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RNA Processing Is a Limiting Step for Murine Tumor Necrosis Factor β Expression in Response to Interleukin-2

D. WEIL, S. BROSSET, AND F. DAUTRY*

Laboratoire d'Oncologie Moléculaire, Institut Gustave Roussy, Centre National de la Recherche Scientifique UA 1158, Villejuif, France

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We have previously reported that tumor necrosis factor β (TNFβ) expression is induced by interleukin-2 (IL-2) in the murine lymphocytic T-cell line CTLL-2. In this study, we have characterized the nuclear and cytoplasmic TNFβ transcripts and assessed their role in TNFβ gene expression. A unique feature of TNFβ expression was the accumulation of nuclear precursors, which reflected a slow nuclear RNA processing. As a consequence, there was a delay in the appearance of cytoplasmic messengers after the transcriptional induction of TNFβ by IL-2. We also found that two messengers, the fully spliced messenger and an intron 3-retaining messenger, were exported to the cytoplasm and actively translated. The same pattern of expression was observed in concanavalin A-stimulated splenocytes, although the level of expression was much lower than in CTLL-2 cells. The simple genetic structure and the high level of accumulation of nuclear precursors make TNFβ a particularly attractive model system to use for studies of RNA processing and cytoplasmic transport of partially spliced messengers.

Regulation of mRNA accumulation in mammalian cells has been studied mostly within the context of cell stimulation by growth factors and hormones. Although these factors induce complex and pleiotropic responses, the expression of only a limited set of genes increases during the early part of the response. Some proto-oncogenes have been shown to belong to this group of immediate-early genes, most notably *fos* (19), *myc* (27), and *jun* (45), and differential screening has been used to identify others. Most of these genes appear to code for transcription factors or secreted growth regulators (43). Although gene transcription, RNA processing, and degradation could be involved in the control of induction of these genes, only gene transcription and cytoplasmic mRNA stability have been extensively studied (43). Indeed, direct observation of nuclear RNA processing and cytoplasmic transport is, in most cases, hardly feasible because of the low level of nuclear RNA precursors. Nevertheless, when measurements of gene transcription and mRNA stability fail to account for mRNA accumulation, a role for other post-transcriptional regulations has been postulated (20, 23, 40, 41).

We have studied early-gene expression in response to interleukin-2 (IL-2) in a lymphocytic T-cell line, CTLL-2 (10). These cells can be synchronized by IL-2 starvation and then stimulated to proliferate by addition of IL-2. Having characterized the expression of several proto-oncogenes (10), we used the differential screening of a cDNA library to identify other immediate-early genes of the IL-2 response. This led us to the isolation of a tumor necrosis factor β (TNFβ; lymphotoxin) cDNA and to the observation that the expression of TNFα, TNFβ, and gamma interferon is induced by IL-2 (50). Although TNFα and TNFβ have only limited sequence homologies, they share the same wide spectrum of biological activities, which include cytotoxicity, tumor necrosis, stimulation of growth or differentiation, antiviral activity, and immunomodulation (4, 37, 44, 48), and the two factors compete for the same receptor (1). Expression of TNFβ appears to be restricted to lymphocytes (38), whereas TNFα is expressed in both monocytes and lymphocytes (4). The expression of TNFα and TNFβ does not correlate with a cytotoxic activity of T lymphocytes (25, 50), and the induction of these factors by IL-2 could be associated with autocrine or paracrine regulatory loops in the immune system.

One TNF locus, which comprises the TNFβ and TNFα genes organized in tandem, has been described in the human and murine genome (16, 36). So far, only one type of murine TNFβ cDNA has been cloned, which is generated by the splicing of three introns (34, 50). In contrast with this simple structure of the TNFβ gene, we have reported the detection by Northern (RNA) blots of two major TNFβ transcripts that accumulated with different kinetics during the IL-2 stimulation of CTLL-2 cells (50). In this study, we report the structure, localization, and kinetics of accumulation of the different TNFβ transcripts. These results establish that RNA processing is a limiting step for TNFβ expression and that a second TNFβ messenger, coding for a distinct protein, is generated by the transport to the cytoplasm of a partially spliced transcript.

MATERIALS AND METHODS

Cell culture. CTLL-2 clone G4 was derived by single-cell cloning from the lymphocytic T-cell line CTLL-2 (3). The cells were routinely maintained as described elsewhere (10). For RNA preparations, the cells were deprived of IL-2 for 16 h and cultured with a 5-U/ml (42 pM) concentration of recombinant human IL-2 (Amgen) for times shorter than 4 h and with 10 U/ml (84 pM) for longer times.

Splenocytes from BALB/c mice were obtained by dissociation of the spleen, washed twice with phosphate-buffered saline, and incubated at 2.5×10^6 cells per ml in RPMI 1640 supplemented with 5% nonmitogenic decomplemented fetal calf serum, 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.3), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, penicillin (50 U/ml), streptomycin (50 μg/ml), and concanavalin A (5 μg/ml; Pharmacia). The

* Corresponding author.

efficiency of stimulation was assessed by measuring RNA and DNA synthesis as described previously (27).

RNA extraction. Cellular RNA was extracted by the guanidine thiocyanate technique (7). For the purification of nuclear and cytoplasmic RNA, cells were washed three times in ice-cold phosphate-buffered saline and lysed by incubation in ice-cold 0.5% Nonidet P-40 (NP-40)–10 mM Tris hydrochloride (pH 7.4)–10 mM NaCl–10 mM $MgCl_2$ (1 ml for 75×10^6 cells) for 5 min. This lysate was spun for 5 min at $500 \times g$ and 4°C. The nuclear pellet was suspended, and the cytoplasmic supernatant was diluted in guanidine thiocyanate. Both were processed as described above. In the case of splenocytes, the cells were first centrifuged over a metrizamide cushion, and an inhibitor of RNase (Vanadyl RiboC) was added to the lysis buffer.

Polysome fractionation was achieved by a modification of the method of Aziz and Munro (2). The cells were washed in ice-cold phosphate-buffered saline and lysed by incubation in a solution containing ice-cold 0.5% NP-40, 20 mM HEPES (pH 7.3), 250 mM KCl, 10 mM $MgCl_2$, 20 mM dithiothreitol (DTT), 10 U of RNase inhibitor (InhibitACE) per ml, and 150 μg of cycloheximide per ml (for Fig. 7A) or 0.5% NP-40, 20 mM HEPES (pH 7.3), 300 mM KCl, 2 mM $MgCl_2$, 20 mM DTT, and 10 mM Vanadyl RiboC (For Fig. 7B). This lysate was spun for 5 min at $500 \times g$ and 4°C. For Fig. 7A, the cytoplasmic supernatant was spun as described previously (2) in a 10 to 50% sucrose gradient buffered with 20 mM HEPES (pH 7.3)–250 mM KCl–20 mM $MgCl_2$–2 mM DTT–500 μg of heparin per ml. Then 13 fractions were collected and ethanol precipitated. The pellets were resuspended in guanidine thiocyanate and processed in the manner used for other RNAs. For Fig. 7B, the cytoplasmic supernatant was incubated for 15 min at 0°C followed by 15 min at 37°C with or without 10 mM puromycin and then spun on a 10 to 50% sucrose gradient buffered with 20 mM HEPES (pH 7.3)–300 mM KCl–10 mM $MgCl_2$–2 mM DTT–10 mM Vanadyl-ribonucleoside complexes–500 μg of heparin per ml. Then 11 fractions were collected, diluted in guanidine thiocyanate, and processed in the manner used for other RNAs.

Poly(A) tail digestion. RNA (4 μg) was hybridized with 0.5 μg of 20-mer oligo(dT) and digested with 1 U of RNase H (Boehringer) as described by Erster et al. (12) except that the RNase H buffer was 40 mM Tris hydrochloride (pH 8)–50 mM KCl–10 mM $MgCl_2$–1 mM DTT.

Northern blot. A 4-μg sample of RNA or, for the polysome study, 1/50 of each fraction was electrophoresed through 1.5% formaldehyde-agarose gels and transferred to uncharged nylon as described previously (10). Hybridization with riboprobes was performed at 60°C in 50% formamide–750 mM NaCl–150 mM Tris hydrochloride (pH 8)–200 mM sodium phosphate buffer (pH 6.8)–10 mM EDTA–0.1% sodium dodecyl sulfate (SDS)–500 μg of heparin per ml, followed by washing for 45 min at 68°C in 0.5× SPE (1× SPE is 180 mM NaCl, 10 mM phosphate buffer [pH 6.8], and 1 mM EDTA)–0.1% SDS. Hybridization with the nick-translated DNA probe was performed at 68°C in 4× SPE–0.2% SDS–500 μg of heparin per ml, followed by washing for 45 min at 68°C in 1× SPE–0.1% SDS. ^{32}P-labeled RNA probes (pEx4, pInt3, pInt2, probe A, and pBACT5; specific activity, 3×10^8 cpm/μg) were obtained by runoff transcription using T3 or T7 RNA polymerases. A ^{32}P-labeled DNA probe (pInt1; specific activity, 8×10^8 cpm/μg) was obtained by nick translation.

The exon- or intron-specific TNFβ probes have the following structures, according to the genomic sequence of Semon et al. (46): pEx4, nucleotides 2222 to 2741; pInt3, nucleotides 2018 to 2226; pInt2, nucleotides 1813 to 1894; pInt1, nucleotides 1165 to 1327; and probe A, nucleotides 1733 to 2226. All were cloned in Bluescript. pBACT5 is the *TaqI-PstI* fragment of a murine β-actin cDNA cloned in Bluescript.

RNase mapping. ^{32}P-labeled RNA (specific activity, 6×10^7 cpm/μg) was obtained by in vitro runoff transcription with T3 RNA polymerase. Hybridization of 100 pg of labeled RNA with RNA samples (5, 10, or 20 μg), digestion with RNases A and T_1, and electrophoresis on 5% polyacrylamide-urea gels were performed as described elsewhere (N. Modjtahedi, H. Haddada, C. Lavialle, E. Lazar, T. Lamonerie, and O. Brison, submitted for publication).

Probe A contains nucleotides 1733 to 2226, probe B contains nucleotides 1165 to 1760, and probe C contains nucleotides 1962 to 2341 of the TNFβ genomic sequence (46).

Nuclear run-on. Isolation of nuclei and in vitro elongation of the transcripts were performed as described previously (19). Purification of labeled RNA and DNA blotting (pLTI, pTNFα, pCOII, and pBT) were performed as described previously (10). Hybridization was for 72 h at 42°C in 50% formamide–4× SPE–0.1% SDS–500 μg of heparin per ml–1.2×10^6 cpm of labeled RNA per ml. Filters were washed for 45 min at 68°C in 0.5× SPE–0.1% SDS. pLTI contains a cDNA of murine TNFβ starting at nucleotide 1733 and ending at nucleotide 3205, pTNFα contains a partial cDNA of murine TNFα encompassing nucleotides 31 to 1110 (39), and pCOII contains a partial cDNA of murine cytochrome oxidase II encompassing nucleotides 7070 to 7531, all cloned in Bluescript (pBt).

RESULTS

Nuclear and cytoplasmic TNFβ transcripts. Figure 1A summarizes some of our previous results on TNFβ expression after IL-2 stimulation of CTLL-2 cells. The expression of two transcripts of 1.7 and 1.4 kb is induced by IL-2 within 1 h, the former accumulating before the latter. Accumulation of these transcripts reaches a maximum after 8 h. A faint band at 2.2 kb and a smear between 1.4 and 1.7 kb suggest the presence of other transcripts.

To obtain some structural data on these transcripts, we constructed a cDNA library from total RNA of CTLL-2 cells that had been stimulated for 2 h by IL-2, at which time the levels of accumulation of the 1.4- and 1.7-kb transcripts were similar. Twenty-four clones were isolated and analyzed by restriction enzyme mapping and partial sequencing. All were derived from the previously characterized TNFβ gene. The only structural difference was the presence of introns 2 and 3 in one and of intron 3 in two of the seven clones encompassing this region. This finding indicated the presence of partially spliced TNFβ transcripts.

We therefore analyzed by Northern blot nuclear and cytoplasmic RNAs of CTLL-2 cells that had been stimulated for 8 h by IL-2 (Fig. 1B). To improve the sharpness of the different bands, the same samples were also run after poly(A) tail digestion. Analysis of untreated RNA (Fig. 1B, left panel) suggested the existence of four TNFβ transcripts, the two larger ones being restricted to the nucleus and the two smaller ones being present in both the nuclear and cytoplasmic RNA fractions. The TNFβ probe used in this experiment begins in exon 2 and contains intron 2, exon 3, and intron 3 (probe A; see Fig. 3). It detected the largest species more efficiently than did the exon 4 probe used in Fig. 1A. Poly(A) tail digestion confirmed the presence of

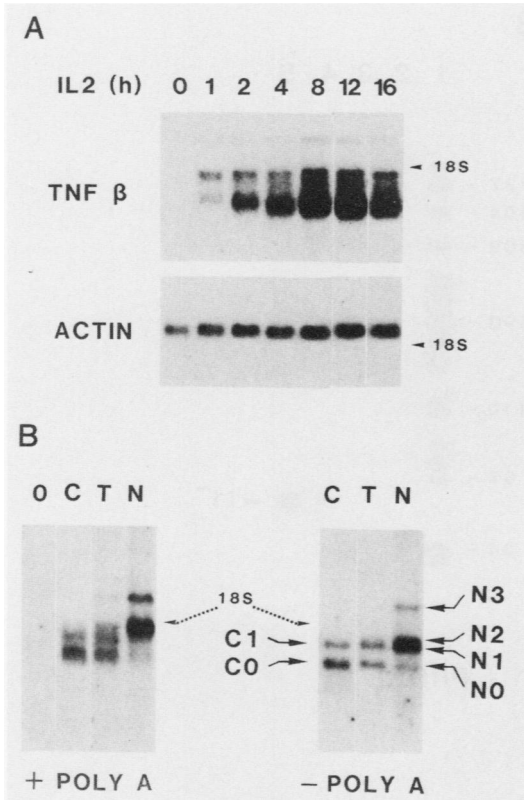

FIG. 1. Northern blot analysis of TNFβ expression during IL-2 stimulation of CTLL-2 cells. (A) CTLL-2 cells were stimulated with IL-2 for 0, 1, 2, 4, 8, 12, or 16 h. Total RNA (4 μg per lane) was hybridized with the TNFβ exon 4 probe (upper panel), and rehybridized with a β-actin probe (lower panel). (B) CTLL-2 cells were stimulated with IL-2 for 8 h. Total RNA was extracted from one half, and nuclear and cytoplasmic RNAs were extracted from the other half. A 4-μg sample of nuclear (N), total (T), or cytoplasmic (C) RNA, before (left panel) or after (right panel) poly(A) tail digestion with RNase H, was hybridized with TNFβ probe A. An unstimulated control (0) is present on left panel. C0, C1, N0, N1, N2, and N3 designate the different TNFβ transcripts detected in cytoplasmic or nuclear RNA after poly(A) tail digestion. The migration of 18S rRNA is indicated.

four different transcripts (Fig. 1B, right panel). We will designate the cytoplasmic transcripts C0 and C1 and the nuclear transcripts N0, N1, N2, and N3, as indicated in Fig. 1B. N1 and N2 appeared as a doublet but were clearly distinguished in later experiments. Poly(A) tail digestion increased the mobility of all of the transcripts but to different extents. In particular, the poly(A) tail of N1 appeared to be up to 100 residues longer than that of C1 (Fig. 1B, left panel).

These data suggest the existence of four polyadenylated TNFβ transcripts. Two are specifically nuclear, N3 and N2, whereas two others, N1-C1 and N0-C0, are both nuclear and cytoplasmic. Furthermore, these results indicate that the two major RNA species previously detected on Northern blots (50; Fig. 1A) are in fact composed of N2, N1, and C1 and of N0 and C0, respectively.

Structures of the TNFβ transcripts. We further analyzed these samples after poly(A) tail digestion by hybridization of Northern blots with exon- or intron-specific probes (Fig. 2). An exon 4 probe hybridized with all of the nuclear and cytoplasmic RNA previously detected with probe A. An intron 3 probe hybridized with N1, N2, and N3 and with C1. An intron 2 probe hybridized with N2 and N3. Thus, the

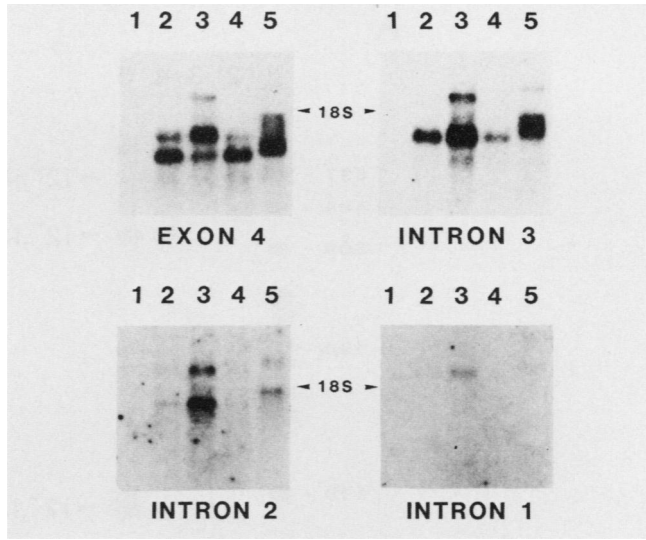

FIG. 2. Northern blot analysis of TNFβ transcripts with exonic and intronic probes. A 4-μg sample of total RNA from untreated CTLL-2 cells (lane 1) or of total (lane 2 and 5), nuclear (lane 3), or cytoplasmic (lane 4) RNA from 8-h-stimulated CTLL-2 cells, after (lanes 1 to 4) or before (lane 5) poly(A) tail digestion, was hybridized with the TNFβ exon 4, intron 3, intron 2, or intron 1 probe.

N1-N2 doublet could be resolved by comparing the patterns obtained with the intron 2 and the intron 3 probes. As discussed later, our observations on the structure of the murine TNFβ gene suggest that intron 1 extends from nucleotides 1218 to 1708 of the genomic sequence of Semon et al. (46). To avoid the long TC stretch that lies in the middle of intron 1, we chose a probe extending from nucleotides 1165 to 1327. This probe hybridized only to N3.

In summary, N3 hybridized with the three intronic probes and with the exon 4 probe, N2 hybridized with the intron 2, intron 3, and exon 4 probes, N1 and C1 hybridized with the intron 3 and exon 4 probes, and N0 and C0 hybridized only with the exon 4 probe.

These hybridizations could have resulted either from the complete retention of the introns or from alternative splicing. We analyzed by RNase mapping the nature of the intronic sequences present in nuclear and cytoplasmic RNA. Mapping of introns 2 and 3 was achieved with probe A (Fig. 3A). Splicing of both introns would result in the protection of two fragments of 82 and 100 nucleotides, corresponding to a part of exon 2 and to exon 3. Splicing of only intron 2 or intron 3 would result in the protection of a 265- or a 332-nucleotide fragment, corresponding respectively to exon 2-intron 2-exon 3 or to exon 3-intron 3-exon 4, whereas retention of both introns would result in the protection of a 497-nucleotide fragment. The data show the presence in the nucleus of three classes of transcripts corresponding to the presence of introns 2 and 3, intron 3 alone, or neither of these introns. Their relative abundances were approximately 35, 55, and 10%. In the cytoplasm, we detected RNA that contained intron 3 alone or was fully spliced, with a relative abundance of 15 or 85%, respectively. We detected no signal corresponding to the presence of intron 2 alone.

Mapping of intron 1 was achieved with probe B. Although the murine TNFβ locus has been completely sequenced (16, 17, 46), there is no direct information on the 5′ end of intron 1, which had been tentatively located by analogy with the structure of the human TNFβ gene. We propose that intron 1 starts at nucleotide 1218, and therefore that exon 1 is 17 to

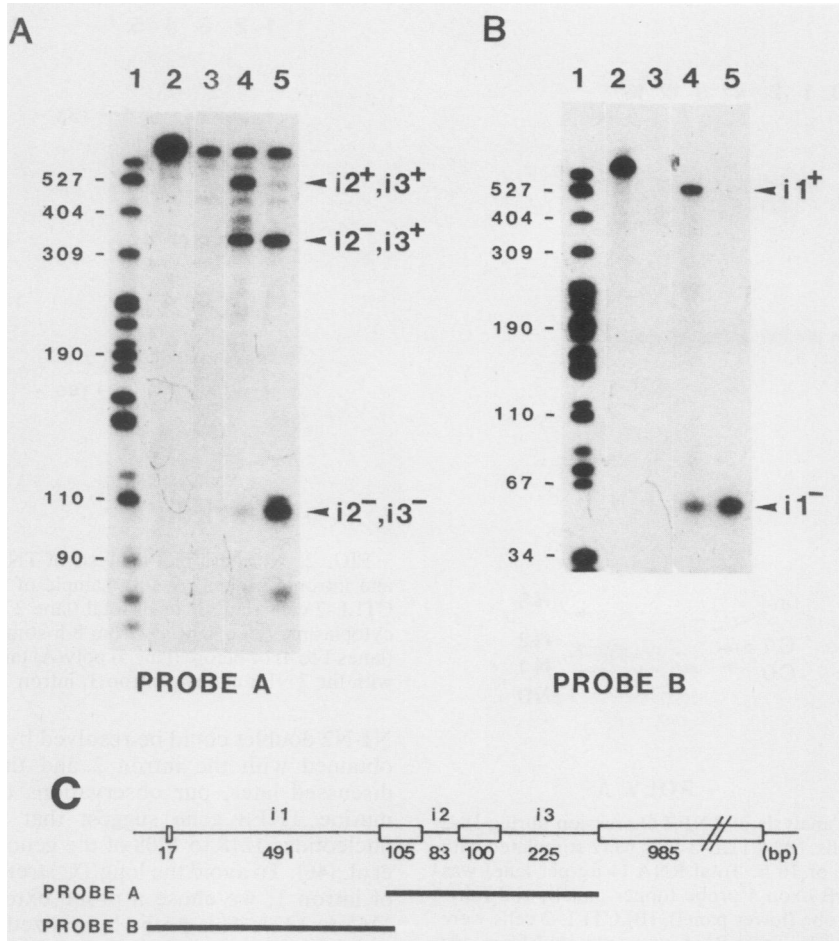

FIG. 3. RNase mapping analysis of intronic sequences in TNFβ transcripts. A 10-μg sample of each RNA was hybridized with 100 pg of TNFβ probe A (A) or with 100 pg of TNFβ probe B (B), digested with RNases A and T_1, and migrated through a 5% urea-polyacrylamide gel. Lanes: 1, molecular weight markers (*Msp*I-digested pBR322 DNA); 2, 7 pg of undigested probe; 3, total RNA from untreated CTLL-2 cells; 4 and 5, nuclear (lane 4) and cytoplasmic (lane 5) RNAs from 8-h-stimulated CTLL-2 cells. $i2^{+/-}$ and $i3^{+/-}$ indicate the fragments protected by RNA having or having not retained intron 2 or 3. (C) Schematic representation of the TNFβ gene showing exons and introns and the locations of probes A and B.

21 bp long (upon the exact site of initiation of transcription) instead of 145 bp (16, 17, 46), for the following reasons: (i) a computer search for donor splice sites identified nucleotide 1218 as the best candidate, (ii) our longest cDNA has a structure consistent with the usage of this splice site, and (iii) extension of a primer complementary to nucleotides 1743 to 1760 generated a fragment of 65 to 67 nucleotides (data not shown). With use of probe B for RNase mapping, the presence of intron 1 should result in the protection of a 564-nucleotide fragment, whereas in its absence a fragment of 52 nucleotides corresponding to exon 2 should be protected. The data (Fig. 3B) indicated that intron 1 was present only in nuclear RNA. Furthermore, the absence of a specific fragment corresponding to exon 1 indicated that exon 1 was smaller than 27 nucleotides, in agreement with our splice site assignment.

Thus, the signals observed on Northern blots with intronic probes resulted not from alternative splicing but from the complete retention of the corresponding introns. Taken together, these results suggest the following scheme for the structure of the TNFβ transcripts (Fig. 4). N3 is the primary unspliced transcript, and N2 is a nuclear precursor in which intron 1 has been spliced out. N1 results from the splicing out of introns 1 and 2, and N0 results from the splicing out of

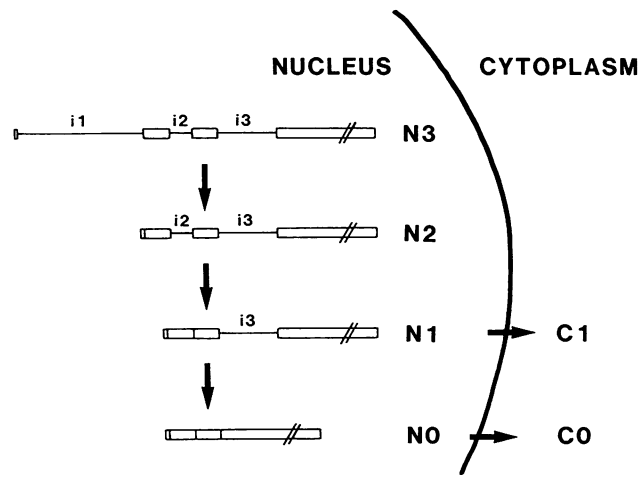

FIG. 4. Proposed model for the structure of TNFβ transcripts and their maturation. i1, i2, and i3, Introns 1, 2, and 3.

the three introns. Both N0 and N1 can be exported to the cytoplasm, giving rise to C0 and C1. This interpretation takes into account the following points: (i) of seven cDNA clones containing exons 2 and 3, three also contained either introns 2 and 3 or intron 3 alone; (ii) the differences in migration of the four transcripts are compatible with the intron sizes (intron 1, 491 nucleotides; intron 2, 83 nucleotides; and intron 3, 225 nucleotides); (iii) the relative abundances of the different species identified by Northern blot and RNase mapping are in agreement; and (iv) we found no indication for other structural differences such as alternative splicing, polyadenylation site, or transcription initiation site.

Regulation of TNFβ transcript accumulation. In view of the accumulation of TNFβ messenger precursors in the nucleus and of the existence of two cytoplasmic products, regulation by IL-2 of TNFβ gene expression can take place at several levels: transcription of the gene, excision of introns 1 and 2, excision of intron 3 versus exportation to the cytoplasm of N1, and degradation of each of these RNAs.

To study TNFβ transcription, we performed nuclear run-on experiments after IL-2 stimulation of CTLL-2 cells for 0, 1, 4, and 8 h (Fig. 5A). In quiescent cells, no transcription was detectable. Transcription was induced by 1 h and increased slightly between 1 and 8 h. Transcription of TNFα, which is also regulated by IL-2 (50), was detected in quiescent cells and induced during stimulation. Transcription of cytochrome oxidase II was stable during the stimulation and served as an internal control. Therefore, there is transcriptional control of TNFβ expression by IL-2.

To investigate the posttranscriptional regulation of TNFβ expression, we used dactinomycin to inhibit transcription. After 1 or 8 h of stimulation by IL-2, CTLL-2 cells were treated with dactinomycin for 0, 30, 60, and 90 min, and nuclear and cytoplasmic RNAs were analyzed by RNase mapping with probe A (Fig. 5B). Considering first the results after 8 h of stimulation by IL2, we observed the following. (i) In the absence of transcription, N3-N2 (which were not distinguished by probe A) had a half-life of less than 10 min. N1 was stable during the first 30 min and then disappeared, with a half-life of about 20 min. N0 accumulated during the first 60 min and then decreased. (ii) In the cytoplasm, the abundance of C1 and C0 continuously decreased, indicating similar half-lives of about 90 min.

The finding that N0 could accumulate during the dactinomycin treatment indicated that both its degradation by nucleases and its export to the cytoplasm were slow on the time scale used (30 min). The biphasic kinetics of N1 accumulation can be explained by assuming that it is rapidly generated from N3 and N2 by splicing out of introns 1 and 2 and then converted to N0 by splicing out of intron 3. Thus, each of the nuclear precursors is finally converted into N0 in the absence of any significant degradation.

After 1 h of stimulation, the behavior of nuclear RNA was similar to that observed after 8 h: processing of N3, N2, and N1 resulted in half-lives of less than 10 min for N3-N2 and about 20 min for N1, whereas N0 accumulated during the dactinomycin treatment. In the cytoplasm, C1 was stable and C0 increased slightly during the 90-min dactinomycin treatment, indicating that transport to the cytoplasm of N0 occurred in the presence of dactinomycin, albeit very slowly. Furthermore, this accumulation strongly suggested that C0 was more stable after 1 h than after 8 h of stimulation.

In summary, the splicing rates of TNFβ introns in the presence of dactinomycin were similar after 1 and 8 h of stimulation. Splicing, rather than degradation, appeared to be the principal mechanism controlling nuclear precursors half-lives.

Comparative study of C0 and C1. We have demonstrated the export to the cytoplasm of two TNFβ transcripts, C0 and C1. We analyzed their relative abundance during IL-2 stimulation of 0 to 16 h, using RNase mapping with probe C, which gave a lower background than did probe A (Fig. 6). C0 should protect a fragment of 123 nucleotides instead of 384 nucleotides for C1. The two transcripts accumulated in the cytoplasm with the same kinetics, reaching a maximum after 8 to 12 h of stimulation, C0 being about 8- to 11-fold more abundant than C1. It should be noted that probe A reproducibly yielded higher values for the C1/C0 ratio than did probe C, probably reflecting differences in the kinetics of hybridization to both RNA species. Thus, our previous studies by Northern blot analysis (50; Fig. 1A), which indicated that the accumulation of N2, N1, and C1 preceded that of N0 and C0, reflected the accumulation of nuclear precursors rather than that of cytoplasmic messengers.

To study the translation level of C1 and C0, we fractionated on a sucrose gradient the cytoplasmic extract of CTLL-2 cells that had been stimulated for 8 h by IL-2. The RNA of each fraction of the gradient was analyzed by Northern blot with exon 4 and intron 3 probes (Fig. 7A). Both C0 and C1 were predominantly present in the polysomal fractions. To confirm that this rapid sedimentation was due to an association with ribosomes, we investigated the effect of puromycin, which is known to induce the dissociation of polyribosomes in vitro (5). We analyzed by RNase mapping with probe C the gradient fractions collected after incubation with or without puromycin (Fig. 7B). In the absence of puromycin, both C0 and C1 were found in the polysomal fractions. Intron 3-containing transcripts were more abundant in the monosomal fractions in this experiment than in the previous one, probably because of a higher contamination of the cytoplasmic extract by nuclear RNA, as indicated by Northern blot analysis (Fig. 7C). After puromycin treatment, both C0 and C1 were chased into the monosomal fractions. Thus, both C0 and C1 were actively translated.

The open reading frame of C1 differs from that of C0 by the presence of intron 3. We sequenced intron 3 in two independent cDNA clones of our CTLL-2 library and found an additional C at nucleotide 2163 with respect to the genomic sequence of Semon et al. (46; Fig. 8). According to our sequence, C1 codes for a protein of 127 amino acids, composed of the first 66 amino acids of TNFβ followed by 61 amino acids encoded by intron 3. Thus, this new protein encoded by the TNFβ gene contains the signal sequence of the TNFβ protein and should be secreted. Accordingly, the C1 message, as well as C0, was found associated exclusively with membrane-bound polyribosomes (data not shown).

Expression of TNFβ transcripts in murine splenocytes. The detection of several TNFβ transcripts is not restricted to the cell line CTLL-2. We previously reported the presence of two major RNA species in another murine lymphocytic T-cell line, B6.1, although at much lower levels than in CTLL-2 (50). To extend this observation, we stimulated murine splenocytes with concanavalin A and analyzed the accumulation of TNFβ transcripts on Northern blots with exon 4 and intron 3 probes (Fig. 9A). Expression of TNFβ was detectable after 4 h and increased until 48 h. As in CTLL-2, several diffuse bands could be observed, the upper ones being predominant during the early part of the response. Hybridization with an intron 3 probe confirmed that the upper bands contained intronic sequences.

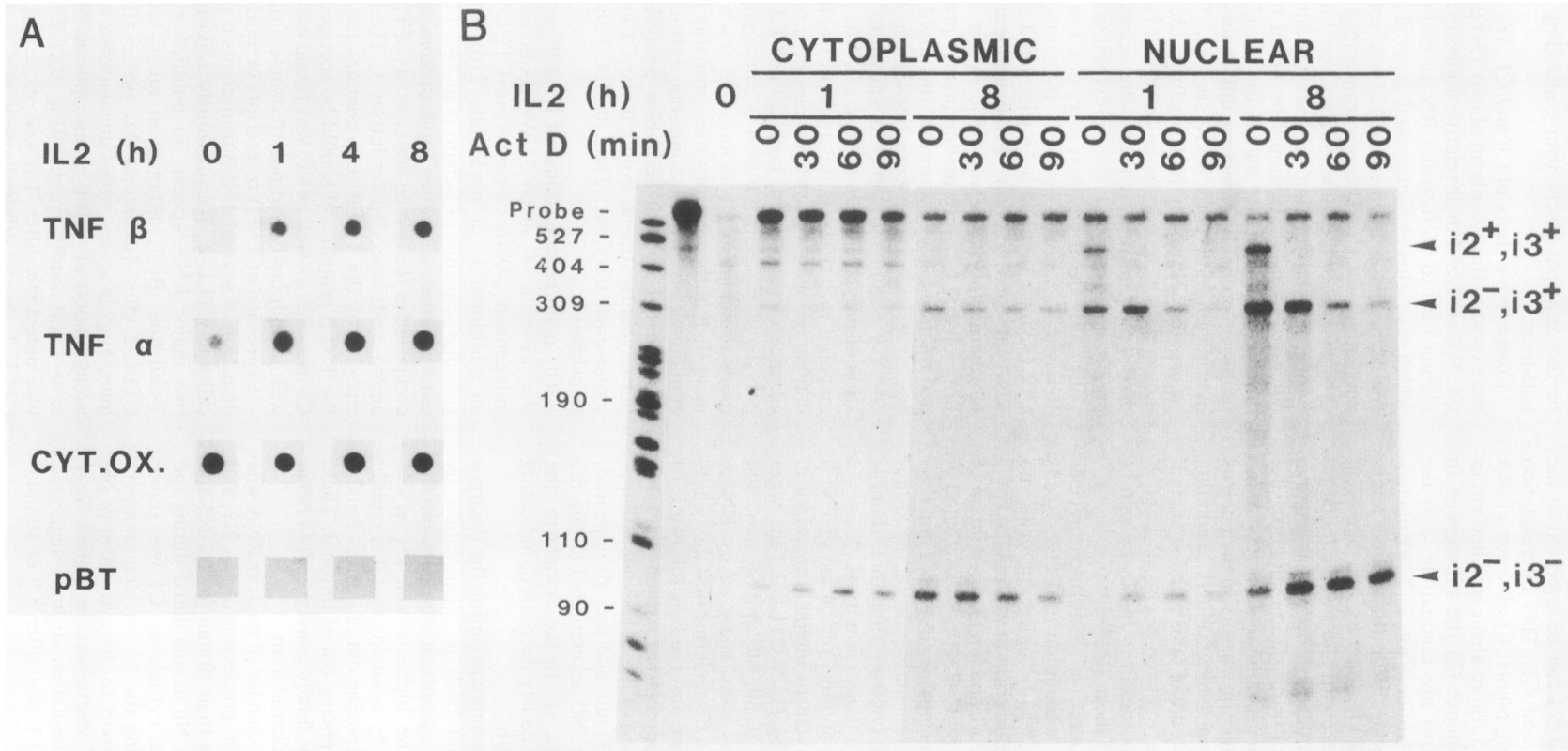

FIG. 5. Analysis of the regulation of TNFβ expression in CTLL-2 cells. (A) Nuclear run-on transcription assays. CTLL-2 cells were stimulated with IL-2 for 0, 1, 4, and 8 h, and nuclei were prepared and used for in vitro run-on transcription. Samples (5 μg) of denatured TNFβ, TNFα, cytochrome oxidase II (CYT.OX), and plasmid pBt were dotted on nitrocellulose and hybridized with the products of run-on transcription. (B) RNase mapping analysis of the stability of TNFβ transcripts. CTLL-2 cells were stimulated with IL-2 for 1 and 8 h and then treated with dactinomycin (10 μg/ml) for 0, 30, 60, and 90 min. Nuclear and cytoplasmic RNAs were extracted at each time point, and 10 μg was analyzed by RNase mapping as for Fig. 3 with TNFβ probe A. An unstimulated sample is included (lane 0). Protected fragments are designated on the right as for Fig. 3.

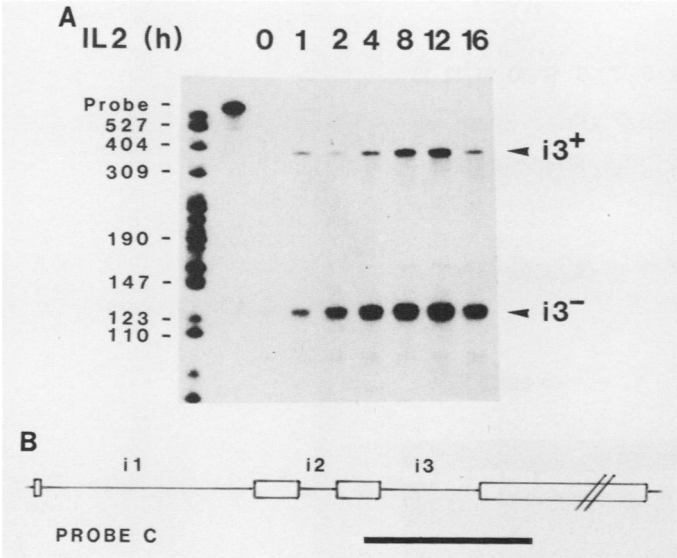

FIG. 6. (A) RNase mapping analysis of cytoplasmic TNFβ transcripts during IL-2 stimulation of CTLL-2 cells. CTLL-2 cells were stimulated with IL-2 for 0, 1, 2, 4, 8, 12, and 16 h. Cytoplasmic RNA was prepared at each time point and analyzed by RNase mapping with probe C. (B) Structure of probe C.

To distinguish between nuclear precursors and cytoplasmic messengers, we analyzed by RNase mapping with probe C the cytoplasmic RNA from splenocytes that had been stimulated for 6 or 48 h with concanavalin A (Fig. 9B). Both C0 and C1 were detected, and their levels increased between 6 and 48 h of stimulation. Their relative abundances were the same as in CTLL-2 cells. Therefore, the RNAs detected by Northern blot analysis with the intron 3 probe after 6 h were mostly nuclear, whereas those detected after 48 h were mostly cytoplasmic.

Thus, concanavalin A induced expression of the TNFβ gene in splenocytes, leading to the accumulation of nuclear precursors and of two cytoplasmic messengers, C0 and C1, as in CTLL-2 cells.

DISCUSSION

In this study, we used a combination of cDNA cloning, Northern blots, and RNase mapping to characterize four TNFβ transcripts that are expressed during the IL-2 stimulation of CTLL-2 cells. Two of them are restricted to the nucleus: N3, the primary transcript with its three introns, and N2, which lacks intron 1. The two others are both nuclear and cytoplasmic: N1-C1, which contain intron 3, and N0-C0, which are fully spliced. C0 is the mature messenger previously described (34, 50) and codes for the TNFβ protein, whereas C1 codes for a protein with a different C-terminal moiety.

One striking feature of TNFβ expression is the abundance of nuclear precursors, since for most genes such precursors are not easily detectable on Northern blots. There are, nevertheless, a few precedents, such as the ovalbumin and ovomucoid (49), the phosphoenolpyruvate carboxykinase (22), the thymidine kinase (20), and the p55 IL-2 receptor (14) genes and the *fgr* proto-oncogene (33). However, characterization of these precursors has been hampered either by lack of information on the gene structure (ovalbumin and phosphoenolpyruvate carboxykinase genes) or the number and the length of the introns (thymidine kinase, p55 IL-2 receptor, and *fgr* genes) or by the presence in the introns of highly repetitive elements (thymidine kinase gene). Thus, TNFβ, with only three short introns, constitutes a particularly convenient model for the study of RNA maturation. Our study indicates a well-defined sequence of events in the processing of the TNFβ transcripts. All of the nuclear TNFβ precursors are polyadenylated, establishing that splicing occurs after polyadenylation of the initial transcript. Our assignment of a specific structure to each of the nuclear precursors further indicates that splicing proceeds in an ordered manner, from intron 1 to intron 3. Although such a sequence of events in RNA processing is generally assumed, there are indications that other orders are possible, depending on the gene under consideration. For instance, splicing can precede polyadenylation (31), and several pathways of splicing have been observed to coexist for the ovomucoid transcripts (49). The high level of TNFβ nuclear precursor accumulation makes it possible to consider the question of their stability. It is usually assumed that nuclear RNA is rapidly degraded because only a fraction of the sequences present in heterogeneous nuclear RNA are present in cytoplasmic RNA (9, 32). Our results indicate that in the case of TNFβ, splicing rather than degradation is the factor limiting nuclear RNA half-life.

The detection of precursors N3, N2, and N1 implies that excision of the TNFβ introns is sufficiently slow to allow their accumulation. Using dactinomycin, we could evaluate splicing rates in vivo of the different introns. Introns 1 and 2 are spliced out at least twice as rapidly as intron 3. Such differences in splicing rate have also been described for the thymidine kinase gene (20). The absolute values of these rates are to be considered cautiously, since the dactinomycin treatment might affect splicing. We have observed no clear variation of these splicing rates during the cell cycle, although such a regulation of RNA processing has been proposed for the thymidine kinase gene during the G_1/S transition of fibroblasts (20). More detailed investigations will require the uncoupling of gene transcription from the cell cycle. Our main results on the regulation of TNFβ expression can be summarized as follows: (i) IL-2 increases transcription of the TNFβ gene most markedly before 1 h, (ii) the C0 half-life decreases slightly between 1 and 8 h of stimulation, and (iii) C0 accumulation reaches a maximum only after 8 to 12 h of stimulation. Taken together, these results indicate that maturation is a limiting step in the accumulation of C0. This view is in agreement with the observed accumulation of N3, N2, and N1 during the stimulation (this study; 50). Therefore, although the primary level of TNFβ regulation by IL-2 is transcriptional, RNA processing is a key factor controlling the kinetics of TNFβ mRNA accumulation.

We proposed a scheme for the maturation of the TNFβ messengers whereby all of the nuclear transcripts derive from each other by splicing. It is the simplest model based on the structures of N3, N2, N1, and N0 and on the results of dactinomycin treatment. Demonstration of the precursor-product relationship between these different nuclear RNAs would require pulse-chase experiments, which have been performed only for highly expressed and stable RNAs (e.g., ovalbumin RNA; 49).

Among the TNFβ nuclear precursors, N1 has a special status since it can also be exported to the cytoplasm as a mature message despite the presence of intron 3. This can be considered the simplest model of alternative splicing (6). Such a situation is central to the life cycle of retroviruses (51) and of influenza virus (28) but is rarely encountered for

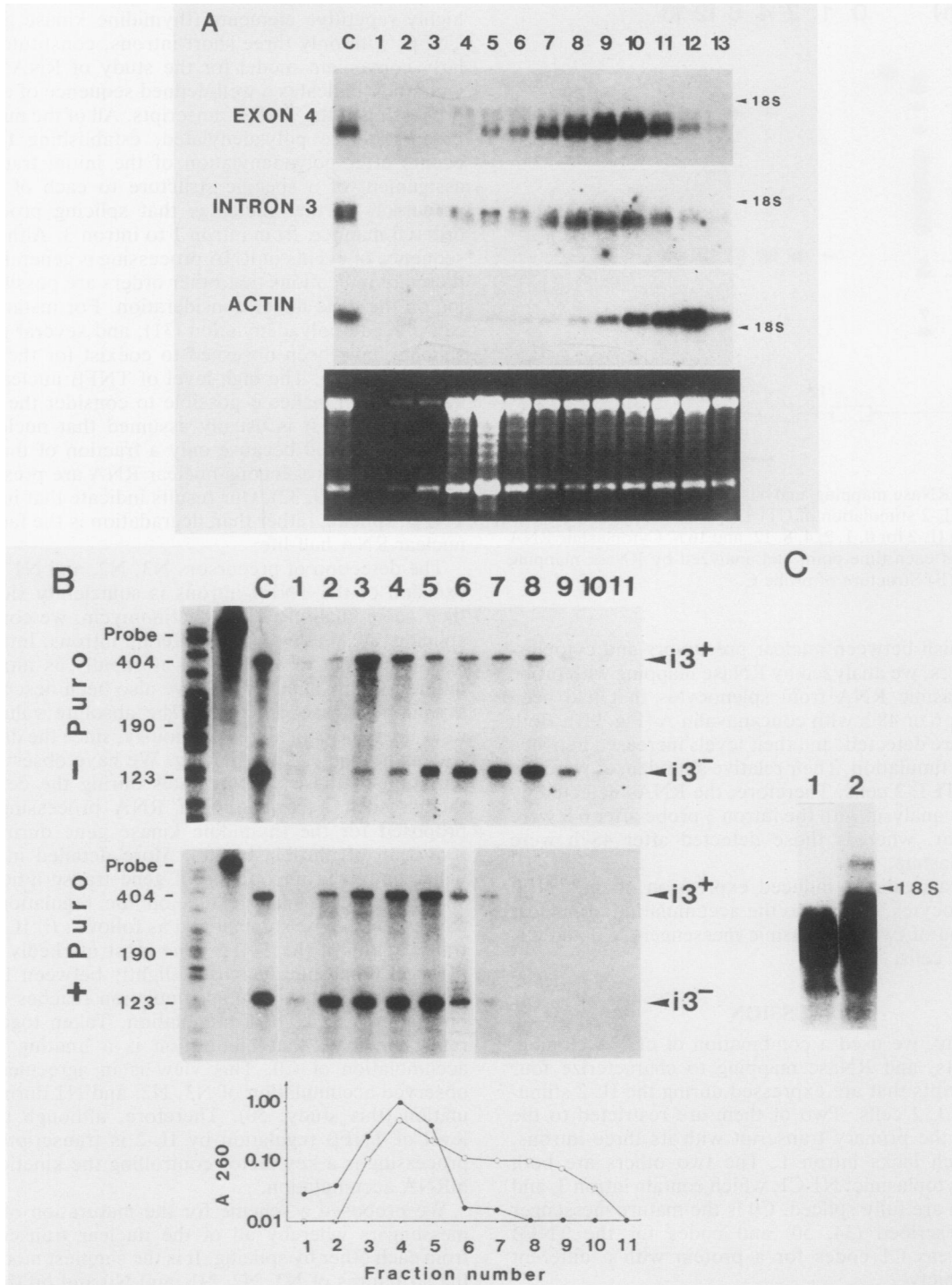

FIG. 7. Association of TNFβ messages with ribosomes. (A) Northern blot analysis of TNFβ messages in polyribosomes. CTLL-2 cells were stimulated with IL-2 for 8 h. After lysis with NP-40, the cytoplasmic supernatant was fractionated through a sucrose gradient before RNA extraction. A 4-μg sample of total RNA (lane C) or a constant amount of each fraction (lane 1 corresponding to the top and lane 13 corresponding to the bottom of the gradient) was hybridized with the TNFβ exon 4 or intron 3 probe. The upper filter was rehybridized with a β-actin probe. Ethidium bromide fluorescence of this filter before the hybridization is shown in the bottom panel. (B) RNase mapping analysis of TNFβ messages after polysome dissociation by puromycin. After lysis with NP-40, the cytoplasmic supernatant was incubated without (upper panel) or with (middle panel) puromycin (Puro). RNA was then fractionated through a sucrose gradient and extracted. A 10-μg sample of cytoplasmic RNA (lane C) or a constant amount of each fraction of the gradient was analyzed by RNase mapping with TNFβ probe C. The graph shows the A_{260} of the fractions collected after incubation without (○) or with (●) puromycin. (C) Northern blot analysis of the cytoplasmic supernatants. Samples (4 μg) of unfractionated cytoplasmic supernatants from the experiments shown on panels A (lane 1) and B (land 2) were analyzed by Northern blot with the TNFβ exon 4 probe.

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1966
.. GGC ATC CTG AAA CCT GCT GCT CAC CTT GTT G:GT AAA CTT
.. Gly Ile Leu Lys Pro Ala Ala His Leu Val G:ly Lys Leu

CTG CCT CCA GAG GAG AGG TCC AGT CCC TGC CTT TTG TCC TAC
Leu Pro Pro Glu Glu Arg Ser Ser Pro Cys Leu Leu Ser Tyr

TTG CCC AGG GGC TCA GGC GAT CTT CCC ATC TCC CCA CAC CAA
Leu Pro Arg Gly Ser Gly Asp Leu Pro Ile Ser Pro His Gln

CTT TTC TTA CCC CTA AGG GCA GGC ACC CCA CTC CCA TCT CCC
Leu Phe Leu Pro Leu Arg Ala Gly Thr Pro Leu Pro Ser Pro

TAC CAA CCA TCC CAC TTG TCC AGT GCC TGC TCC CTC AGG GAT
Tyr Gln Pro Ser His Leu Ser Ser Ala Cys Ser Leu Arg Asp

GGG GAC CTC TGA TCT TGA TAG CCC CCC AAT GTC TTG TGC CTC
Gly Asp Leu TER
                                                    2252
TTC CCA G:GG TAC CCC AGC AAG CAG AAC TCA CTG CTC TGG ..
          ly Tyr Pro Ser Lys Gln Asn Ser Leu Leu Trp ..
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FIG. 8. Sequence and predicted protein product of the TNFβ intron 3 region. Two independent clones containing intron 3 were sequenced by the dideoxy technique. Nucleotides are numbered according to the genomic sequence of Semon et al. (46); the intron 3 sequence is boxed. The predicted protein encoded by C1 is in bold type; that encoded by C0 is underlined.

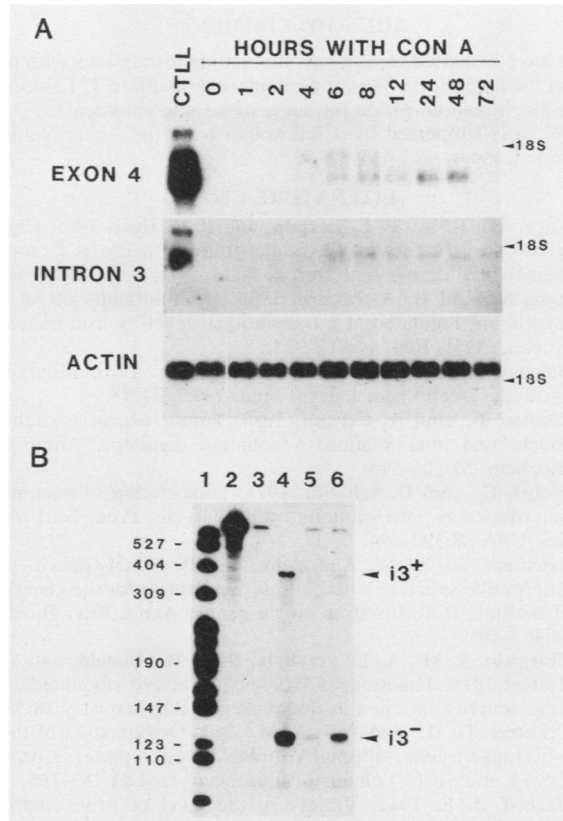

FIG. 9. TNFβ expression in concanavalin A-stimulated splenocytes. (A) Northern blot analysis of TNFβ expression during stimulation with concanavalin A (CON A). Murine splenocytes were stimulated for 0, 1, 2, 4, 6, 8, 12, 24, 48, and 72 h with concanavalin A. Then 4 μg of total RNA from 8-h IL-2-stimulated CTLL-2 cells (lane CTLL) or from concanavalin A-stimulated splenocytes (as indicated) was hybridized with the TNFβ exon 4 or intron 3 probe. The upper filter was rehybridized with a β-actin probe. (B) RNase mapping analysis of cytoplasmic TNFβ transcripts. Murine splenocytes were stimulated with concanavalin A for 6 and 48 h. Cytoplasmic RNA was extracted and analyzed with probe C. Lanes: 1, molecular weight markers (pBR322 digested with *Msp*I); 2, 7 pg of undigested probe; 3, cytoplasmic RNA from unstimulated CTLL-2 cells (5 μg); 4, cytoplasmic RNA from CTLL-2 cells stimulated for 8 h with IL-2 (5 μg); 5, cytoplasmic RNA from splenocytes stimulated for 6 h with concanavalin A (20 μg); 6, cytoplasmic RNA from splenocytes stimulated for 48 h with concanavalin A (20 μg).

cellular genes. Three cases have been well documented; the rat γ fibrinogen (8), the *Drosophila* P-element (29, 42), and the human L-*myc* (24, 26) genes. In these three cases, the spliced and partially spliced mRNAs are translated into proteins differing in physical characteristics (γ fibrinogen gene), function (P-element gene), or cellular localization (L-*myc*). Similarly, the alternative TNFβ protein (TNFβ*) shares only 66 amino acids with the product of the fully spliced message, suggesting that the two proteins could have different biological activities. It should be noted that TNFα interacts with its receptor as a trimer (47). If the same is true for TNFβ, then a relatively modest level of TNFβ* (15%) could modify the characteristics of about half of the trimers, leading to an amplification of the TNFβ* contribution.

The presence of an intron in a cytoplasmic mRNA is difficult to reconcile with models of RNA maturation involving two distinct pathways, splicing for precursors and exportation for intronless transcripts (30, 35). An underlying question is whether the export of mRNA to the cytoplasm is passive or under active control, as has been shown for tRNA (52) and 5S rRNA (21). The ability of glucocorticoids to regulate the expression of liver messengers by permitting their exportation to the cytoplasm (15) indeed suggests the existence of a cellular control of mRNA transport. This view is also supported by our observation that dactinomycin treatment partially blocks exportation to the cytoplasm of C0 and C1. A particularly interesting model system is provided by human immunodeficiency virus, wherein the virus-encoded protein Rev is necessary for transport in the cytoplasm of partially spliced viral env RNA (11, 13, 35). The existence of such a specific transport mechanism for a partially spliced RNA has been taken as an indication that the expression of cellular genes could also use similar mechanisms (18), and TNFβ provides us with a probe to investigate this question.

We have observed a similar pattern of TNFβ expression in concanavalin A-stimulated murine splenocytes. In particular, we observed, as in CTLL-2 cells, the accumulation of partially spliced transcripts and the presence of C0 and C1 in the cytoplasm. Although it is not possible to relate directly the IL-2 stimulation of CTLL-2 cells with the cascade of events occurring during concanavalin A stimulation of splenocytes, the patterns of TNFβ transcript maturation appear to be similar in cell lines and in ex vivo lymphocytes. A remaining question is whether this pattern of transcript maturation is cell type specific. Resolution of this question will require placing expression of the TNFβ gene under the control of a different promoter.

An increasing number of studies are suggesting that RNA processing could be an important component of gene regulation. However, the low level of nuclear precursors has hampered direct investigations of this hypothesis. The accumulation of the different TNFβ precursors, which results from limiting steps in TNFβ messenger maturation, opens the possibility of using this gene as a probe for factors controlling RNA processing.

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