsed in water for 30 min and the RNA transferred in 20 × SSC (1 × SSC = 0.15 M NaCl/0.015 M Na citrate) by capillary action to Hybond N membrane. Following the transfer the filters were rinsed briefly in 2 × SSC, air-dried, UV-crosslinked, and baked at 80°C for 2 h. The filters were blocked for at least 2 h at 42°C in a prehybridization/hybridization solution containing 50% formamide, 5 × SSC, 50 mM Na phosphate, pH 6.5, 0.1% SDS, 100 μg/ml sonicated salmon sperm DNA (Sigma), and 5 × Denhardt's (1 × Denhardt's = 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrolidone). The salmon sperm DNA was heated at 98°C for 10 min prior to its addition to the prehybridization mix. A cDNA probe spanning nucleotides 97–1321 of 11c1 was radiolabeled as described [Feinberg and Vogelstein, 1983]. The probe that had been heated at 98°C for 10 min in 1–2 ml prehybridization/hybridization solution, to which a further 50 μg/ml sonicated salmon sperm DNA had been added, was then added to the existing prehybridization/hybridization solution and the filters incubated at 42°C for 12 h. The final probe concentration was 5 ng/ml hybridization solution. The filters were then washed 2 times at room temperature in 2 × SSC, 0.1% SDS, and at 60°C for 30–60 min in 0.2 × SSC, 1% SDS. Excess moisture was removed and 11c1 mRNA visualized by autoradiography. As an internal standard for RNA loading the filters were stripped and re-probed with a cDNA encoding GAPDH (kindly provided by Dr. Ora Bernard, Walter and Eliza Hall Institute, Melbourne).

Chromosome Localization by In Situ Hybridization

A cDNA probe spanning nucleotides 97–1321 of 11c1 was tritium labeled to a specific activity of approximately 1.3 × 10^8 cpm/μg DNA and hybridized, as described previously [Sutherland et al., 1988], to metaphases from two normal males at a concentration of 0.1 μg/ml, and exposed for 33–39 days. All silver grains touching chromosomes were counted to determine the pattern of the hybridization of the probe.

RESULTS

Overlapping clones identified a full-length cDNA of 1,902 bp, excluding the poly (A) tail. The cDNA was termed 11c1; however, a search of DNA and protein data bases revealed that the nucleotide and predicted amino acid sequence of 11c1 was identical, except for an additional 91 bp in the 5′ untranslated region, to that of ETR101, a gene isolated by Shimizu et al. [1991] from an HL60 promyelocytic cDNA library (Fig. 1). As a consequence the 11c1 gene was renamed ETR101. As noted by Shimizu et al. [1991], the coding region of ETR101 shows a high degree of homology to chx1, a murine mRNA recently reported as being rapidly and transiently induced on activated mouse T cells, B cells, and serum-stimulated 3T3 fibroblasts, upon treatment with cycloheximide [Coleclough et al., 1990]. The deduced amino acid sequences of ETR101 and chx1 show 77% homology, as do the coding nucleotides. It is particularly striking that the amino terminus of both proteins is

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  1  GTCGCGGCCGCGACCTGCAGGCGCAGAACTGGTAGGTATGGAAGATCCCTCGAGAT  (J)

 56  CCATTGTGCTCTAAAGCAGTGTCACTGCGTGGGTTGGTTTGTGTAGAGAGGCGTGA  (J)
                                  GGTTTGTGTAGAGAGGCGTGA  (H)

112  GCGAGCCCGTTGTCCGGAGTGCACCTGCTGCCTGTTCTGTCCCTCCCGGGAGCCCC  (J)
     GCGAGCCCGTTGTCCGGAGTGCACCTGCTGCCTGTTCTGTCCCTCCCGGGAGCCCC  (H)

168  CGCCGCTGTCGCCGTCGAGTCGCCATGGAAG ..........................  (J)
     CGCCGCTGTCGCCGTCGAGTCGCCATGGAAG ..........................  (H)
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Fig. 1. Comparison between the 5′ untranslated region of ETR101 cDNA clones isolated from Jurkat and HL60 cells. Shown is the nucleotide sequence of the 5′ untranslated region of ETR101 cDNA clones isolated from Jurkat (J) and HL60 (H) cDNA libraries. Underlined is the start codon of the ETR101 protein.

identical for the first 54 residues, except for a single change at position 27 that converts an aspartate in chx1 to a glycine in ETR101 (Fig. 2A). The change is coded for by a single base change that converts the triplet from GAC to GGC. At the protein level this change could have some significance since it changes the amino acid sequence from arginine-glycine-aspartate (RGD) to arginine-glycine-glycine (RGG). The RGD tripeptide sequence is intimately involved in a wide variety of cell adhesive processes where it serves as a ligand for a family of receptors termed integrins [Ruoslahti and Pierschbacher, 1987]. The sequence is statistically under-represented in known protein sequences but, where present, it is highly conserved through evolution; even within the transactivating protein, tat, of the human immunodeficiency virus, the RGD sequence is highly conserved among viral isolates [Brake et al., 1990]. Since ETR101 cDNA was cloned from a leukemic cell line [as did Shimuzu et al., 1991] the possibility was considered that the single base substitution was a mutation present in leukemic cells, rather than a species difference. This was tested for by carrying out a polymerase chain reaction (PCR) using primers on either side of the predicted mutation, then digesting the PCR product with the restriction enzyme, Hae III, which will cut fragments coding for RGG but not RGD. Six cell types were tested in this way, including peripheral blood lymphocytes, macrophages, and platelets, each isolated from different normal subjects. In every case the PCR yielded fragments of the predicted size that were cleavable with the restriction enzyme (Fig. 2B). Thus the loss of the RGD sequence reflects a true species difference and perhaps suggests that this sequence performs no significant function in chx1.

The chromosome localization of the gene coding for ETR101 was carried out using a cDNA probe spanning nucleotides 97–1321 of the 11c1 clone isolated from the Jurkat library. The in situ hybridization results (Fig. 3) showed that out of 214 grains on 40 metaphases, 25 (11.7% of all silver grains) were on the short arm of chromosome 19 with a peak at 19p13.2–19p13.3. The distribution of the silver grains from these 40 metaphases, and an additional 11 metaphases in which there were silver grains on 19p,

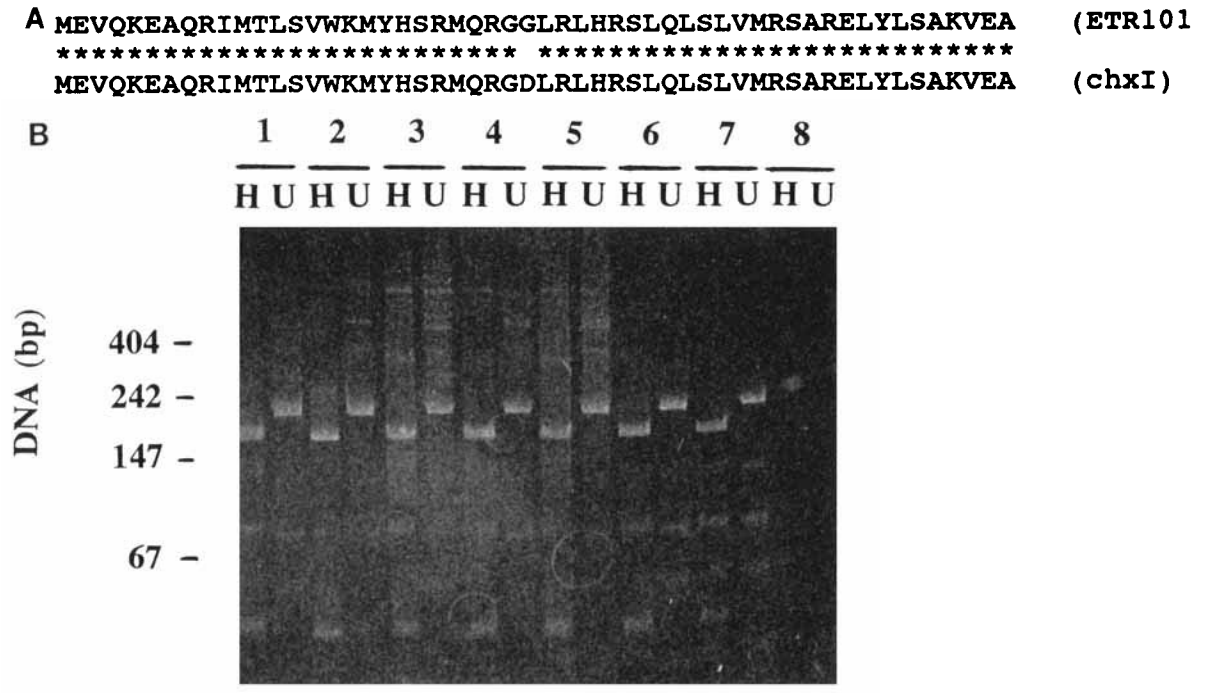

Fig. 2. Investigation of the significance of a single base change in the nucleotide sequence of ETR101 cDNA, when compared to that of its murine homologue, chxI. **A:** Comparison between the first 54 amino acids of ETR101 and chxI proteins. **B:** cDNA reverse transcribed from total RNA isolated from a number of cells and cell lines was used in a PCR to amplify a region of ETR101 DNA spanning nucleotides 100–337 of 11c1 cDNA. An aliquot of the PCR product was digested with Hae III, and the undigested (U) and digested (H) products analyzed on a 6.5% acrylamide gel. 1, LiBr; 2, Jurkat; 3, platelet; 4, U937; 5, PBL; 6, macrophages cultured in GM-CSF; 7, macrophages cultured in M-CSF; 8, control, where no cDNA was added.

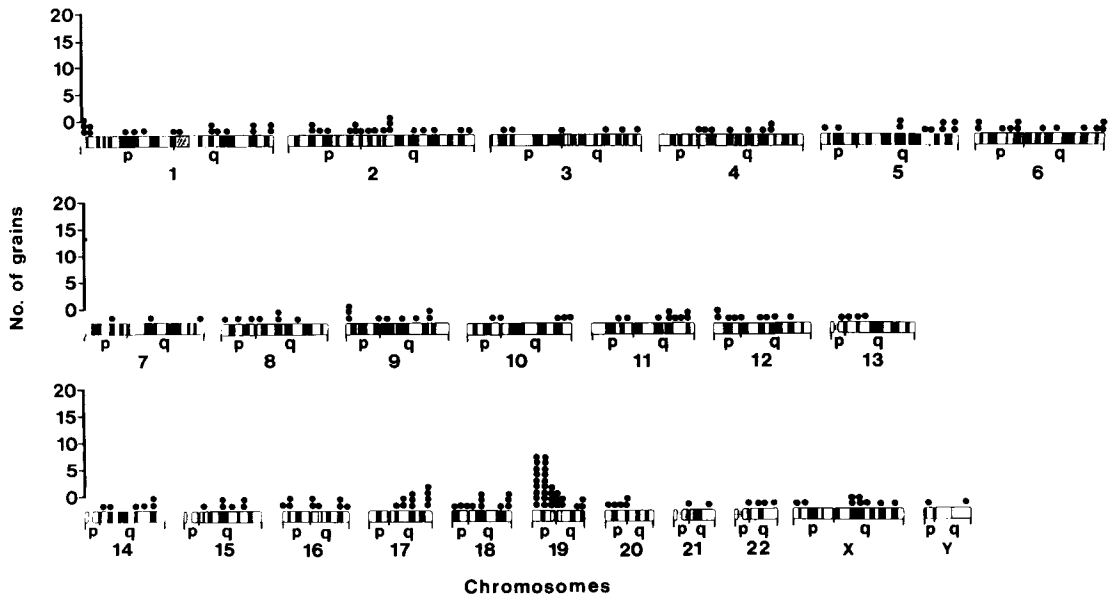

Fig. 3. Distribution of silver grains over all chromosomes in 40 metaphases after in situ hybridization with nucleotides 97–1321 of the 11c1 probe.

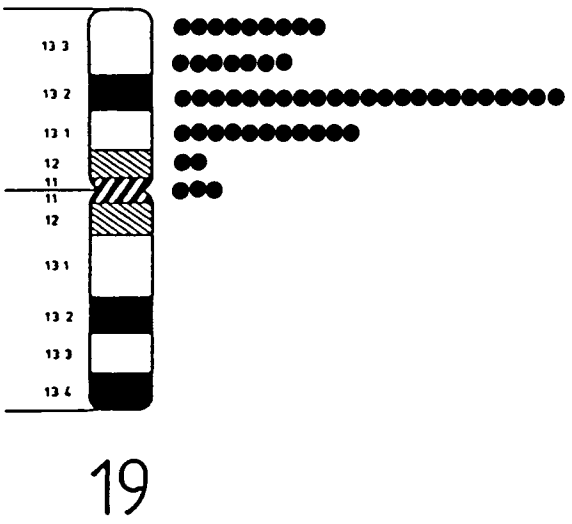

Fig. 4. Distribution of silver grains over the short arm of chromosome 19 from 28 metaphases showing signal on this chromosome arm.

are shown in Figure 4. These data localize ETR101 to the region 19p13.1–19p13.3, but most probably to 19p13.2. A similar result was obtained from the hybridization to the metaphases from the second male (data not shown).

The regulation of ETR101 mRNA levels in Jurkat cells treated with TPA and/or cyclohexamide (chx) was investigated using Northern blot analysis. In a manner similar to that reported for other immediate-early gene products the level of ETR101 message in Jurkat cells rapidly increased (within 10 min) upon stimulation with either TPA or chx and was superinduced in the combined presence of these agents (Fig. 5). Shimizu et al. [1991] also reported a rapid increase in ETR101 gene expression in response to TPA and/or chx treatment. However the regulation of gene expression observed by these authors using HL60 cells differed from that in our own study with Jurkat cells. Whereas the increase in ETR101 gene expression in HL60 cells in response to TPA stimulation was shown by Shimizu et al. [1991] and in our own hands (data not shown) to occur transiently, peaking at 1 h and declining thereafter to undetectable levels, that observed in Jurkat cells was maintained for a period of at least 24 h following the start of stimulation (Figs. 5, 6B). It was noted, however, that in several experiments there was frequently a slight transient decline in ETR101 mRNA levels at 6–12 h post stimulation (e.g., Fig. 6B). The reason for this transient decrease is not known.

The regulation of ETR101 gene expression in Jurkat cells was further investigated at later time points. Figure 6A shows a representative experiment where cells were treated with various agents for 24 h and analyzed by Northern blotting. It is evident that the level of ETR101 message was upregulated by treatment with TPA but not by the presence of an antibody to the CD3 complex, by calcium ionophore, or by forskolin, an activator of cyclic AMP. The lectin, PHA,

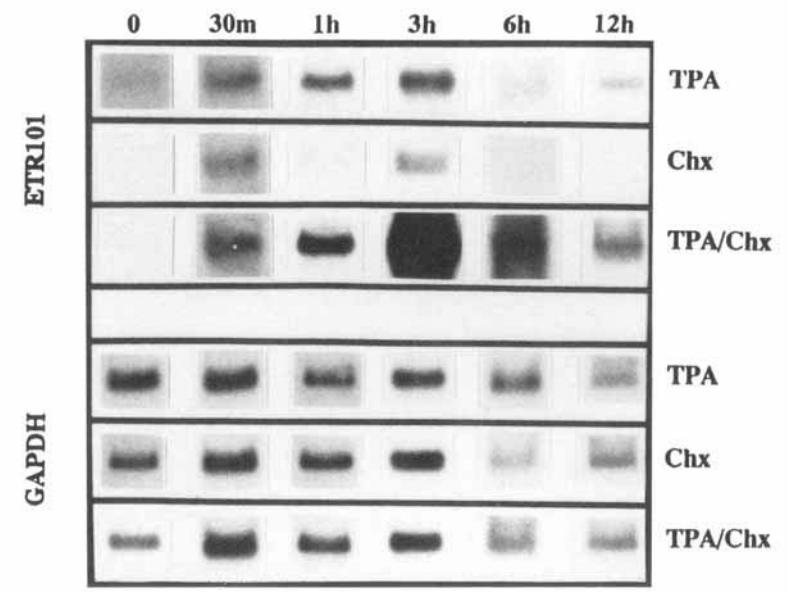

Fig. 5. Regulation of ETR101 expression in Jurkat cells by TPA and cycloheximide. Total RNA isolated from Jurkat cells treated for varying lengths of time (0–12 h) with either TPA (50 ng/ml), cycloheximide (chx; 12 μg/ml), or a combination of TPA and cycloheximide (TPA/Chx) was probed on Northern filters with radiolabeled 11c1 cDNA (nucleotides 97–1321) (ETR101). As a control for sample loading and RNA integrity the filters were reprobed for GAPDH mRNA. Results were visualized by autoradiography.

induced some ETR101 mRNA above unstimulated levels but less than that seen after TPA stimulation. The combined presence of TPA together with anti-CD3 antibody or PHA reduced the level of ETR101 mRNA from that seen with TPA alone, and co-treatment with the ionophore, A23187, abolished the effect of TPA. The combined presence of forskolin had no effect. In repeated experiments carried out between 14 and 20 h of cell stimulation (data not shown), both CD3 and PHA, used alone, stimulated the expression of detectable message but always much less than TPA, and used in combination with TPA both reagents caused variable but consistent inhibition of the TPA response.

Since the combined presence of phorbol ester and ionophore is necessary to induce T cell activation in vitro, the downregulation of ETR101 gene expression in Jurkat cells costimulated with these agents was of considerable interest. For this reason the effect of ionophore, over a wide range of time points, was investigated further. As is shown in Figure 6B costimulation with ionophore inhibits, at all time points, the upregulation of ETR101 mRNA levels induced by TPA alone.

In contrast to the inhibitory effect of ionophore on TPA-induced ETR101 mRNA expression, A23187 did not inhibit the upregulation of ETR101 message induced by treatment with chx alone (data not shown). The ionophore also failed to inhibit the superinduction of ETR101 induced by both TPA and chx (Fig. 7).

DISCUSSION

Rabbit antiserum raised to a T cell and platelet activation antigen, termed PTA1, was used to screen a Jurkat T cell cDNA library. A number of clones were isolated, four of which were identical. Northern analyses, using the cDNA isolates as probes, revealed that the expression of the cloned gene product was regulated in an identical manner to that of PTA1. Full-length cDNA clones, obtained by reprobing T cell cDNA libraries yielded a final cDNA of 1,902 bp. Sequencing showed this clone to code for an immediate-early protein identical to ETR101 cloned by Shimizu et al. [1991] from HL60 promyelocytic leukemia cells. There were, however, differences in the regulation of ETR101 expression in response to stimulation with the phorbol ester, TPA, between the two cell types. Where the increase in ETR101 mRNA levels in response to TPA in Jurkat cells was maintained for at least 24 h, the positive response in ETR101 expression to TPA stimulation shown in HL60 cells was transient [Shimizu et al., 1991] [Scott, unpublished data]. The activation and subsequent proliferation and differentiation of T cells in vivo is a 7–10 day process, during which time

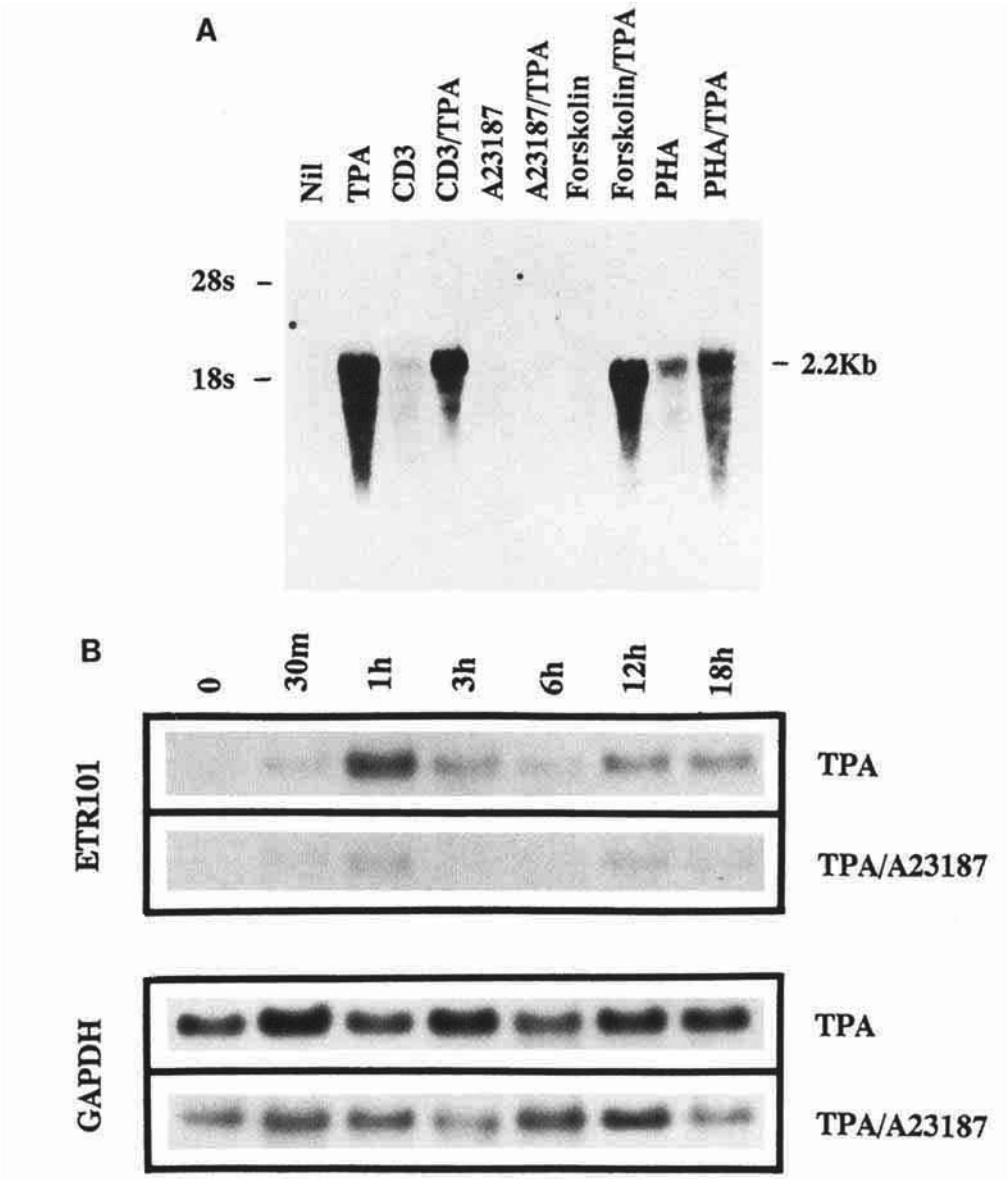

Fig. 6. Regulation of ETR101 expression by agents that induce T cell activation. **A:** Total RNA isolated from Jurkat cells treated for 24 h with different agents used either alone or in combination; TPA, 20 ng/ml; PHA, 2 μg/ml; anti-CD3 mAb (OKT3), 1:500 ascites; A23187, 300 nM, and forskolin, 20 μM, were probed on a Northern filter with ^{32}P-labeled 11c1 (nucleotides 97–1321) cDNA and visualized by autoradiography. Ethidium bromide staining of the gel used for blotting was utilized to demonstrate equal sample loading. **B:** Jurkat cells were incubated for 0–18 h in the presence of either TPA alone (50 ng/ml) or a combination of TPA and A23187 (300 nM), total RNA isolated, and Northern analysis using 11c1 cDNA as a probe carried out. As an internal control for RNA loading and transfer the filters were reprobed with a cDNA encoding GAPDH.

the expression of a large number of molecules is specifically regulated. Two discrete signals are required for T cell activation and these can be provided in vitro by the combined presence of phorbol ester and calcium ionophore [Truneh et al., 1985]. By contrast, the treatment of HL60 cells with phorbol ester alone can induce growth arrest and macrophagic differentiation [Nathan et al., 1979]. Since ETR101 mRNA expression is differentially regulated in Jurkat and HL60 cells following cellular activation, it is possible that the ETR101 protein plays some role in determining the different responses of these cells to a common activation signal. The role of ETR101 was investigated further by studying the regulation of ETR101 mRNA expression in Jurkat cells, by agents that induce T cell activation. When these agents were used over time periods

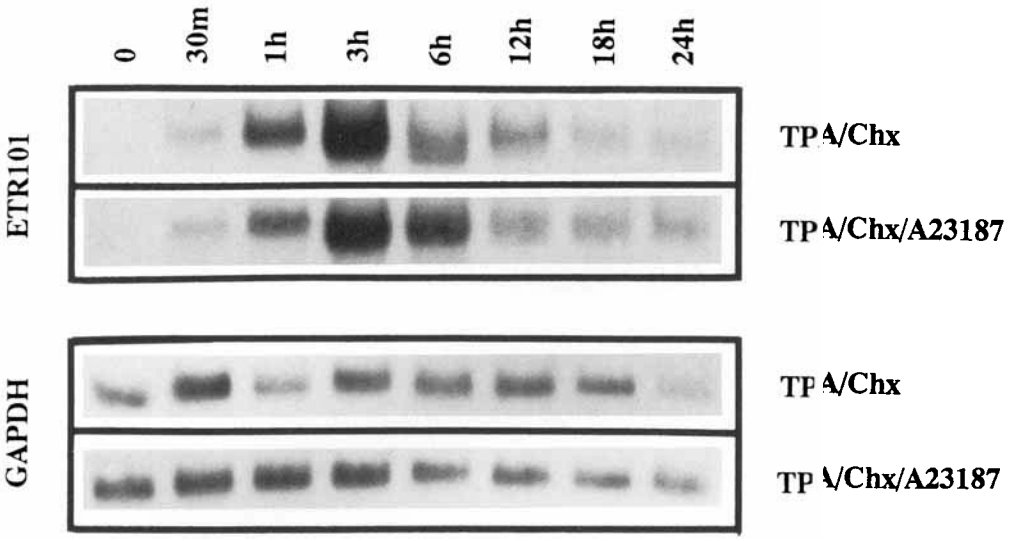

Fig. 7. Regulation of TPA and ionophore-induced ETR101 expression by cycloheximide. Total RNA isolated from Jurkat cells treated for 0–24 h with a combination of either TPA (50 ng/ml) and cycloheximide (chx; 12 μg/ml), or TPA, cycloheximide, and A23187 (300 nM), was probed on Northern filters with radiolabeled 11c1 cDNA (nucleotides 97–1321) (ETR101). As an internal control for RNA loading and transfer, filters were reprobed with GAPDH cDNA. Results were visualized by autoradiography.

of 14–24 h, other than the strong positive response to TPA and a slight increase in ETR101 mRNA levels in the presence of PHA, a lectin that acts through the T cell receptor, the other T cell activators, anti-CD3 antibody and the calcium ionophore A23187, had no effect on ETR101 expression. However, when used in combination with TPA, therefore providing the dual signal required for T cell proliferation, they were found to markedly attenuate the expression of ETR101 mRNA induced by TPA. Further investigation also revealed that the inhibitory effect of ionophore occurred over a wide range of time points. Interestingly, A23187 did not inhibit the expression of ETR101 message induced by chx or superinduced by cotreatment with TPA and chx. These results may indicate that the two agents induce ETR101 gene expression by different mechanisms, one that is inhibitable by ionophore and one that is not.

Using a probe that spanned the coding region and parts of the 5′ and 3′ untranslated sequence of ETR101 cDNA, the gene coding for this protein was shown to map to human chromosome 19p13.2. When Coleclough et al. [1990] isolated the murine homologue of ETR101, chx1, they were able to demonstrate patches of homology between chx1 and the mouse *Jun* D gene product. Although the homology between human *Jun* D and ETR101 is less pronounced it is interesting to note that the ETR101 gene localizes to the same region on chromosome 19 as the human *jun* B and *jun* D proto-oncogenes [Mattei et al., 1990]. Translocations involving this region of chromosome 19 have been observed frequently. In some cases of acute non-lymphoblastic leukemia (ANLL) or acute lymphoblastic leukemia (ALL) this region is linked to chromosome 11q23, while in other cases of ALL, to chromosome 1q23 [Trent et al., 1988]. In a case of megablastic leukemia (MEL), chromosome 1q12 was linked to this locus [Trent et al., 1988]. Whether or not the biological consequences of such translocations might implicate expression of the ETR101 gene is purely speculative since the function of the ETR101 protein product is not known at this time. However, the data reported here that ETR101 mRNA expression is downregulated when T cells receive the two signals required for proliferation but upregulated upon stimulation with a single differentiative signal, suggest the possibility that this protein may be involved in regulatory function.

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