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Regulation of Interleukin 2 Gene Expression by CD28 Costimulation in Mouse T-Cell Clones: both Nuclear and Cytoplasmic RNAs Are Regulated with Complex Kinetics

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T-cell receptor (TCR) signalling is required to induce expression of the interleukin 2 (IL-2) gene in mouse T cells. Additional costimulation through CD28 augments IL-2 production by 30- to 100-fold. Using IL-2 RNA accumulation and transcription reporter assays, we have addressed potential mechanisms of CD28 regulation at various time points of stimulation. The kinetic regulation of IL-2 mRNA by TCR and CD28 signals is complex: (i) at the earliest detectable time point, CD28 signalling causes a 20-fold increase compared with TCR signalling alone; (ii) both groups rapidly accumulate mRNA for the first 4 h; (iii) IL-2 mRNA then disappears from cells stimulated through the TCR alone but plateaus or increases slightly in cells costimulated through CD28; and (iv) after 8 h, the mRNA disappears in cultures with the anti-CD28 antibody. Transcription reporter assays did not show a specific effect of CD28 signalling on IL-2 enhancer driven transcription. This was true for either a 353- or a 1.9-kb enhancer, over a broad range of kinetics and TCR occupancy, and with several TCR signal mimics. The early component of CD28 costimulation is nuclear, however, since the initial enhancement of mRNA is also found in unspliced IL-2 RNA. Between 2 and 6 h, there is a marked difference in the rates of decay of IL-2 mRNA in the presence and absence of the CD28 signalling. Rapid decay of IL-2 mRNA commences after 8 h even in the presence of CD28 signals, although the decay occurs at a rate slower than that seen after 4 h of anti-TCR stimulation alone. This complexity suggests the existence of two interesting molecular mechanisms by which CD28 costimulates lymphokine gene expression.

The efficacy of antigen-specific signals in lymphocytes is regulated significantly by non-antigen-specific signals. While some of these additional signals are soluble, others are delivered to lymphocytes via ligands that are differentially expressed on antigen-presenting cells (APC). These signals are called costimulation. In particular, models using costimulation were proposed to explain the dichotomy between tolerizing and activating signals delivered via the same antigen-specific receptor (6, 11). Empirical evidence for such signals began to emerge from studies of in vitro stimulation of T-cell clones. It was discovered that a T-cell receptor (TCR) signal alone, delivered for example by major histocompatibility complex (MHC) and peptide antigen on a chemically fixed APC, did not result in optimal lymphokine production and proliferation (22, 30). For some T cells, the lymphokine interleukin 1 (IL-1) can provide costimulatory signals (32). For other T cells, which lack an IL-1 receptor, the addition of allogeneic APC to fixed syngeneic APC and peptide can augment lymphokine secretion by cell-cell contact (21). Cell surface molecules that deliver costimulatory signals were then widely sought (33).

The CD28 molecule on the surface of T cells (19, 23) has proved the most interesting costimulatory molecule to date because antibodies to CD28 synergize strongly with TCR signals for the production of cytokines such as IL-2 in $CD4^+$ T cells (24, 37). CD28 is a 44-kDa glycoprotein member of the immunoglobulin superfamily and is found as a homodimer on the surface of a major population of human and all mouse T cells. A ligand of CD28 called B7 (10, 16) was cloned as an activation marker on B cells and dendritic cells. Costimulation of TCR signals can also be provided by B7-transfected cells, demonstrating a functional role for its interaction with CD28 (10, 16). Additional homologs of B7 have recently been identified (5, 17, 18, 20, 26). A homolog of CD28, CTLA-4, has been cloned, and its protein product has been identified on activated T cells. A fusion construct of CTLA-4 with the Fc domain of immunoglobulin binds strongly to B7 and has proved a useful tool for characterizing costimulation processes in vitro and in vivo.

Preliminary studies have tested some of the potential mechanisms by which CD28 mediates its effects on lymphokine production. In studies using human T cells and activating antibodies against CD28, two mechanisms of action have been described: Lindsten et al. (27) discovered an effect on stabilization of IL-2, gamma interferon, tumor necrosis factor alpha, and granulocyte-macrophage colony-stimulating factor mRNAs, while Fraser et al. (13, 14) identified an effect on transcription initiated at the IL-2 enhancer.

The responsiveness to costimulation of the IL-2 gene in mouse T-cell clones is well characterized. Therefore it seemed appropriate to characterize and quantitate molecular effects of CD28 signalling in these clones. In analyzing the kinetics of IL-2 mRNA accumulation, we have uncovered evidence for two mechanisms of CD28 enhancement. An early mechanism is nuclear in origin but is apparently distinct from transcription initiation via the IL-2 enhancer. A second mechanism is cytoplasmic RNA stabilization. The complexity of the kinetic regulation of these mechanisms is striking and may be coordinated, resulting in tight regulation of IL-2 secretion.

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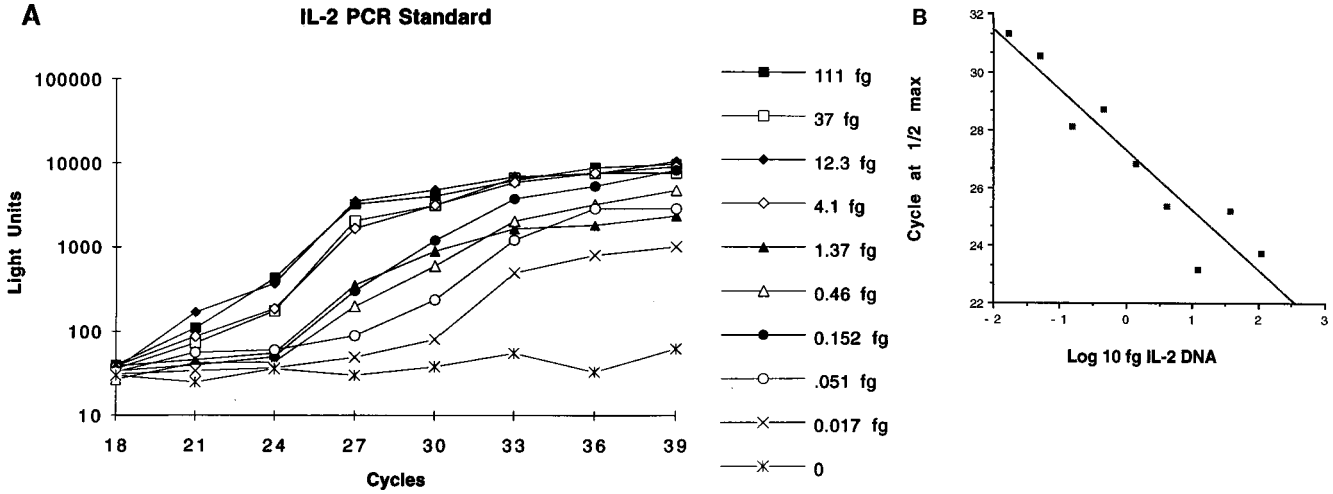

FIG. 1. Quantification of IL-2 RNA levels by RT-ase-mediated PCR. Gel-purified PCR product, previously amplified from IL-2 cDNA, was titrated into a series of PCRs. For each DNA amount, samples were removed from the same wells at the indicated cycles and analyzed for PCR product by chemiluminescence as described in Materials and Methods (A). Data from the luminometer were fit to a logistic equation, and the parameters were used to calculate the C_{50} of amplification. C_{50} values were plotted against the $\log_{10}$ (input DNA concentration) (B), and the best-fit line was used to estimate experimental cDNA amounts.

MATERIALS AND METHODS

T-cell stimulation. The mouse Th1 T-cell clones A.E7 and 3R3.11 were propagated as described previously (4). Briefly, cells were stimulated with peptide antigen and irradiated splenic APC, grown in IL-2, and then rested for 3 to 5 weeks. Cells were harvested and treated with anti-MHC class II antibodies (10.2.16 and Y17) and complement (Low Tox-M Rabbit Complement; Cedarlane Laboratories, Hornby, Ontario, Canada) before experimental stimulation. Except where noted, experimental stimulation consisted of culturing 10^6 T cells in 10 ml of medium (4) in a T-25 flask (Costar) coated for at least 2 h with purified H57-597 (H57) anti-TCR β antibody (hereafter referred to as anti-TCR) at a concentration of 10 μg/ml in phosphate-buffered saline (PBS). For the experiment shown in Fig. 5B, 2×10^7 cells were stimulated in a T-162 flask (Costar) in 100 ml of medium. Where indicated, the hamster anti-mouse CD28 monoclonal antibody 37.51 (developed in the laboratory of James Allison; hereafter referred to as anti-CD28) was added to the medium at a 1:5,000 dilution of ascites fluid. Cells were harvested at indicated time points with 0.05% trypsin–0.02% EDTA (Biofluids, Rockville, Md.), quenched with medium, and washed with cold PBS (Biofluids). For RNA preparation, cells were frozen at −70°C and directly resuspended in RNAzol. For luciferase assays, cells were resuspended in 250 mM Tris-Cl (pH 7.5 at 25°C) and then frozen at −70°C.

IL-2 protein assay. IL-2 protein activity was assayed as described previously (2) by measuring the proliferation of the IL-2-dependent CTLL cell line (American Type Culture Collection, Rockville, Md.). IL-2 units presented in Fig. 2 are $\log_{10}$ relative units in which the units from each of three experiments was normalized by dividing each time point by the peak value for anti-TCR alone. Units were then graphed as the mean of the $\log_{10}$ normalized values. Values at 1.5 h are duplicates from one experiment; values at 16 h are duplicates from two experiments; all other values are duplicates from three experiments.

Plasmids and primers. Mouse IL-2 promoter and enhancer fragments were subcloned from plasmid pJY1 (31) into the pGL2 luciferase vector (Promega, Madison, Wis.). The −353 to +38 fragment was prepared by PCR using primers that created a 5′ *Xho*I site and a 3′ *Hin*dIII site. These sites were used for overhang ligation into pGL2 to create p353IL2luc. The final plasmid was sequenced to verify that no mutations were introduced in the process of cloning. The 1.9-kb enhancer was created by ligating the *Eco*RI-to-*Acc*I fragment from pJY1 into the *Sma*I-to-*Acc*I fragment of p353IL2luc, using a blunt ligation on the 5′ end and an overhang ligation on the 3′ end. This plasmid was named p1.9kbIL2luc. Plasmid pIL-2luc containing the human IL-2 enhancer was the gift of Gerald Crabtree. To produce stable transfectants, reporter DNA was cut out by using *Xho*I and *Bam*HI for p353IL2luc and *Hpa*I for p1.9kbIL2luc. Digested DNA was gel purified by electroelution. Plasmid pSV2neo, digested with *Xmn*I, was used as a selection marker. For transient transfections, supercoiled reporter DNA alone was used.

Primers and probes for PCR and luminometry were designed by using Macvector software. Primer sites that span introns thus reducing amplification of genomic DNA were chosen. The following primers and probes were used for PCR: IL-2 5′ primer (GAACCTGAAACTCCCCAGGATGCT); IL-2 3′ primer (GGTACATAGTTATTGAGGGCTTGT); IL-2 probe (CATGCCGCAGAGG TCCAAGTTCA); glyceraldehyde phosphate dehydrogenase (GAPDH) 5′ primer (GGTGAAGGTCGGTGTGAACGGA); GAPDH 3′ primer (TGTTAG TGGGGTCTCGCTCCTG); GAPDH probe (CACTGCAAATGGCAGCCC); IL-2 intron 5′ primer (contained in intron B) (CCCCCTTTACAGAGGACA GGGAGT); IL-2 intron 3′ primer (contained in intron C) (TGCACAGAAC-CCATCAAAGACCAG) (intron primer PCR products were probed with the same probe as IL-2 mRNA); MHC K^k intron 5′ primer (CTGGTTGTCCTT GGAGCTGCAA); MHC K^k intron 3′ primer (CCCCATTGATGAGCAGA CGCA); and the MHC K^k probe (TGCCCTTTCCTACCTGTGT). Primers were synthesized by several vendors (including Synthecell, Operon, Geneosys, and Bioserve) with similar results.

Quantitative PCR. mRNA levels were quantitated by using a reverse transcriptase (RTase)-mediated PCR assay (1). RNA samples were prepared from 10^6 cells by using RNAzol (Tel-Test, Friendswood, Tex.). Samples were resuspended in 10 μl of diethyl pyrocarbonate-treated water and stored at −20°C. For unspliced RNA quantitation in Fig. 5B, RNA was treated with DNase (RQ1; Promega) and phenol extracted before use. Before reverse transcription, the samples were denatured by heating to 95°C for 10 min and quickly cooled on ice. DNA copies were created by using a random hexamer primer and avian myeloblastosis virus RTase in a reaction mixture containing 10 mM Tris-Cl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100, 7 mM $MgCl_2$, 0.25 mM each deoxynucleoside triphosphate (dNTP), 0.25 U of RNAsin (Promega) per μl, 2 mM dithiothreitol, 0.625 ng of random hexamer primer (Promega) per μl, and 0.2 U of avian myeloblastosis virus RTase (Promega) per ml. The total reaction volume was 20 μl, and each reaction mixture contained 2 μl of RNA sample. The reaction mixture was incubated at 23°C for 10 min and 42°C for 1 h and then heated to 95°C for 10 min. DNA copies were stored at −20°C. Reaction mixtures containing no RNA and RNA but no RTase were always included as controls for contamination and were negative by PCR analysis as described below.

PCRs were performed under the following conditions: 10 mM Tris-Cl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.05 mM each dNTP, 0.25 μM each primer, and 0.03 U of *Taq* polymerase (Promega) per μl. Each reaction mixture contained a total of 50 μl with 2 μl of cDNA. A hot start technique was used to reduce primer artifacts as follows. Primer and dNTP were separated from *Taq* and sample by Ampliwax (Perkin-Elmer Cetus, Norwalk, Conn.) until the sample was placed onto a heated PCR machine. Samples were amplified in 96-well plates, using a PCR machine from MJ Research (Watertown, Mass.). Aliquots of 5 μl were removed every three or four cycles for quantitation, starting at cycle 18.

Quantitation was performed by using a modified enzyme-linked immunosorbent assay as follows. One primer for each gene was labeled with a 5′ biotin moiety, allowing capture of the PCR products on an avidin-coated plate. The second strand was denatured by treatment with NaOH (0.1 M), and the captured strand was probed with an oligonucleotide labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, Ind.). Bound probe was detected via an anti-digoxigenin-11-dUTP antibody coupled to alkaline phosphatase and a substrate, disodium 3-(4-methoxyspiro[1,2 dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}] decan]-4-yl)phenyl phosphate (CSPD; Tropix, Bedford, Mass.), which emits light after dephosphorylation. Luminesence was measured by using a luminometer (model ML1000 or ML3000; Dynatech, Chantilly, Va.) on cycle mode with medium gain.

To compare data from different groups, experimental samples were matched to a standard curve generated by amplifying serially diluted product in the same PCR (Fig. 1A). For greater accuracy, comparisons were made by using the

following strategy. Logarithms of the light units (Y) corresponding to the raw data cycles (X) were fitted to a four-parameter logistic curve of the form $(Y) = C + (D - C)/(1 + \exp(-A - BX))$. A and B are curve parameters describing the intercept and slope of the line, $A + BX$ {which is the logit transform of $(Y - C)/(D - C)$, that is, $\log_e [(Y - C)/(D - Y)] = A + BX$}, and C and D are the lower and upper plateaus of the logistic curve. The half-maximum point of the curve occurs at $Y^* = (C + D)/2$, corresponding to a cycle value of $X^* = -A/B$. The four parameters were determined by nonlinear least-squares analysis using Systat 5.2 on a Macintosh computer. Most curves fit with a residual standard deviation of 0.02 or less. The cycle value at the half-maximum point (C_{50}) was determined as $-A/B$. Values of C_{50} were plotted against the corresponding input DNA concentrations to generate a line which was fit by the linear least-squares function in Microsoft Excel (Fig. 1B). Amounts of cDNA were then determined by using this equation and the experimental C_{50} values. To correct for variability in RNA recovery and efficiency of reverse transcription, GAPDH cDNA was amplified and quantitated from each cDNA preparation. In Fig. 5B, unspliced RNA levels were normalized for nuclear RNA recovery by using MHC K^k intron primers. Data for IL-2 mRNA amounts are expressed relative to GAPDH mRNA or K^k unspliced RNA equivalents and were internally normalized in each experiment in order to calculate geometric means. For kinetic experiments, values were normalized to the average peak of IL-2 mRNA in anti-TCR-stimulated cells for a given experiment. For mRNA stabilization experiments, values were normalized to the average of the values at the time of transcription inhibitor addition (time zero) for each experiment. Under the experimental stimuli and kinetics used, there was no consistent variation of GAPDH mRNA or unspliced K^k RNA amounts between groups. All error bars shown are standard errors of the means.

Transfection. To generate stable lines, T cells were stimulated with peptide antigen and APC for 48 h and then subjected to electroporation (3, 25). Electroporations were performed with a Bio-Rad (Richmond, Calif.) Gene Pulser at 270 V and 960 mF. Cells were resuspended at 5×10^7 cells in 0.2 ml in RPMI and 20% fetal calf serum (Biofluids) with 10 μg of reporter DNA and 2.5 μg of DNA encoding neomycin resistance (both linearized) in a 4-mm cuvette. Following electroporation, cells were incubated for 48 h in mouse recombinant IL-2 (10 U/ml; Biosource, Camarillo, Calif.) and then with the drug G418 (Gibco-BRL), also in IL-2. After 7 to 10 days of rest, the live cells were restimulated with antigen and APC and the selection process was repeated. Stable lines were established after 5 to 6 weeks. Cells for transient transfections were propagated in IL-2 (20 U/ml) and were split 1:2 or 1:4 every 2 or 3 days. Transient transfections were performed with a BTX (San Diego, Calif.) electroporator at 250 V, 2,000 μF, and 186 ohms. Cell density, electroporation cuvette, and medium were the same as for stable transfections, but 50 μg of supercoiled reporter DNA was used with 5×10^7 cells. Cells recovered from the cuvette were rested for 18 h without IL-2. Live cells were then collected on a Ficoll-Hypaque density gradient and stimulated as described above.

Luciferase assays. Cell extracts were prepared by three rounds of freeze-thaw in 250 mM Tris-Cl (pH 7.5) followed by centrifugation to remove cell debris. Supernatants were assayed for luciferase activity by using the Promega luciferin substrate system and a Dynatech luminometer (model ML1000 or ML3000) on integrate flash mode on high gain. Values presented are arbitrary light units minus light units of wells with buffer alone.

RESULTS

Mouse T-cell clones are strongly costimulated by CD28 signals. We have assessed the molecular effects of CD28 signalling on IL-2 gene regulation in the mouse T-cell clones A.E7 and 3R.3.11. Both of these clones are of the Th1 phenotype. These clones have previously been characterized with regard to their responsiveness to TCR signalling without CD28 stimulation; both clones make small amounts of IL-2, proliferate poorly, and are subsequently anergized. In the present experiments, TCR and CD28 signals were initiated by the use of two monoclonal antibodies, H57 (anti-TCR β) and 37.51 (anti-CD28). In general, cells were stimulated at low density in flasks coated with anti-TCR at 10 μg/ml. Where indicated, 37.51 was added in soluble form as a dilution of ascites fluid. In this work, potential effects of CD28 signalling on two regulation mechanisms were assessed quantitatively. Levels of nuclear (unspliced) and cytoplasmic (spliced) IL-2 RNAs were determined by quantitative PCR, and transcription initiation was measured in a transfection reporter assay.

As shown in Fig. 2A, clone A.E7 secretes measurable amounts of IL-2 in the presence of anti-TCR cross-linking alone. Under these conditions, IL-2 production is frequently detectable by 2 h and reaches a peak of 0.2 to 4 U/ml by 6 to 8 h and then declines slightly, possibly because of consumption. This amount of IL-2 is probably not due to residual CD28 costimulation because the T-cell population was depleted of residual APC by antibody and complement treatment (see Materials and Methods) and because the addition of CTLA-4–immunoglobulin does not block IL-2 production (8a). Adding anti-CD28 to the culture has a dramatic effect on IL-2 production. IL-2 levels routinely increased 30- to 100-fold above the

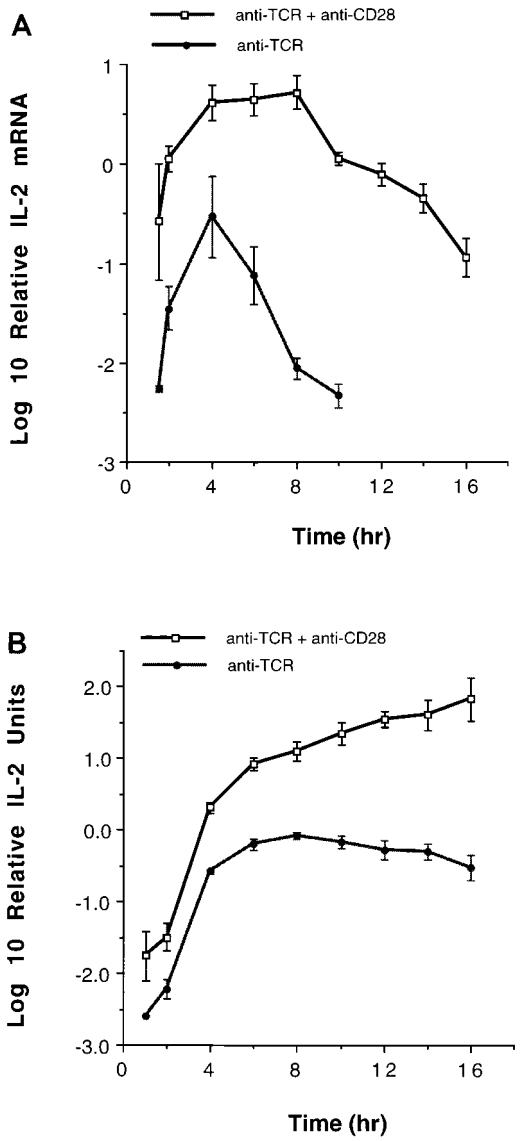

FIG. 2. Kinetics of IL-2 mRNA accumulation and protein secretion: effects of anti-CD28 costimulation on anti-TCR-stimulated cells. A.E7 cells (10^6) were cultured in anti-TCR-coated flasks in 10 ml of medium alone or with anti-CD28. Supernatants were collected for IL-2 protein assay (A), and cells were harvested for mRNA assay (B) at various times after the beginning of the culture. IL-2 protein is expressed as $\log_{10}$ of normalized units per milliliter. mRNA is expressed as $\log_{10}$ normalized femtograms of IL-2 cDNA. Points plotted are the means of three experiments with duplicate time points except those for 1 and 1.5 h, which are duplicate points from one experiment each. Normalization was performed as follows: for IL-2 protein, the concentration in units per milliliter from each point was divided by the peak units (average of duplicate values) of anti-TCR alone (1.43, 3.89, and 0.17 U/ml, respectively). For IL-2 mRNA, the amount in femtograms was divided by femtograms of GAPDH, and then all points were divided by the average peak of anti-TCR alone (8.75, 16.49, and 2.81 copies per cell, respectively).

level produced by cells stimulated through the TCR alone. The peak of IL-2 production was reached between 10 and 16 h. It should be noted, however, that the rate of increase of IL-2 was greatest over the first 6 to 8 h and that the amount of IL-2 in cultures with anti-CD28 was significantly greater even at the earliest time points measured. The effect of anti-CD28 on IL-2 production was also seen with clone 3R.3.11 and with other TCR signalling mimics such as concanavalin A (ConA) and phorbol myristate acetate (PMA) plus ionomycin (data not shown).

CD28 signals enhance IL-2 mRNA accumulation. In contrast to the steady accumulation and plateau of secreted IL-2 protein, IL-2 mRNA increases for the first 4 to 8 h and then decreases in both groups (Fig. 2B). There is already a 20-fold-higher amount of IL-2 mRNA in the groups treated with anti-CD28 for 1.5 h, the earliest time point at which IL-2 mRNA is detectable by our methods. This difference is maintained during the rapidly increasing phase, which lasts for 2.5 h. After that point, the cells stimulated through the TCR alone rapidly lose IL-2 message. A similar rapid decline in IL-2 mRNA levels is seen in the costimulated cells but with a delay of 4 h compared with cells receiving TCR signals alone. Between 4 and 8 h, the cells triggered through TCR and CD28 slightly increase their levels of IL-2 mRNA. These observations are consistent with the fact that the rate of production of IL-2 protein is highest during the early time points (2 to 8 h) when mRNA is accumulating or on plateau. Lacking a mathematical model for integrating mRNA amounts into protein amounts, we cannot rule out regulation of translational or posttranslational processes by CD28. The observed effects of anti-CD28 on mRNA accumulation could be due to regulation of processes such as transcriptional initiation, transcriptional elongation, splicing, mRNA transport, and mRNA stability. Effects of anti-CD28 antibodies on human cells have been described for both mRNA stabilization (27) and transcription initiation (14, 15).

Transcription reporter assays show little effect of CD28 signalling. The 20-fold enhancement of IL-2 mRNA by CD28 at the earliest detectable time point would seem most likely to be mediated by a nuclear event such as the initiation of transcription from the IL-2 enhancer. While a transcription run-on assay would seem to be the method of choice for analysis of this mechanism, it is a relatively insensitive assay in nontransformed cells and does not assay only transcription initiation (39). In particular, the mouse IL-2 gene contains a serine codon repeat near its 5′ end which makes probe design problematic (7). Therefore, to assess the possible effects of CD28 signals on transcription initiation of the IL-2 gene, transcription reporter assays were used. The luciferase gene was chosen as a reporter because of the sensitivity and ease of the enzyme assay. The mouse IL-2 enhancer and promoter sequences between −353 and +38 (p353IL2luc) were used for most experiments, while key results were duplicated with a 1.9-kb enhancer and promoter sequence (p1.9kbIL2luc). Both regions were cloned upstream of the luciferase gene in the vector pGL2 (see Materials and Methods). In addition, a human IL-2 enhancer construct (pIL-2luc [13]) was tested in some experiments. Both stable and transient transfections were performed by electroporation in both clone 3R3.11 and clone A.E7.

Stimulation of stable transfectants was performed as described above. Cells were cultured in anti-TCR-coated flasks with and without anti-CD28. Cells were harvested at indicated times, and extracts were prepared for luciferase enzyme assays (see Materials and Methods). The kinetics of luciferase induction by TCR stimulation (Fig. 3A) are similar to the kinetics of induction of secreted IL-2 protein (Fig. 3B). The luciferase

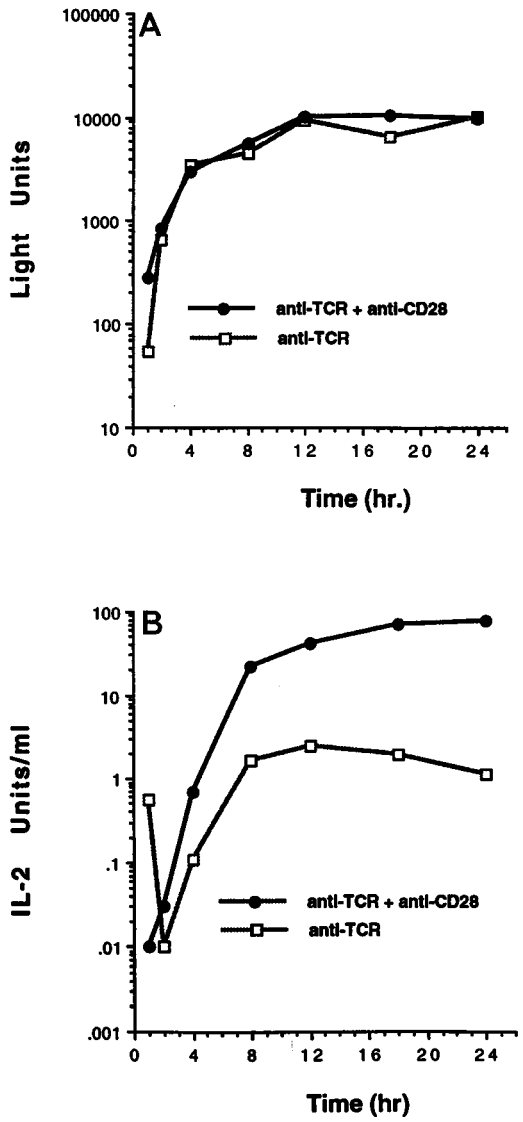

FIG. 3. Kinetics of luciferase and IL-2 production: effects of anti-CD28 costimulation on anti-TCR-stimulated cells. 3R.3.11 cells (10^6) stably transfected with p353IL-2luc were cultured in anti-TCR-coated flasks in 10 ml of medium alone or with anti-CD28. Cells were harvested for luciferase assay (A), and supernatant was collected for IL-2 protein assay (B) at various times after the beginning of the culture. Luciferase extracts were diluted to correct for differences in cell recovery. Luciferase activity is expressed as arbitrary light units minus background units of buffer alone.

activity in unstimulated cells is generally not significantly above background readings made without cell extract. In TCR-stimulated cells, the light units peak between 500- and 1,000-fold above the level found in unstimulated cells. Addition of anti-CD28 does not enhance luciferase activity in many experiments. In other experiments, the effect is at most between 1.5- and 2-fold above the level seen with anti-TCR alone. This is in marked contrast to the observed IL-2 production found in the supernatant from the same cells, in which anti-CD28 antibody costimulates IL-2 production approximately 30-fold (Fig. 3B). It is also noteworthy that luciferase activity remains at plateau in these cells until at least 24 h. This is true despite the fact that the luciferase enzyme is found in the cytoplasm and decays with a half-life of approximately 3 h (data not shown), suggest-

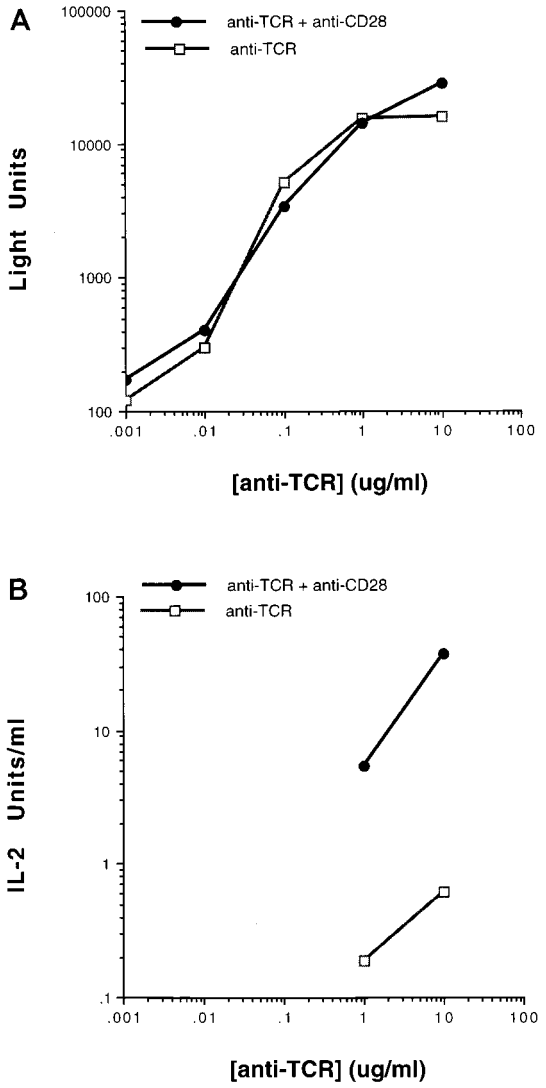

FIG. 4. Luciferase induction in cells exposed to various anti-TCR concentrations with and without anti-CD28. Culture flasks were coated with various concentrations of anti-TCR. 3R3.11 cells (10^6) stably transfected with p353IL-2luc were cultured in these flasks in 10 ml of medium alone or with anti-CD28. Cells were harvested for luciferase assay (A), and supernatant was collected for IL-2 protein assay (B) at 8 h after the beginning of the culture.

ing that the IL-2 enhancer continues to initiate transcription well past the point of maximal IL-2 secretion.

To test whether the small enhancement of luciferase activity by anti-CD28 was specific for the IL-2 enhancer, stable transfectants were made in which luciferase transcription was controlled by the β-actin enhancer. These transfectants constitutively expressed luciferase at levels comparable to that of TCR-induced IL-2 luciferase transfectants. At late time points after TCR stimulation (>8 h), the luciferase levels in a β-actin enhancer reporter increase approximately 10-fold. At all time points, however, the effect of anti-CD28 on the cells transfected with the β-actin enhancer construct was the same as that for the IL-2 enhancer, 1.5- to 2-fold (data not shown).

Since anti-TCR stimulation alone induces luciferase expression to high levels, we were concerned that the reporter assay might saturate at the relatively high dose of plate-bound anti-TCR. Therefore, we carried out a titration of the TCR signal in which the flasks were coated with serial threefold dilutions of the anti-TCR stock while a constant amount of soluble anti-CD28 was added to half of the samples. The results (Fig. 4A) indicate that regardless of the concentration of anti-TCR used, CD28 signalling did not significantly increase luciferase expression. This is true despite the apparent logarithmic relationship between luciferase expression and anti-TCR concentration and the roughly 60-fold effect of anti-CD28 on IL-2 production (Fig. 4B). Notably, the luciferase assay is more sensitive than the CTLL bioassay for IL-2 production in this experiment since luciferase activity is detectable in groups that produced no detectable IL-2.

In transient transfection experiments, levels of luciferase expression in unstimulated cells were generally higher, probably because of the larger number of DNA copies that are present shortly after transfection. Luciferase expression was still induced 10- to 100-fold by overnight culture in H57-coated flasks, while effects of antibody 37.51 were again less than 2-fold. Despite continuous growth in IL-2 prior to electroporation, the IL-2 production from the cells was still augmented more than 50-fold by anti-CD28 (data not shown). Nearly identical results were obtained with either p353IL2luc or p1.9kbIL2luc (data not shown).

Although we believe that H57 cross-linking is a suitable mimic of TCR signalling by MHC and peptide, other commonly used mimics include ConA and PMA plus ionomycin. Using both ConA and PMA plus ionomycin, we have found that the IL-2 enhancer drives the expression of luciferase at comparable levels in cells treated with the TCR mimic alone or with anti-CD28 in addition (38), thus confirming our results with anti-TCR. We have also tested our transfectants with class II MHC-bearing cells (DCEK fibroblasts) and peptide antigen and have found induction of luciferase activity similar or identical to induction upon anti-TCR stimulation. Thus, we conclude that anti-CD28 costimulation has no effect on the initiation of transcription via the IL-2 enhancer and promoter used to construct these reporter elements.

Steady-state levels of unspliced IL-2 RNA are affected by CD28 and are kinetically regulated. Nuclear events other than transcription initiation might be responsible for the early effect of CD28 on IL-2 mRNA accumulation. To investigate this point, we amplified and quantitated unspliced IL-2 RNA from our samples. In general, IL-2 nuclear RNA was detected at levels roughly 30- to 50-fold below the level of spliced RNA from the same sample. This is probably due to rapid processing and transport of the unspliced RNA, as indicated by a rapid loss of nuclear IL-2 RNA after inhibition of transcription as discussed below. Using samples from Fig. 2, we could amplify unspliced RNA from almost all samples treated with anti-TCR and anti-CD28, but groups treated with anti-TCR alone were generally below the limit of sensitivity of our assay with this particular primer pair (Fig. 5A). When the stimulation was repeated with 2×10^7 cells per datum point, unspliced IL-2 RNA was quantitatable under conditions of anti-TCR stimulation as well as anti-TCR plus anti-CD28 (Fig. 5B). In this experiment, after 4 h of stimulation, the effect of anti-CD28 was eightfold. This result confirms the hypothesis that a least one component of the regulation of IL-2 gene expression by CD28 occurs in the nucleus. In addition, as seen in Fig. 5A, there is a kinetic regulation of the steady-state level of unspliced IL-2 RNA in cells stimulated with both anti-TCR and anti-CD28. Unspliced RNA was first detected at 90 min, before it was detectable in spliced form in the cytoplasm. Accumulation of unspliced IL-2 RNA reached a plateau between 4 and 6 h. After 6 h, the RNA disappeared rapidly.

CD28 signals enhance the stability of IL-2 mRNA. To measure mRNA decay, the production of new transcripts must be

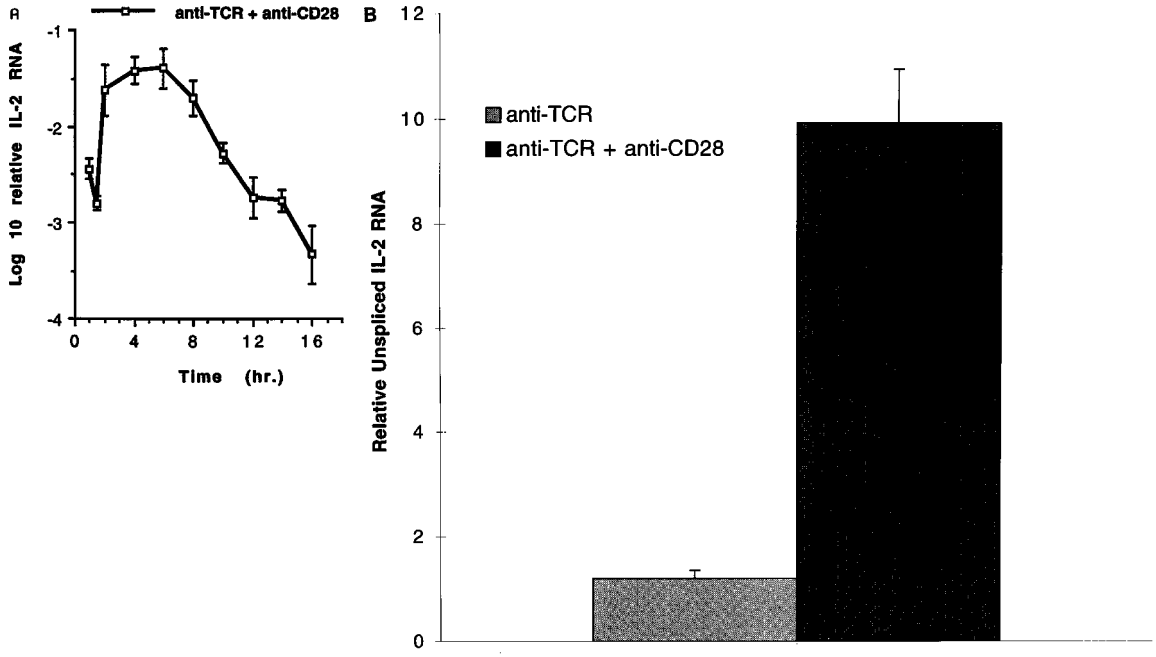

FIG. 5. Unspliced IL-2 RNA levels: anti-TCR with and without anti-CD28. (A) Samples from Fig. 2B were amplified with primers contained within IL-2 introns B and C (see Materials and Methods). Values are $\log_{10}$ normalized femtograms as in Fig. 2. Unspliced RNA values were divided by GAPDH mRNA values and then the average peak of spliced IL-2 RNA for each time point. The means of the 1- and 1.5-h points are from duplicate values from single experiments. All other means are of duplicate samples from three experiments. (B) A.E7 cells (2×10^7) were cultured in 100 ml of medium in anti-TCR-coated flasks for 4 h with and without anti-CD28. Cells were harvested, and RNA was prepared and quantitated as described in Materials and Methods. IL-2 unspliced RNA was amplified and quantitated by using intron primers and an exon probe. Values were normalized for unspliced RNA recovery by using MHC K^k primers and probe and further normalized to the lowest value for anti-TCR alone for each of two experiments. The anti-TCR values are the averages of two points each from two experiments. The anti-TCR and anti-CD28 values are the averages of a total of three points from two experiments.

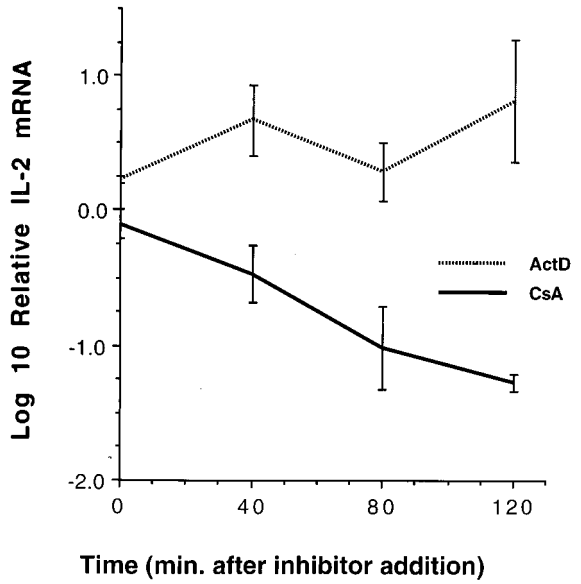

FIG. 6. Effect of CsA and ActD on IL-2 mRNA stability. A.E7 cells (10^6) were precultured in 10 ml of medium in anti-TCR-coated flasks for 4 h. CsA and ActD were added at final concentrations of 200 ng/ml and 10 μg/ml, respectively. Cells were harvested for IL-2 mRNA assay at time points after inhibitor addition (time zero). Values are from two experiments, with duplicate time points expressed as $\log_{10}$ normalized femtograms. IL-2 cDNA values were first normalized by GAPDH and then divided by the higher of duplicate values at time zero. Points are the geometric means of duplicate points from two experiments.

stopped. This is usually performed by using a general transcription inhibitor such as actinomycin D (ActD). Effects of the addition of ActD on mRNA decay, however, have been described both for proto-oncogenes such as c-*fos* and for cytokine genes such as the IL-2 gene (34, 36). Therefore, we were concerned about the use of ActD and compared its effects to that of cyclosporin A (CsA), which specifically blocks the transcription of genes induced by TCR signals (8, 12). In this experiment, transcription was initiated by preculture of the cells for 4 h in anti-TCR-coated flasks. The transcriptional inhibitor was then added, and samples were taken at the indicated times (Fig. 6). From these results, it is clear that ActD stabilizes IL-2 mRNA such that the normal decay observed after 4 h does not occur (compare with Fig. 2B). To demonstrate that ActD was in fact blocking transcription, cells were treated with ActD and the level of β-actin mRNA was quantitated by Northern (RNA) blotting. The level of β-actin mRNA drops within 2 h of ActD addition and has declined threefold in 3 h (data not shown). In contrast, CsA addition does not prevent the decrease in IL-2 mRNA seen in the unperturbed system; indeed, the rate of decay is faster than normal, suggesting that additional input is blocked. The resulting decay rate will be discussed below. To further demonstrate that CsA is effective at blocking ongoing IL-2 transcription, cells were stimulated with anti-TCR and anti-CD28 for 3 h and then CsA was added. Amplification of unspliced RNA indicated that nuclear RNA levels dropped 10-fold within 15 min after CsA addition and below the limit of sensitivity (>20-fold) within 30 min (data not shown). Thus, for subsequent experiments, CsA was used to block input of new mRNA.

Using the stimulus conditions described above, we performed several experiments with duplicate time points to

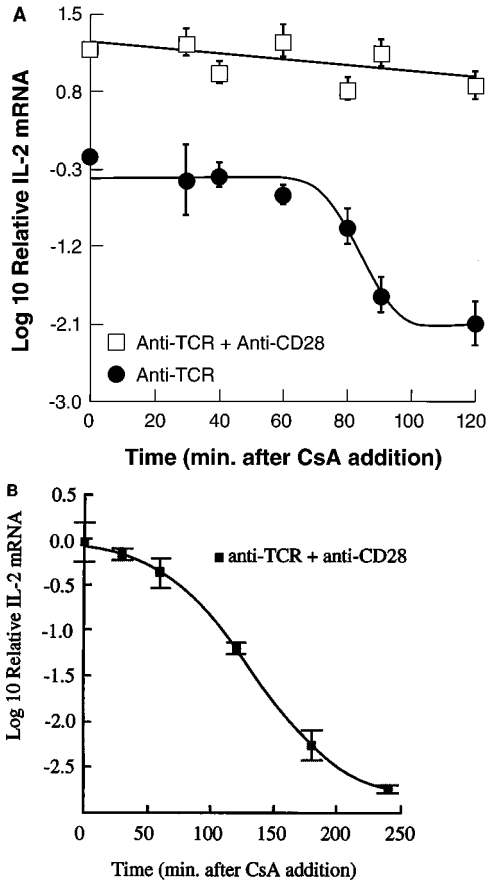

FIG. 7. IL-2 mRNA decay: effect of anti-CD28 costimulation on anti-TCR-stimulated cells. A.E7 cells (10^6) were precultured in anti-TCR-coated flasks in 10 ml of medium alone or with anti-CD28 for the indicated times. CsA was added at a final concentration of 200 ng/ml. Cells were harvested for mRNA assay at time points after inhibitor addition (time zero). (A) Preculture for 4 h before CsA addition; (B) preculture for 8 h before CsA addition. Values are expressed as $\log_{10}$ normalized femtograms, dividing IL-2 mRNA levels first by GAPDH and then by the average value at the time (zero) of inhibitor addition. Values plotted in panel A are the geometric means of duplicate points from two (40- and 80-min points), five (30-, 60-, and 90-min points), and seven (0- and 120-min points) experiments. Values plotted in panel B are triplicate points from one experiment.

quantitate IL-2 mRNA decay in the presence or absence of anti-CD28. As seen in Fig. 7A, there is a significant difference in the rates of decay between the groups with and without the anti-CD28 antibody when CsA is added at 4 h. After addition of CsA, the IL-2 mRNA decays more rapidly in the absence of the CD28 signalling than in its presence. In addition, the mathematical functions which describe the decay curves are different: the cells costimulated with anti-CD28 lose IL-2 mRNA in a single-order exponential decay ($t_{1/2}$ = 76 min), while the cells treated with anti-TCR alone are subject to a more complex decay pattern. This decay function is better fit by a logistic exponential function, as plotted in Fig. 7A, than a simple exponential or pair of exponential curves. Using a four-parameter nonlinear curve fit (as described for our PCR quantitation; see Materials and Methods), we found that the slope of the tangent line at the midpoint of decay is 35-fold higher than the slope of the decay line in the presence of anti-CD28 signalling. Similar decay curves were observed for cells stimulated only 2 h before CsA treatment. This difference in stability is not due simply to the large difference in the amount of starting mRNA, since addition of anti-CD28 to cells precultured in anti-TCR-coated flasks only an hour before addition of CsA caused only a minimal increase in IL-2 mRNA levels, while stabilization was still observed (data not shown). This stabilization effect does not last: if CsA is added to cells treated with anti-TCR and anti-CD28 for 8 h, the decay is more rapid than the decay in the cells treated with anti-TCR and anti-CD28 at 4 h, as shown in Fig. 7B. This result is consistent with the unperturbed kinetics in which IL-2 mRNA disappears after 8 h even in the presence of anti-CD28. As in the anti-TCR-stimulated cells at 4 h, this function is logistic. The rate of decay in cells treated with both antibodies for 8 h is less than that of cells treated with anti-TCR alone for 4 h, however, by approximately fivefold ($P < 0.02$).

DISCUSSION

In this study, we have attempted to characterize the mechanisms of CD28 costimulation of IL-2 production in mouse T-cell clones. These clones are highly responsive to CD28 costimulation, exhibiting a 30- to 100-fold increase in IL-2 secretion above that of cells stimulated through the TCR alone (Fig. 2A). The kinetic regulation of IL-2 mRNA accumulation is quite complex (Fig. 1B). (i) At 1.5 h, mRNA becomes detectable in both groups and there is already a 20-fold difference between cells stimulated with anti-TCR alone and those stimulated with anti-TCR and anti-CD28. (ii) IL-2 mRNA increases until 4 h in both groups; it then begins to disappear from cells stimulated via TCR alone and plateaus or increases slightly in CD28-costimulated cells. (iii) At 8 h, the mRNA begins to disappear from CD28-treated cells as well. The kinetic of IL-2 mRNA accumulation by itself is consistent with regulation at any step from transcriptional initiation up to and including mRNA decay, since accumulation is a function of both production and loss. Because of the limited availability of assays with sufficient sensitivity, however, to date we have been able to address only the mechanisms of transcription initiation and mRNA decay.

It is notable from the results presented above that CD28 signalling has an effect on IL-2 mRNA accumulation from very early time points. The abrupt appearance of the initial difference suggests that a mechanism of nuclear regulation must be involved. We have not, however, seen a specific effect of CD28 signalling on transcription in transfection reporter assays. This is true for both the 353-bp and 1.9-kb mouse enhancer and the 370-bp human enhancer, over a broad range of kinetics (Fig. 2) and TCR signal strength (Fig. 3), and with several TCR signal mimics (38) and in transient as well as stable transfectants. This result is in apparent contrast with reports from human cells (13, 14) in which an element in the IL-2 enhancer (CD28 response element [CD28RE]) is reported to specifically respond to CD28-mediated pathways (13, 14). The CD28 specificity of the effect reported by Fraser et al. (13, 14) is in some dispute, however, as Civil et al. (9) have reported efficient transactivation of the CD28RE via anti-CD3 and PMA treatment alone. We have found that a protein complex that binds to the mouse version of the CD28RE can be induced by strong TCR signals alone (38). Several explanations for the discrepancy are possible, among them that human and mouse T cells use different mechanisms to mediate CD28 effects, or that T-cell clones, such as those used in this study, differ from transformed cells or PHA-activated peripheral blood lymphocytes. These results are based on transcription reporter assays and electrophoretic mobility shift assays and are subject to the caveat of selectivity: sequences outside of those chosen for reporter constructs or binding probes may be involved in tran-

scriptional regulation by CD28 but would not be detected. Experiments are currently under way to examine sequences of the IL-2 coding region, introns, and 3′ untranslated region for CD28-specific enhancer activity.

The early effect of CD28 on IL-2 mRNA accumulation is clearly a nuclear event, since there is an eightfold difference in unspliced IL-2 RNA (Fig. 5B). In addition, a striking level of kinetic regulation of unspliced IL-2 RNA is revealed by these experiments (Fig. 5A). The causes of the CD28 and kinetic effects are unknown: mechanisms such as transcriptional elongation, nuclear RNA stability, and regulated rates of splicing and/or transport need to be investigated. Interestingly, the kinetics of steady-state levels of unspliced IL-2 RNA do not match those of the IL-2–luciferase reporter. Unspliced IL-2 RNA begins to disappear by 6 h, whereas luciferase activity, also at a steady state of synthesis and decay, remains on plateau from 6 to 24 h. On the basis of the kinetics of the transcription reporter (Fig. 3A), and consistent with the results of Shaw et al. (35), we believe that IL-2 enhancer-driven transcription remains active past 10 h after initiation of TCR signalling. Therefore, we suspect that an additional mechanism reduces the rate of input of unspliced IL-2 RNA after 6 h. This mechanism may either be identical to or distinct from that which limits IL-2 transcripts without CD28.

During the late period of mRNA accumulation, we have found a difference in the rates and functions of mRNA decay between cells stimulated through the TCR and those stimulated through the TCR and CD28. This finding agrees with those of a study using human cells and the general transcriptional inhibitor ActD (27). We have found in our system, though, that ActD on its own causes a stabilization of IL-2 mRNA, which otherwise disappears under unperturbed conditions (compare Fig. 6 and 2B after 4 h). Using CsA as a cytokine-specific transcriptional inhibitor, we were able to block ongoing IL-2 transcription without stabilizing mRNA (Fig. 6). Furthermore, because of the rapid kinetics of IL-2 mRNA decay in the TCR-stimulated cells after 4 h in the absence of CsA, we do not believe that CsA accelerates message degradation (28). It should be taken into account that decay curves contain within them the addition of new mRNA via splicing and transport of nuclear RNA present at the time of the transcriptional block. The amount of such unprocessed material, however, is small (<30-fold of spliced RNA).

Results obtained with use of this inhibitor after either 2 or 4 h of stimulation revealed that in the presence of TCR signal alone, IL-2 mRNA decayed with complex kinetics which could be fit by a logistic exponential curve. This finding is consistent with an autocatalytic function, suggesting that an RNase activity might be further activated by the liberation of a *cis* element (perhaps in the form of a stem-loop) from degraded IL-2 message. At the same time points, however, IL-2 mRNA decay in cells treated with both TCR and CD28 antibodies is relatively slow and single order. Interestingly, when the effect of CD28 on message stabilization wanes at 8 h, the observed decay rate is also logistic. This finding suggests that the same mechanism is involved in decay as in the cells treated with anti-TCR alone. The rate of decay at 8 h with anti-CD28 is not as rapid though, perhaps because the concentration of a limiting degradation component is lower under these conditions.

In summary, there are at least two kinetic components of CD28 enhancement of TCR-induced IL-2 mRNA accumulation. There is a nuclear mechanism probably distinct from transcription initiation from a 1.9-kb IL-2 enhancer which requires further characterization. There is also the stabilization of IL-2 mRNA. Perhaps most striking, however, is the kinetic regulation of these mechanisms: the nuclear effect is present only from the beginning of detectable RNA synthesis until 4 to 6 h, while mRNA stabilization begins earlier than 2 h and lasts until 8 h. It is possible that a single *trans*-acting factor could produce these two effects, such as an RNA shuttle protein which could protect IL-2 RNA from sequence-specific degradation in both the nucleus and the cytoplasm (29). Alternatively, distinct mechanisms could operate in the nucleus (such as transcription elongation) and the cytoplasm (such as competition for RNA binding between an RNase and a stabilizing protein). The end result of these complex kinetics is that in the presence of both TCR and CD28 signals, IL-2 mRNA increases rapidly for 4 h, plateaus for 4 h as the rate of input drops off and the existing mRNA is stabilized, and then declines as the decay rate increases again. These kinetics indicate that both the nuclear and cytoplasmic effects are subject to complex regulation.

As we have suggested in a recent review (38), different mechanisms of regulating IL-2 secretion may have biological significance for immune function. In our simplified experimental system, we find that a TCR signal alone is enough to initiate maximum levels of transcription of a reporter gene via the IL-2 enhancer. Optimal secretion of IL-2, however, requires a costimulatory signal such as the one delivered by CD28. If, as we report here, the CD28 signal transduction pathway acts downstream of transcription initiation, a rheostatic control can be exerted to continuously modulate the final readout of the on/off switch of transcription initiation. In addition, if two separate mechanisms are involved in CD28 costimulation, the possibility exists to alter the coordinate regulation via other signal pathways. A third signal might blunt mRNA stabilization, for example without affecting the nuclear component, thus shifting both the kinetics and peak of IL-2 secretion. Since many cells in the immune system use IL-2 as a primary growth factor, the regulation of IL-2 secretion can have an exponential effect on the level of effector response. Thus, similar strengths of signal through the TCR can have different immunological consequences depending on the level of costimulatory ligands on APC. These ligands in turn can be modified by inflammatory hormones which are released by tissue damage due to infection.

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