T Cell Receptor (TCR) Engagement Leads to Activation-induced Splicing of Tumor Necrosis Factor (TNF) Nuclear Pre-mRNA

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Summary

Inducible gene expression is primarily regulated at the level of transcription. Additional steps of "processing" pre-mRNA, involved in the regulation of induced gene expression, have not been previously reported. Here we report a novel mechanism of "activation-induced splicing" of preexisting tumor necrosis factor (TNF) message (pre-mRNA) in naive T lymphocytes after engagement of the T cell receptor (TCR), which still occurs after inhibition of transcription. Expression of TNF has been previously demonstrated to be regulated at both the transcriptional and translational levels. However, neither the large pool of TNF mRNA observed in activated T cells nor TNF protein production, which peaks very shortly after activation, can be solely attributed to increased transcription. Evidence is presented that activation-induced splicing of TNF pre-mRNA plays a significant role in the rapid production of TNF seen in activated T cells. Activation triggers processing of TNF pre-mRNA that has accumulated in naive T cells (before activation-induced transcription), and the mature TNF mRNA is translocated to the cytoplasm for rapid translation and protein production. This novel form of activation-induced splicing of TNF may allow T cells to mount an immediate response to activation stimuli under physiological conditions.

Key words: T cells • TNF • transcription • RNA splicing • pre-mRNA

TNF is one of the most potent cytokines and plays a critical role in host defense, immune regulation, and inflammatory responses. For more than two decades, extensive studies have revealed an intriguing regulatory mechanism for TNF expression. Like many other cytokines, TNF production can be detected after activation of various cell types (including macrophages and T and B lymphocytes) by mitogens such as LPS or PMA/ionomycin (1–3). However, a striking feature that distinguishes TNF from other cytokines is that TNF production can very rapidly reach a high level in the bloodstream in response to activation stimuli. Endotoxins such as LPS induce a massive production of TNF by macrophages in a very short time which may lead to lethal shock. Anti-CD3 antibody administration causes severe clinical symptoms in both humans and animals due to the rapid and massive release of TNF by activated T lymphocytes (4–6). However, the mechanism(s) that allows this rapid, massive production of TNF is not fully understood.

The expression of TNF is regulated at multiple levels. Activation increases transcription of the TNF gene. Activation also triggers posttranscriptional regulatory mechanisms. T cell TNF mRNA is stabilized by "costimulatory" signaling through CD28 molecules during T cell activation (7). The translation of TNF mRNA in macrophages is controlled by an endotoxin-responsive element (8). However, the three- to fivefold peak increase in transcription of the TNF gene detected in both activated macrophages and T cells, in addition to mRNA stabilization detected in T cells, cannot explain the 100-fold increase in TNF mRNA or the massive protein production seen in these activated cells (9, 10). Another previous observation was that resting T lymphocytes express TNF mRNA at low levels (11, 12), although TNF protein is not detectably produced (13, 14). These studies suggested the possibility of a posttranscriptional regulatory mechanism distinct from mRNA stability.

Splicing of pre-mRNA has been described as a potential regulatory mechanism for the expression of IL-1β and IL-2 (15, 16). Both IL-1β and IL-2 mRNA can be superinduced by cycloheximide, a protein synthesis inhibitor that facilitates the processing of pre-mRNA to mature spliced mRNA. Cycloheximide similarly superinduces TNF mRNA (17). Since the TNF gene consists of three introns and four exons (18), the transcript must be processed for protein production (see Fig. 1 *A*). In studies reported below, we describe a novel form of posttranscrip-

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tional regulation involved in the rapid TNF protein production seen after activation of T cells.

Materials and Methods

$CD4^+$ T Cell Purification, Activation, and Fractionation. $CD4^+$ T cells were purified from spleens and lymph nodes of 12-wk-old female BALB/cJ mice using MiniMacs magnetic separation (Miltenyi Biotec Inc., Sunnyvale, CA). $CD4^+$ T cells were cultured at 3×10^6 cells/ml for the indicated times with or without antibodies as indicated. For activation, the T cells were first incubated with purified anti-CD3 antibody (145.2C11) and anti-CD28 antibody (whenever indicated; PharMingen, San Diego, CA) for 30 min on ice. The cells were then washed, incubated with anti–hamster IgG antibody (PharMingen) for 30 min on ice, and activated at 37°C for the times indicated. Antibodies were used at a concentration of 10 mg/ml. PMA (10 ng/ml) and ionomycin (1 mM) were added at the time of activation.

For the experiments of [^{3}H]uridine incorporation, $CD4^+$ T cells were incubated with 2 mCi [^{3}H]uridine (Du Pont-NEN, Boston, MA) in triplicate wells in a 96-well plate. In the indicated wells, actinomycin D (Act.D;[1] Sigma Chemical Co.), 2 mg/ml, and/or PMA/ionomycin were added. For transcription inhibition, the T cells were first incubated with 10 mg/ml Act.D for 1 h in a 37°C incubator. After cooling down on ice, the cells were incubated with anti-CD3 antibody followed by anti–hamster IgG antibody cross-linking at 4°C. Activation time began when the cells were incubated at 37°C after cooling.

For fractionation of nuclei and cytoplasm, T cells were suspended in a solution containing 50 mM KCl, 80 mM NaCl, 2 mM $MgCl_2$, 4% glycerol, and 10 mM Hepes, pH 7.4. 0.05% NP-40 was added on ice for 3 min. Nuclei were spun down gently and nuclear RNA was prepared using the RNeasy kit (QIAGEN, Inc., Chatsworth, CA). The supernatant was cleared by full-speed centrifugation on a microfuge and mixed with an equal volume of 7 M urea, 10 mM EDTA, 0.35 M NaCl, and 20 mM Tris-HCl, pH 8.0. The cytoplasmic RNA was prepared by phenol extraction from the mix.

Reverse Transcription–PCR and Northern Blot Analysis. Equal numbers of $CD4^+$ T cells were harvested and total RNA was prepared using the RNeasy kit (QIAGEN, Inc.). The RNA concentration was determined for each sample and 2.5 mg total RNA was used in the reverse transcription (RT) reactions. Equal amounts of cDNA were then used in PCR reactions. PCR primers used for TNF mRNA detection were (*a*) 5′ end: ATGAGCACAGAAAGCATGATCCGCGAC; and (*b*) 3′ end: TCACAGAGCAATGACTCCAAAGTAGACCTG. The PCR reactions were performed under the following conditions: 94°C, 1.5 min; 62°C, 2 min; 72°C, 2 min for 32 cycles and PCR products were analyzed on a 2% agarose gel. To further confirm the specificity of the PCR products, the bands on the gels were transferred to nylon membranes and hybridized with a ^{32}P-labeled probe, GTGCTCCTCACCCACACCGTC. RT-PCR for expression of the *HPRT* gene (hypoxanthine phosphoribosyltransferase) was performed to demonstrate the relative quantity of mRNA in each sample. PCR primers for *HPRT* mRNA were (*a*) 5′ end: GTAATGATCAGTCAACGG; (*b*) 3′ end: CCAGCAAGCTTGCAACC. The sequence for the *HPRT* hybridization probe was CAAGCTTGCAACCTTAAC. PCR primers for *bcl*-2 gene were (5′ primer) TTGAAGTGCCATTGGTAC and (3′ primer) GCTGGGGCATATAGTTCCACA-AAGGCATC. For Northern blot analysis, 20 mg of total RNA for each sample was used. A PCR fragment containing a 252-bp exon 4 sequence of the TNF gene was purified, nick translated, and used as the probe.

Results

TCR Engagement Induced Both Transcription and Splicing of TNF Pre-mRNA. To determine pre- and mature TNF mRNA in naive and activated T cells, RT-PCR analysis was used with primers designed in the first and the fourth exons (from the ATG translation initiation codon at the 5′ end and the TGA stop codon at the 3′ end of the TNF gene; see Fig. 1 *A*). The expected PCR products include both unprocessed transcripts and spliced mRNA. $CD4^+$ T cells were isolated from BALB/cJ mice and activated by cross-linking with anti-CD3 and anti-CD28. RT-PCR was then performed with cDNA prepared from both naive and activated $CD4^+$ T cells. Two major TNF PCR products were detected. In the absence of TCR engagement, the dominant PCR product was a 1.7-kb cDNA fragment. Within 15 min after activation, high-level expression of a 0.7-kb fragment, representing mature TNF mRNA, was detected (Fig. 1 *B*). Expression of the 0.7-kb fragment peaked 3 h after activation and decreased gradually thereafter. Interestingly, the 1.7-kb fragment was detected with decreased intensity immediately after activation; however, the intensity of the 1.7-kb fragment was slightly increased 24 h after TCR engagement (the intensity of the 0.7-kb fragment had greatly diminished by 24 h). In contrast, PCR assays demonstrated a consistent expression of HPRT mRNA during the 24 h after T cell activation. Since the PCR protocol allowed a proportional amplification of both the 1.7- and 0.7-kb fragments (Fig. 1 *C*), the reciprocal changes of intensity between these two bands reflected changes in the two RNA transcripts after activation.

Both the 1.7- and 0.7-kb cDNA fragments were cloned and sequenced, and the data obtained demonstrated that the 1.7-kb fragment contained all four exons and three introns of the TNF gene (pre-mRNA, 1688 bp; reference 18), whereas the 0.7-kb fragment contained only the exon sequences of the TNF gene (708 bp). The 1.7-kb fragment was not detected by PCR when RNA of naive T cells was processed in the absence of reverse transcriptase; thus, the 1.7-kb fragment represented TNF pre-mRNA accumulated in naive T cells. The decrease in the 1.7-kb fragment and the marked increase in the 0.7-kb fragment after TCR engagement indicated that there was a preexisting accumulation of TNF pre-mRNA in naive $CD4^+$ T cells, and that activation of the T cells induced splicing of this TNF pre-mRNA, in addition to transcription of the TNF gene. This activation-induced splicing of TNF pre-mRNA disappeared within 24 h of activation.

Since anti-CD28 treatment has been shown to stabilize mature TNF mRNA (7), the increased level of mature TNF mRNA in activated T cells may be partially due to the accumulation of "stabilized" mRNA. To determine

[1] *Abbreviations used in this paper:* Act.D, actinomycin D; HPRT, hypoxanthine phosphoribosyltransferase; RT, reverse transcription.

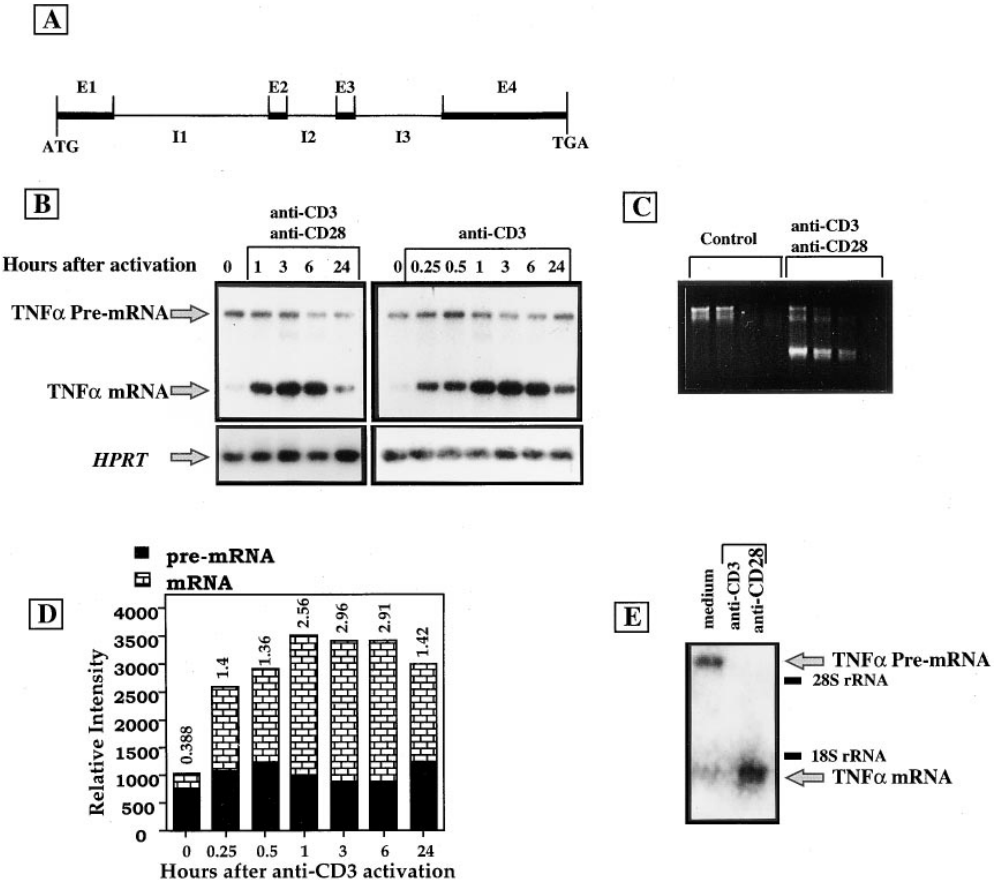

Figure 1. (*A*) Schematic representation of the structure of TNF pre-mRNA which includes four exons (*thick lines*: exon 1, 186 bp; exon 2, 55 bp; exon 3, 48 bp; exon 4, 419 bp; total 708 bp of coding region) and three introns (*thin lines*: intron 1, 509 bp; intron 2, 177 bp; intron 3, 294 bp; total 980 bp). (*B*) Detection of TNF mRNA by RT-PCR in $CD4^+$ T cells activated by anti-CD3/anti-CD28 or by anti-CD3 alone. In naive T cells, a significant amount of TNF pre-mRNA was detected with a trace amount of mature mRNA. Anti-CD3 antibody cross-linking induced a marked increase in mature mRNA and decreased pre-mRNA. (*C*) Twofold serial dilutions of RNA were used in the PCR reactions and resulted in a proportional decrease of PCR product of both pre- and mature TNF mRNA, thus the density of PCR products reflected the amount of pre- or mature TNF mRNA without preferential amplification in each reaction. (*D*) Densitometry analysis showed that mature mRNA increased ~10-fold after anti-CD3 activation whereas the total amount of TNF mRNA increased only 3.5-fold. The numbers on the top of the columns are the ratio of mature/pre-mRNA. (*E*) Northern blot analysis of total RNA of purified $CD4^+$ T cells incubated for 4 h with medium alone or anti-CD3 and anti-CD28 antibodies. Without antibody cross-linking, a strong band of TNF pre-mRNA and a weak band of mature TNF mRNA were detected. The antibody cross-linking led to a large amount of mature TNF mRNA. It was not possible to detect decreased TNF pre-mRNA by Northern blot analysis after activation.

the role of the activation signal in posttranscriptional processing in the absence of mRNA stabilization, $CD4^+$ T cells were activated by cross-linking with anti-CD3 antibody alone. Mature spliced TNF mRNA was markedly increased within 15 min after anti-CD3 cross-linking (Fig. 1 *B*). Within 6 h, the amount of mature TNF mRNA had increased 10 times (Fig. 1 *D*). In contrast, total TNF mRNA (mature + pre-mRNA) increased only threefold. The change in total mRNA reflected the transcriptional activity and a threefold increase is consistent with the results of nuclear run-on studies (9). The ratio of mature to pre-mRNA changed from >0.4 to ~3 (Fig. 1 *D*). Thus, the increased amount of mature TNF mRNA was not due solely to stabilization and accumulation, but also to efficient processing (in addition to increased transcription) after activation.

Northern blot analyses were performed to further confirm the accumulation of TNF pre-mRNA in naive $CD4^+$ T cells and the splicing of TNF pre-mRNA induced by activation. The probe used in the Northern analyses contained the exon 4 sequence which allowed detection of both TNF pre-mRNA and mature mRNA. Consistent with the PCR analysis, Northern analysis detected two distinct TNF bands from the total RNA of $CD4^+$ T cells (Fig. 1 *E*). In naive T cells, the major band detected by Northern blot analysis was of TNF pre-mRNA, which migrated slightly slower than 28S rRNA. A small amount of mature TNF mRNA, which ran slightly faster than 18S rRNA, was also detected. However, 4 h after TCR engagement a dramatic increase in mature TNF mRNA was seen; however, TNF pre-mRNA was barely detected. Once again, the increase of mature TNF mRNA after activation was concomitant with the decrease of TNF pre-mRNA, indicating that the increase of mature TNF mRNA was the result of the combination of increased transcription and splicing of TNF pre-mRNA.

An Activation Signal Generated by TCR Engagement Is Required for Efficient Splicing of TNF Pre-mRNA. Various antibodies and mitogens were tested for induction of splicing of TNF pre-mRNA in $CD4^+$ T cells. Among the antibodies tested, only treatment with anti-CD3 induced splicing of TNF mRNA, whereas anti-CD28 antibody (as well as anti-CD4 or anti–H-$2K^d$) treatment alone was unable to induce splicing of TNF pre-mRNA above the basal level

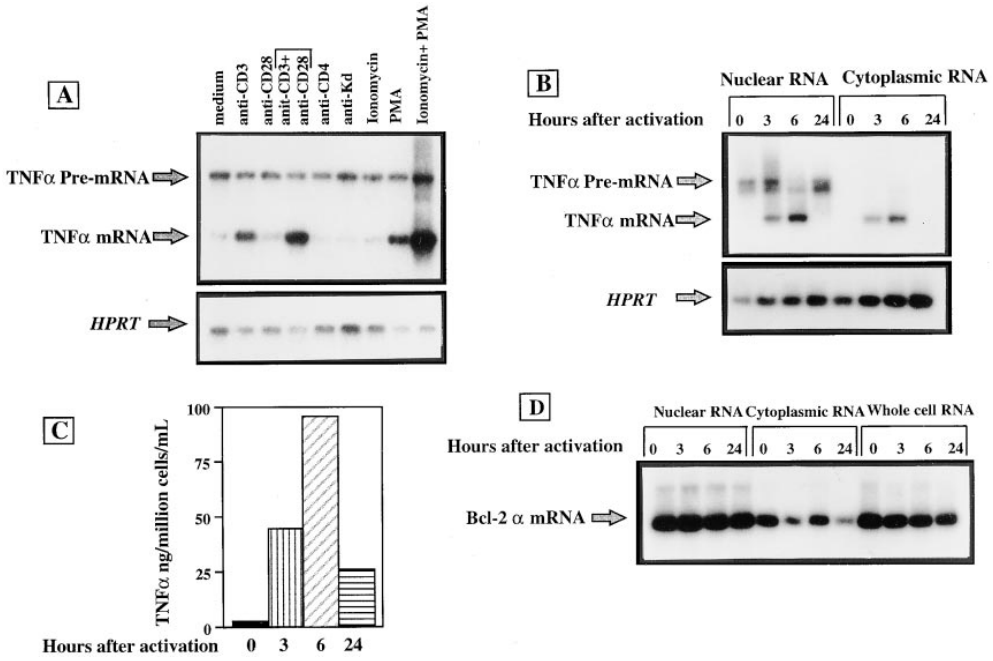

Figure 2. (*A*) CD4+ T cells were incubated with medium or various antibodies and reagents (anti-CD3 [145. 2C11, 10 mg/ml], anti-CD28 [37.51, 10 mg/ml; PharMingen], anti-CD4 [GK1.5, 5 mg/ml], anti–H-2K^d [SF1-1.1, 10 mg/ml; PharMingen], ionomycin [1 mM], and PMA [10 ng/ml]) for 4 h and harvested for detection of TNF expression by RT-PCR. Anti-CD3 and PMA treatment induced splicing of TNF pre-mRNA. Anti-CD28 or ionomycin was unable to induce efficient splicing of TNF pre-mRNA although a weak band of mature mRNA indicated a basal level of processing during the incubation. (*B* and *C*) Expression of TNF in CD4+ T cells activated by PMA/ionomycin was determined at the RNA and protein levels. The purified CD4+ T cells were incubated with PMA (10 ng/ml)/ionomycin (0.5 μM) for the times indicated. Total RNA was separately prepared from nuclear and cytoplasmic fractions for RT-PCR analysis. TNF protein in the culture medium was determined with the TNF-sensitive cell line L929. TNF protein production coincided with the expression of mature TNF mRNA. (*D*) Expression of the α forms of Bcl-2 mRNA in the CD4+ T cells (nuclear and cytoplasmic fractions) activated by PMA/ionomycin for the times indicated. The same RNA samples were used as in *B*. A large amount of mature Bcl-2 mRNA was consistently detected with or without activation of the cells. Only small amounts of pre-mRNA were detected in the nuclear fractions (*upper bands*), indicating a constant and efficient splicing of Bcl-2 pre-mRNA in CD4+ T cells.

(Fig. 2 *A*). Anti-CD3 activation of T cells is known to trigger calcium influx and induce a cascade of protein phosphorylation events (19). It has been demonstrated that calcium influx affects alternative splicing of ATPase mRNA (20). However, incubation of T cells with ionomycin alone, which triggers a strong calcium influx, was unable to induce splicing of TNF pre-mRNA. In contrast, mature TNF mRNA was increased when T cells were incubated with PMA. The combination of PMA and ionomycin greatly increased both transcription and splicing of TNF pre-mRNA; the intensity of both the TNF pre- and mature mRNA signals more than tripled (Fig. 2 *A*).

When RNA was prepared separately from nuclear and cytoplasmic fractions of resting CD4+ T cells, unspliced TNF mRNA (pre-mRNA) was the predominant form seen in the nuclear fraction. No TNF mRNA of either form was seen in the cytoplasmic fraction of resting CD4+ T cells (Fig. 2 *B*) and no TNF protein production was detected in the culture medium (Fig. 2 *C*). Activation of resting CD4+ T cells by PMA/ionomycin strongly induced splicing of TNF pre-mRNA (Fig. 2 *A*). After PMA/ionomycin activation, mature TNF mRNA was detected in both the nuclear and cytoplasmic fractions. The mature TNF mRNA in the cytoplasmic fraction increased with time after activation, mirrored by a concomitant decrease in the TNF pre-mRNA in the nuclear fraction (Fig. 2 *B*). A marked increase in TNF protein production was also detected after activation (Fig. 2 *C*). 24 h after activation, cytoplasmic TNF mRNA was no longer detected, although TNF pre-mRNA was again seen in the nuclear fraction (Fig. 2 *B*). The concentration of TNF protein in the culture medium had greatly decreased by 24 h after activation (Fig 2 *C*). These results demonstrated that there was nuclear accumulation of TNF pre-mRNA but that splicing of nuclear TNF pre-mRNA in resting CD4+ T lymphocytes was a slow and inefficient process. The "basal" level of splicing of TNF pre-mRNA in naive (nonactivated) T cells did not generate sufficient mature TNF mRNA for protein production. However, the processing of TNF pre-mRNA was profoundly (but transiently) upregulated after TCR engagement. The rapid, efficient processing of nuclear TNF pre-mRNA induced by T cell activation was accompanied by translocation of mature TNF mRNA to the cytoplasm and protein production.

To ask whether TCR engagement and resultant T cell activation may simply reflect heightened efficiency of pre-mRNA splicing machinery in general, expression of the *bcl*-2 gene was examined in resting and activated T cells. The *bcl*-2 gene contains a single intron (917 bp) and encodes two proteins, Bcl-2a and Bcl-2b. Production of Bcl-2a requires splicing, whereas Bcl-2b is derived from unspliced mRNA (21). RT-PCR was performed to examine both spliced and unspliced Bcl-2a mRNA from nuclear and cytoplasmic fractions of activated and resting CD4+ T cells. The expected PCR fragment for the unspliced mRNA was 1552 bp and for the spliced mRNA was 635 bp. As shown in Fig. 2 *D*, the spliced Bcl-2 mRNA, Bcl-2a, maintained similar levels in the T cells before and after activation. A

weak but consistent band representing the unspliced Bcl-2 mRNA (containing a 917-bp intron) was also detected in the nuclear fraction. Thus, the splicing machinery was functioning at a similar efficiency for Bcl-2 pre-mRNA in $CD4^+$ T cells with or without activation; activation-induced splicing was specific to the TNF pre-mRNA.

Activation-induced Splicing of TNF Pre-mRNA Can Be Dissociated from Transcription. Activation-induced splicing of pre-mRNA is accompanied by activation-induced transcription; however, as demonstrated above, TNF pre-mRNA accumulated in the nuclei of naive $CD4^+$ T cells without being spliced until TCR engagement. This observation suggested that transcription could be dissociated from activation-induced splicing, and it was possible that activation-induced splicing was independent of transcription. It was also possible that a threshold of TNF pre-mRNA had to be reached for initiation of efficient splicing. In that case, the splicing event should occur only after activation-induced transcription. To distinguish between these two potential mechanisms, we asked whether activation-induced transcription and splicing of TNF pre-mRNA in $CD4^+$ T cells could be dissociated. Act.D had been demonstrated to inhibit TNF gene transcription even in cells that had been activated (8, 9, 14). This was confirmed in our studies when we demonstrated that a 1-h incubation of $CD4^+$ T lymphocytes with a low dose (2 mg/ml) of Act.D completely abolished [^{3}H]uridine incorporation even in the presence of the powerful mitogens PMA and ionomycin (Fig. 3 *A*). To ask whether Act.D could block activation-induced TNF pre-mRNA splicing, $CD4^+$ T cells were incubated with a higher dose of Act.D (10 mg/ml) for 1 h and then activated by anti-CD3 cross-linking. Within 5 min, mature TNF mRNA was readily detected in the Act.D–treated cells and had slightly increased by 15 min after activation (Fig. 3 *B*). These data demonstrate that Act.D inhibited activation-induced transcription, but not activation-induced splicing. However, in the presence of transcription inhibition, mature TNF mRNA diminished within 30 min. In contrast, without Act.D, mature TNF mRNA increased steadily after activation (Fig. 3 *C*). Interestingly, with or without the transcription inhibitor, no noticeable increase in the total amount of TNF mRNA, including pre- and mature mRNA, was detected within the first 15 min after TCR-mediated activation, though in both cases, mature TNF mRNA was detected within 5 min of activation (Fig. 3, *B* and *C*). The results of nuclear run-on studies also demonstrated that activation-induced transcription of the TNF gene was detected 20 min after cellular activation (17). Thus, splicing of accumulated TNF pre-mRNA in activated T cells occurs before the initiation of transcription. A similar result was obtained by incubating T cells with a high dose (10 mg/ml) of the RNA polymerase II inhibitor α-amanitin before TCR cross-linking (data not shown). Taken together, these results demonstrate that TCR engagement triggers splicing of TNF pre-mRNA without requiring transcription. However, spliced TNF mRNA was unstable with the anti-CD3 signal alone and degraded rapidly in the presence of the transcription inhibitor, Act.D (Fig. 3 *B*). To determine whether spliced TNF mRNA can be translated in the absence of transcription, T cells were activated by PMA/ionomycin in the presence of Act.D (Fig. 3 *D*). A significant amount of mature TNF mRNA was detected 3 h after activation. Thus, it appears that PMA/ionomycin treatment may not only induce splicing, but may also stabilize TNF mRNA. In addition, the cells activated by PMA/ionomycin in the presence of Act.D produced a substantial amount of TNF protein (Fig. 3 *E*). Splicing of TNF pre-mRNA can be triggered independently of transcription and, when pre-mRNA is processed, protein is produced; thus, activation-induced splicing is physiologically relevant.

Discussion

Splicing serves as a mechanism to regulate gene expression in development and differentiation, and "regulators" have been identified for tissue-specific splicing (22, 23). Although little is known about the role of splicing in regulation of gene expression in acute response to stimuli, it is clear that splicing is involved in gene regulation during cellular activation. For example, alternative splicing of mRNA of certain proteins occurs in response to activation signals (20, 24). This study presents the first clear evidence of a critical role of activation-induced splicing in gene expression after T cell activation. In naive T cells, splicing of TNF pre-mRNA is an inefficient process that results in the accumulation of TNF pre-mRNA in the nucleus. The splicing of all introns of TNF pre-mRNA is triggered rapidly by TCR engagement, and this activation-induced splicing is followed by TNF protein production. Thus, splicing of nuclear pre-mRNA can be a regulating factor in induced gene expression. In addition, activation-induced splicing only occurred for a short time after TCR engagement (possibly as little as 12 h), and activation-induced splicing was apparently reversible. The reversible process and different signal requirements distinguish activation-induced splicing of TNF pre-mRNA from alternative splicing. The mechanism(s) of activation-induced splicing remains unclear. However, in human T cells, splicing of TNF pre-mRNA, but not other cytokine mRNAs, can be specifically inhibited by 2-aminopurine (25), suggesting an mRNA-specific splicing regulator. In naive T cells, this regulator may block the nuclear processing of TNF pre-mRNA. When T cells are activated, the regulator may be released from TNF pre-mRNA allowing the pre-mRNA to be quickly processed. In the later stages of activation, the regulator molecules may again accumulate, bind to TNF pre-mRNA, and stop the splicing of TNF pre-mRNA. Whether such an mRNA-specific splicing regulator can be identified remains an interesting question. It will also be interesting to study whether activation-induced splicing is involved in the regulation of expression of other cytokines, presumably using different regulator(s).

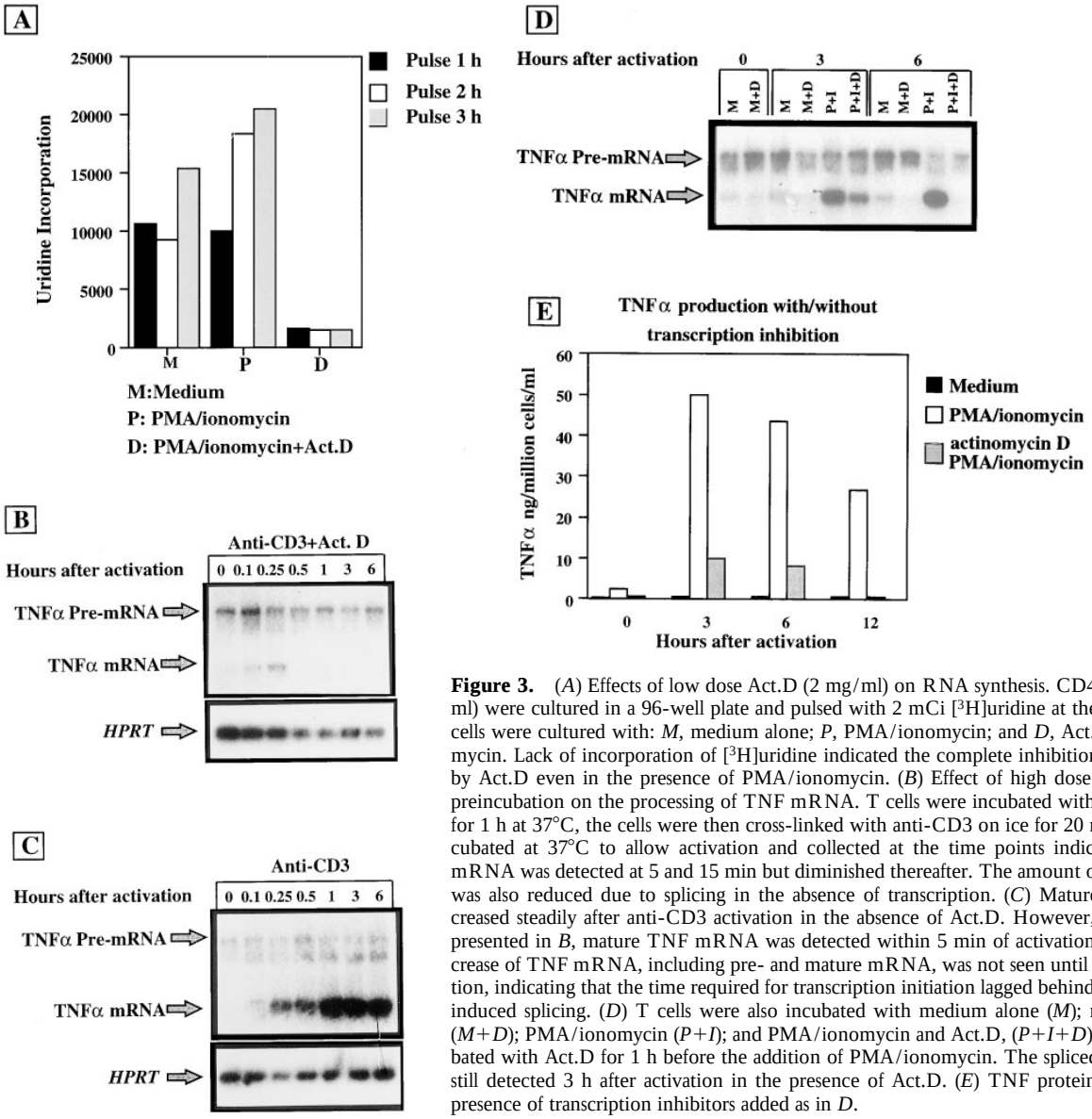

Figure 3. (*A*) Effects of low dose Act.D (2 mg/ml) on RNA synthesis. $CD4^+$ T cells (2×10^6/ml) were cultured in a 96-well plate and pulsed with 2 mCi [^{3}H]uridine at the times indicated. T cells were cultured with: *M*, medium alone; *P*, PMA/ionomycin; and *D*, Act.D and PMA/ionomycin. Lack of incorporation of [^{3}H]uridine indicated the complete inhibition of RNA synthesis by Act.D even in the presence of PMA/ionomycin. (*B*) Effect of high dose Act.D (10 mg/ml) preincubation on the processing of TNF mRNA. T cells were incubated with Act.D (10 mg/ml) for 1 h at 37°C, the cells were then cross-linked with anti-CD3 on ice for 20 min. They were incubated at 37°C to allow activation and collected at the time points indicated. Mature TNF mRNA was detected at 5 and 15 min but diminished thereafter. The amount of TNF pre-mRNA was also reduced due to splicing in the absence of transcription. (*C*) Mature TNF mRNA increased steadily after anti-CD3 activation in the absence of Act.D. However, similar to the data presented in *B*, mature TNF mRNA was detected within 5 min of activation. However, the increase of TNF mRNA, including pre- and mature mRNA, was not seen until 15 min after activation, indicating that the time required for transcription initiation lagged behind that for activation-induced splicing. (*D*) T cells were also incubated with medium alone (*M*); medium and Act.D (*M*+*D*); PMA/ionomycin (*P*+*I*); and PMA/ionomycin and Act.D, (*P*+*I*+*D*). T cells were incubated with Act.D for 1 h before the addition of PMA/ionomycin. The spliced TNF mRNA was still detected 3 h after activation in the presence of Act.D. (*E*) TNF protein production in the presence of transcription inhibitors added as in *D*.

Transcription and splicing are highly coordinated for the expression of many genes. However, transcriptional activity without splicing of pre-mRNA has been observed in the expression of both *IL*-1 and *IL*-2 genes (15, 16). Here we provide evidence that splicing of TNF pre-mRNA in naive $CD4^+$ T cells occurs after an activation signal(s) in the absence of transcription. The coordination and dissociation of transcription and splicing may be controlled by different mechanisms to allow genes to be constitutively active or for genes for which function is transiently required. The mature mRNA of cytokines degrades rapidly due to a conserved AU sequence in the 3′ untranslated region (26, 27). The dissociation of transcription and splicing may result in nuclear accumulation of the cytokine pre-mRNA that is relatively stable and can be released as functionally mature mRNA after appropriate activation stimuli. The mechanism of activation-induced splicing may thus facilitate an immediate response to activation stimuli.

TNF is a cytokine with powerful inflammatory effects involving many autoimmune diseases in animal models (28–30), thus expression of TNF has to be tightly controlled. However, TNF is one of the major players in hyperacute responses (31), mounts the first line of host defense, and triggers a cascade of immune response (32, 33). Particularly in T cells, TNF is the first cytokine produced after activation, followed by many others (34). Thus, whereas TCR-mediated activation-induced splicing of TNF pre-mRNA allows and may enhance the immediate alarm signal after T cell receptor engagement and multiple layers of regulation, including transcription, splicing, RNA stability, translation, and posttranslational events, ensure the accurate regulation of TNF expression.

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