A *cis*-acting element in the 3′-untranslated region of human TNF-α mRNA renders splicing dependent on the activation of protein kinase PKR

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We report a role for the 3′-untranslated region in control of mRNA splicing and show that human *TNF*-α 3′ UTR harbors a *cis*-acting element that renders splicing of precursor transcripts dependent on activation of PKR, the RNA-activated protein kinase that phosphorylates eukaryotic initiation factor 2 (eIF2). When this element, designated 2-APRE, is present, splicing becomes sensitive to inhibition by the PKR inhibitor, 2-aminopurine, or by coexpression of transdominant-negative mutant PKR. Our results reveal that activation of PKR is required for splicing of mRNA when precursor transcripts contain the 2-APRE and that increased expression of wild-type PKR enhances their splicing efficiency. Thus, PKR responds as *trans*-acting factor to the 2-APRE. 2-APRE RNA forms a stable, 17-bp stem–loop structure and strongly activates PKR in vitro, inducing eIF2α phosphorylation. Despite its ability to activate PKR during splicing, the 2-APRE within the 3′ UTR does not affect translation efficiency of the resulting *TNF*-α mRNA in transfected cells. PKR and the 3′ UTR thus interact during mRNA splicing to confer a novel type of regulation on expression of the *TNF*-α gene.

[*Key Words*: Splicing; tumor necrosis factor; *cis*-acting RNA element; 3′-untranslated region; protein kinase PKR]

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Cytokines are encoded by inducible genes, transiently expressed upon their activation. Expression of several cytokine genes is highly regulated at splicing of precursor transcripts (Jarrous and Kaempfer 1994; Gerez et al. 1995; Neel et al. 1995; Umlauf et al. 1995; Jarrous et al. 1996; Yang et al. 1998). Shortly after the onset of induction of human interleukin-2 (*IL-2*) and *IL-1*β genes, the flow of nuclear precursor transcripts into mature mRNA becomes blocked despite the fact that transcription, once activated by a stimulus, continues unabated for an extensive period. Expression of *IL-2* and *IL-1*β mRNA is superinduced by two orders of magnitude in the presence of translation inhibitors, without a significant increase in primary transcription or mRNA stability. Instead, splicing of precursor transcripts is greatly facilitated (Jarrous and Kaempfer 1994; Gerez et al. 1995).

Expression of the human tumor necrosis factor-α (*TNF-α*) gene is also regulated at splicing (Jarrous et al. 1996). 2-Aminopurine (2-AP) blocks expression of *TNF-α* mRNA in primary human lymphoid cells. An adenine isomer, 2-AP inhibits specific kinases that phosphorylate the α-subunit of eukaryotic translation initiation factor 2 (eIF2α)(Samuel 1993), including the RNA-activated protein kinase, PKR (Hu and Conway 1993). Although 2-AP inhibits transcription of the murine *TNF-α* gene (Han et al. 1991), it affects neither transcription nor stability of human *TNF-α* mRNA. Instead, splicing of short-lived *TNF-α* precursor transcripts into mRNA is blocked when 2-AP is present during induction, causing pre-mRNA to accumulate at the expense of mRNA. 2-AP blocks splicing of *TNF-α* precursor transcripts at multiple splice sites. Neither the human *IL-1*β nor *TNF-β* gene shows such regulation. A 2-AP-sensitive component, expressed in functional form before induction, regulates splicing of *TNF-α* mRNA (Jarrous et al. 1996).

PKR, a dsRNA-activated Ser/Thr protein kinase, is a major negative regulator of translation (Farrell et al. 1977; Thomis and Samuel 1993). PKR is expressed constitutively in most cells but is induced by viruses, dsRNA, and interferons (Tanaka and Samuel 1994). 2-AP prevents establishment of the antiviral state in interferon-treated cells (Wathelet et al. 1989). Activation of PKR requires its *trans*-autophosphorylation, which is facilitated by dsRNA (Thomis and Samuel 1993). PKR phosphorylates eIF2α, blocking GDP/GTP exchange

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(Samuel 1993) and preventing the recycling of eIF2 between rounds of initiation of translation (Hershey 1991). Thus, activation of PKR triggers an inhibition of protein synthesis. Dominant-negative mutants of *PKR* induce malignant transformation of mouse cells (Koromilas et al. 1992; Meurs et al. 1993; Donze et al. 1995), thought to result from enhanced translation of mRNAs encoded by growth-control genes that are normally repressed (Donze et al. 1995).

In addition to its role in translational control, PKR functions in signal transduction (Meurs et al. 1993) and activation of immediate early genes c-*myc*, c-*fos*, and JE (Mundschau and Faller 1995). The kinase is involved in transcriptional regulation through activation of NF-κB and IRF-1 (Kumar et al. 1997; Nagai et al. 1997) and activates transcription of *immunoglobulin* κ genes independently of NF-κB (Koromilas et al. 1995).

Activation of PKR depends critically on its binding to RNA (Wu and Kaufman 1997). PKR contains two tandem double-stranded RNA-binding motifs found in diverse proteins such as *Drosophila* Staufen, ribosomal protein S5, and *Escherichia coli* RNase III (Robertson and Mathews 1996). Perfectly matched dsRNA having the A conformation as well as certain other RNAs, including hepatitis delta agent RNA (Robertson et al. 1996), and human *α-tropomyosin* 3′ UTR (Davis and Watson 1996) activate PKR in vitro, whereas adenovirus *VA* RNA (Clarke and Mathews 1995) and *Alu* RNA (Chu et al. 1998) bind to this kinase and thereby inhibit its activation. Both activation of PKR and its inhibition require highly ordered RNA structures rather than specific sequences.

We show here that the human *TNF-α* 3′ UTR contains a *cis*-acting element that renders splicing of precursor transcripts dependent on the activation of PKR. Deletion of this sequence, termed 2-AP response element (2-APRE), or its replacement by *TNF-β* 3′ UTR sequences, frees splicing from a dependence on PKR activation, whereas insertion of the 2-APRE into the *TNF-β* 3′ UTR leads to acquisition of this control. Overexpression of wild-type or transdominant-negative mutant PKR shows that PKR responds as a *trans*-acting factor to the 2-APRE during splicing. Both the level of PKR and its activation regulate splicing of mRNA when precursor transcripts contain the 2-APRE. RNA encoded by the 2-APRE strongly activates PKR and induces eIF2α phosphorylation, supporting this functional link. 2-APRE RNA harbors a stable stem–loop structure with phylogenetically conserved features. These properties of the human *TNF-α* gene reveal a novel role for the 3′ UTR and for PKR in the regulation of splicing.

Results

2-AP inhibits splicing of TNF-α *precursor transcripts in transfected cells*

2-AP inhibits splicing of *TNF-α* precursor transcripts in human peripheral blood mononuclear cells (Jarrous et al. 1996). We examined this response to 2-AP in baby hamster kidney (BHK-21) cells using an exogenous *TNF-α* gene. BHK-21 cells were transfected with an intact human *TNF-α* gene (pTNF-α; Fig. 1C). Transient expression of *TNF-α* precursor transcripts (700-nucleotide band) and spliced mRNA (341-nucleotide band) was quantitated by RNase protection analysis, with an antisense RNA probe that covers part of intron 2, exon 3, intron 3, and part of exon 4 (Fig. 2A). Endogenous *TNF-α* mRNA is not expressed in the kidney cell line and was not detected by this probe, which was fully digested (Fig. 2F, lane 1) unless a human *TNF-α* gene was expressed. Precursor transcripts were abundant at 20 hr after transfection and then declined to low levels. Splicing of these transcripts resulted in the accumulation of mRNA, maximal by 24 hr. Addition of 10 mM 2-AP at 18 hr had a pronounced effect on this pattern of expression, causing a sustained accumulation of precursor transcripts (Fig. 2A,B). In contrast, expression of spliced *TNF-α* mRNA was inhibited (Fig. 2A,C). As a control, expression of a cotransfected *CAT* gene was not affected by 2-AP. The opposite responses of precursor transcripts and mRNA to 2-AP show that it inhibits splicing of *TNF-α* mRNA encoded by a transfected human gene (Jarrous et al. 1996). Sequences within this gene thus confer sensitivity to 2-AP.

A 2-AP-induced shift from spliced *TNF-α* mRNA into unspliced precursor transcripts is shown also in Figure 2D. Here, precursor transcripts increased >10-fold when

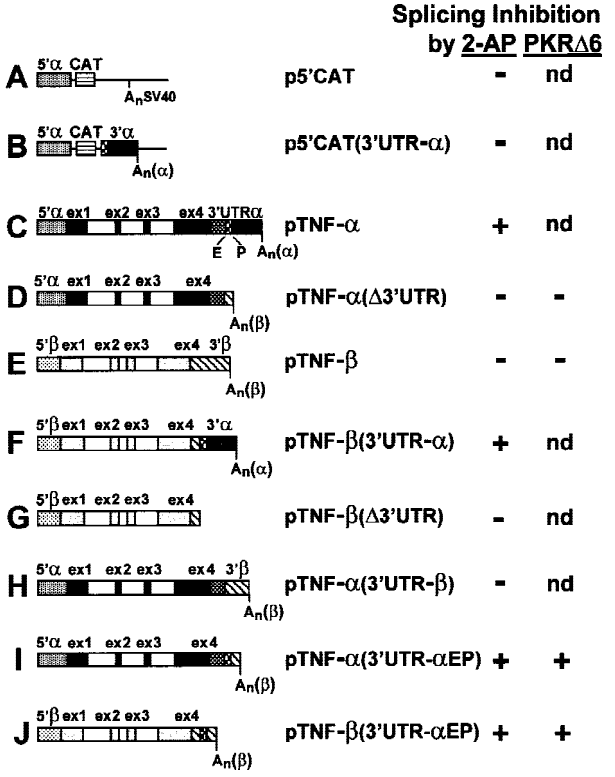

Figure 1. Gene constructs used in this study. (Open boxes) Introns; (ex) exon; (3′UTRα) *TNF-α* 3′-UTR; (A_n) polyadenylation and cleavage site of *TNF-α* gene (α), *TNF-β* gene (β), or SV40; (nd) not determined. 5′α, 3′α, and 3′β are defined in the text. (E) *Eco*RI site; (P) *Pst*I site.

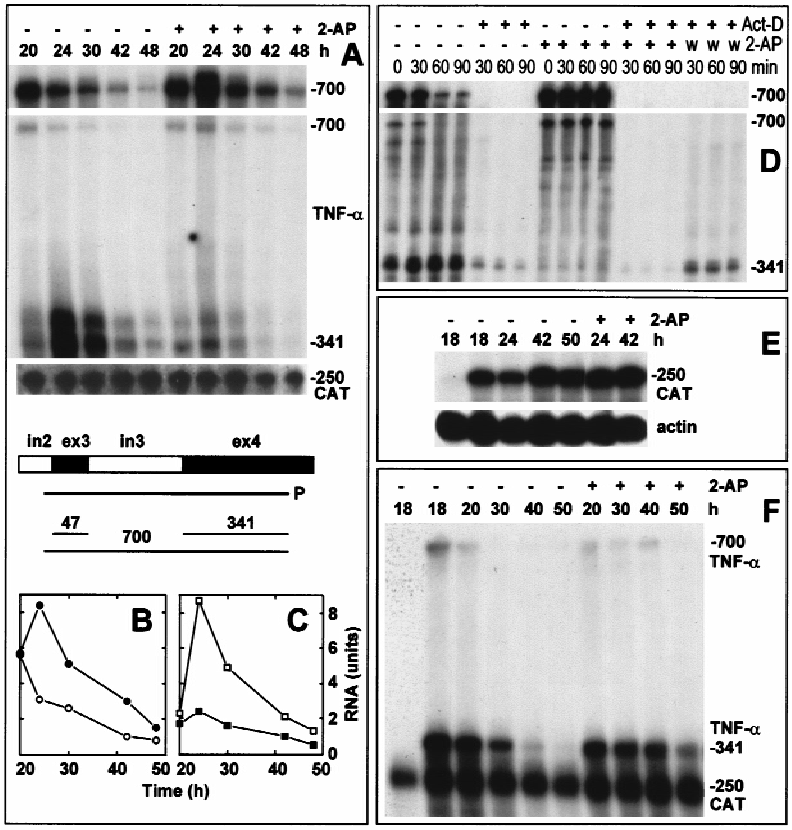

Figure 2. *TNF-α* 3′-UTR sequences are needed for inhibition of mRNA splicing by 2-AP. BHK-21 cells were transfected with pTNF-α (*A–D*), p5′αCAT (*E*) or pTNF-α(Δ3′UTR) DNA (*F*) and cotransfected with pSV_2CAT DNA (*A*,*F*). Where indicated, 2-AP was present at 10 mM from 18 hr after transfection. In *D*, 2-AP was removed 6 hr later by washing the cells (w), and 5 μg/ml of actinomycin D was added at that time as shown. Cell viability remained constant. Total RNA was isolated at times shown after transfection (*D*, after 24 hr), and subjected to RNase protection analysis with ^{32}P-labeled *TNF-α* antisense RNA probe P to quantitate precursor transcripts (700-nucleotide band) or spliced RNA (341-nucleotide band)(*A–D*, *F*). In *A* and *D*, the *top* autoradiograph shows a higher exposure of 700-nucleotide band. For autoradiograph (*A*), intensity of the 700- (*B*) and the 341-nucleotide band (*C*) in the absence (○, □) or presence (●, ■) of 2-AP is plotted; the *top* autoradiograph in *A* is quantitated in *B*. *CAT* mRNA protects 250 nucleotides of probe. α-*Actin* probe detects 215 nucleotides of mRNA (*E*). In *E*, the *left* lane shows untransfected cells; in *F*, cells transfected with pSV_2CAT DNA alone.

2-AP was present, whereas mRNA declined by ~8-fold. Unspliced *TNF-α* precursor transcripts were more unstable than mRNA whether or not 2-AP was present; yet, precursor transcripts that accumulated during 2-AP treatment could be chased effectively into mRNA by washing out 2-AP in the presence of actinomycin D, consistent with a splicing inhibition (Fig. 2D). The chase was complete within 30 min, showing that splicing of *TNF-α* mRNA occurs readily. This agrees with the observation that on induction of the *TNF-α* gene in lymphoid cells, levels of fully spliced mRNA become maximal within 3 hr (Jarrous et al. 1996). In the presence of 2-AP, precursor transcripts and mRNA were not more stable. Accumulation of precursors during 2-AP treatment thus results from continued transcription and inhibition of splicing.

3′-UTR sequences are required for inhibition of TNF-α *mRNA splicing by 2-AP*

To examine whether *TNF-α* gene promoter or 5′-UTR sequences respond to 2-AP, cells were transfected with p5′CAT, in which 821 bp from the *TNF-α* gene, comprising 652 bp preceding the transcription start site, the 5′ UTR and the first six codons of the ORF are abutted to the *CAT* gene (p5′CAT, Fig. 1A). Expression of this hybrid mRNA proceeded unabated in the presence of 10 mM 2-AP in the cell culture (Fig. 2E), a concentration that inhibited the expression of *TNF-α* mRNA from pTNF-α (Fig. 2A,D).

Splicing of *TNF-α* precursor transcripts bearing a truncated 3′ UTR, however, is no longer sensitive to 2-AP. In Figure 2F, BHK-21 cells were transfected with pTNF-α(Δ3′UTR) which lacks the 573 terminal base pairs of the 792-bp *TNF-α* 3′ UTR and ends with a polyadenylation signal of the *TNF-β* gene (Fig. 1D). Expression of precursor transcripts and mRNA transcribed from this truncated gene construct was abundant and as transient as for the complete gene (Fig. 2A), yet expression of mRNA was no longer inhibited by 2-AP, nor was there any enhanced accumulation of precursor transcripts. Removal of certain *TNF-α* 3′-UTR sequences thus resulted in loss of splicing inhibition by 2-AP.

On the other hand, in cells transfected with p5′CAT(3′UTR-α) (Fig. 1B), which carries the 3′-UTR sequences that were deleted in pTNF-α(Δ3′UTR), expression of the resulting hybrid *CAT* mRNA showed no sensitivity to 2-AP (data not shown). This result suggests that when fused to an intronless gene, such as the bacterial *CAT* gene, the *TNF-α* 3′ UTR does not mediate an inhibition of mRNA expression.

2-AP does not inhibit TNF-β *gene expression at splicing*

The above results prompted us to examine a gene that does not respond to 2-AP by inhibition of splicing. We showed previously that in lymphoid cells, increasing doses of 2-AP led to a coordinate decline in *TNF-β* precursor transcripts and spliced mRNA, supporting an in-

hibition at transcription rather than at splicing (Jarrous et al. 1996). Accordingly, we examined the response of the *TNF-β* gene to 2-AP in transfected cells. The entire human *TNF-β* gene (pTNF-β, Fig. 1E) was transfected into BHK-21 cells and expression of *TNF-β* RNA was monitored with an antisense RNA probe covering adjacent segments of intron 3 and exon 4 (Fig. 3A). Unspliced precursor transcripts (637-nucleotide protected fragment) were expressed transiently in large amounts, whereas *TNF-β* mRNA (571 nucleotides) was expressed at lower levels. This ratio attests to slow excision of intron 3 from human *TNF-β* precursor transcripts in BHK-21 cells. Inefficient excision of intron 3 was observed for the murine *TNF-β* gene in the lymphocytic cell line CTLL-2 (Weil et al. 1990). Addition of 2-AP did not elicit any increase in *TNF-β* precursor transcripts; instead, expression of both precursors and mRNA declined in this cell line.

Splicing of remaining exons 1–3 of the transfected *TNF-β* gene was followed next by an antisense RNA probe that overlaps *TNF-β* exons 1–3 and part of intron 3 (Fig. 3B). Primary transcripts protect 169 nucleotides whereas partially spliced ones protect fragments of 169, 324, and 275 nucleotides. A 214-nucleotide fragment became prominent by 24 hr. This fragment is protected by spliced RNA molecules in which exons 2, 3, and 4 are joined. These RNA molecules still retain intron 1 but have an intact ORF because translation starts within exon 2 (Nedwin et al. 1985). These RNA species were also detected in lymphoid cells (Jarrous et al. 1996). Fully spliced mRNA (which protects a 263-nucleotide fragment), although seen in lymphoid cells (Jarrous et al. 1996), was not detected in BHK-21 cells (Fig. 3B). Transfection of pTNF-β yielded transient expression of precursor transcripts whose decline was accompanied by the appearance of spliced RNA (214-nucleotide band). Again, formation of spliced *TNF-β* RNA was relatively slow and the yield was low. Addition of 2-AP led to a decrease in spliced RNA (lane 3 vs. 6) as well as in precursor transcripts (lane 2 vs. 5). This response, seen in Figure 3, A and B, stands in marked contrast to that of the *TNF-α* gene (Fig. 2), an indication that 2-AP failed to inhibit expression of a transfected *TNF-β* gene at mRNA splicing.

2-AP inhibits splicing of TNF-β *precursor transcripts carrying* TNF-α *3′ UTR sequences*

In Figure 3, C and D, transfection was with pTNF-β(3′UTR-α) (Fig. 1F), in which the *TNF-β* 3′ UTR is truncated 160 bp downstream from the stop codon and joined to a 823-bp *TNF-α* gene fragment comprising 573 terminal base pairs of the 3′ UTR, the polyadenylation site, and downstream sequences. Addition of 2-AP led to a shift from spliced RNA (214-nucleotide band) to precursor transcripts, which increased strongly (Fig. 3D, lanes 1–4 vs. 5–7). 2-AP failed to block transcription of the chimeric *TNF-β*(3′UTR-*α*) gene, inhibiting instead the excision of *TNF-β* introns 2 and 3 required for expression of spliced RNA. These findings are corroborated by use

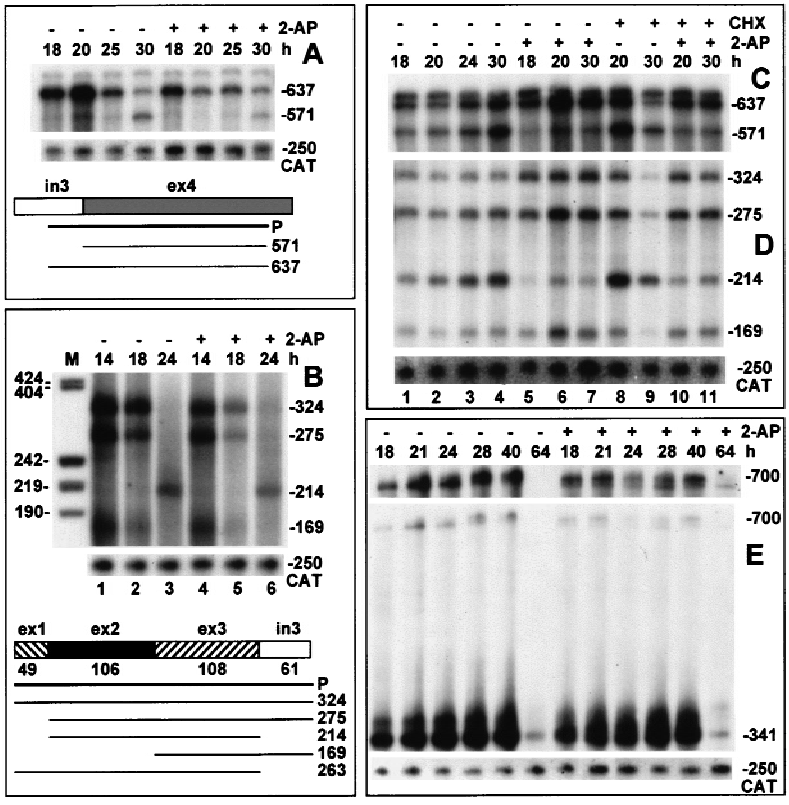

Figure 3. *TNF-α* 3′-UTR sequences suffice to confer splicing control by 2-AP. BHK-21 cells were transfected with pTNF-β (*A*,*B*), pTNF-β(3′UTR-α) (*C*,*D*) or pTNF-α(3′UTR-β) DNA (*E*), and cotransfected with pSV_2CAT DNA. 2-AP was present from 12 (*A*,*B*) or 16 hr after transfection (*C–E*), and 20 μg/ml CHX from 16 hr (*C*,*D*). Cell viability remained constant. Total RNA was isolated at times shown and subjected to RNase protection analysis with ^{32}P-labeled antisense *TNF-β* intron 3/exon 4 RNA probe P to quantitate precursor transcripts (637-nucleotide band) and spliced RNA (571-nucleotide band) (*A*,*C*) or *TNF-β* exon 1/exon 2/exon 3/intron 3 probe P to quantitate precursor transcripts (324-, 275-, and 169-nucleotide bands) and spliced RNA (214-nucleotide band) (*B*,*D*). (M) Size marker. In *E*, *TNF-α* precursor transcripts (700-nucleotide band) and spliced RNA (341-nucleotide band) were analyzed as for Fig. 2A; the *top* autoradiograph shows a higher exposure of the 700-nucleotide band. *CAT* mRNA protects 250 nucleotides of probe.

of the complementary *TNF-β* intron 3/exon 4 probe (Fig. 3C). Again, addition of 2-AP led to a strong increase in precursor transcripts and a concomitant decline in spliced RNA, resulting in a pronounced rise in their ratio (lanes 1–4 vs. 5–7). In contrast, in cells transfected with pTNF-β(Δ3′UTR) (Fig. 1G), lacking the *TNF-α* insert of pTNF-β(3′UTR-α), expression of spliced *TNF-α* RNA was insensitive to 2-AP (F. Osman and R. Kaempfer, unpubl.).

Addition of CHX led, in the pTNF-β(3′UTR-α)-transfected cells, to enhanced expression of both precursor transcripts and spliced RNA (Fig. 3, C and D, lane 2 vs. 8). Expression of spliced RNA was shifted to earlier times, followed by a decline in precursor transcripts and, more slowly, in spliced RNA (lanes 8–9). Here, too, generation of spliced RNA was inhibited by 2-AP (lane 8 vs. 10), whereas precursor transcripts increased (lane 9 vs. 11), the ratio of precursor transcripts to spliced mRNA increasing strongly. We conclude that 2-AP inhibits splicing of *TNF-β* precursor transcripts carrying a *TNF-α* 3′ UTR, and that this inhibitory effect does not require protein synthesis.

Although expression of *TNF-β* mRNA from both pTNF-β and pTNF-β(3′UTR-α) is sensitive to 2-AP, the response to 2-AP is shifted from an apparent inhibition of transcription for pTNF-β to inhibition of splicing for pTNF-β(3′UTR-α). Replacement of part of the *TNF-β* 3′ UTR with *TNF-α* 3′-UTR sequences renders splicing sensitive to 2-AP at multiple sites, shown in Figure 3, C and D, for joining of *TNF-β* exons 2, 3, and 4. The observation that 2-AP fails to inhibit expression of mRNA from p5′CAT(3′UTR-α), yet inhibits mRNA expression from pTNF-β(3′UTR-α) at splicing, emphasizes a requirement for exon/intron junctions in addition to *TNF-α* 3′-UTR sequences. However, there is no requirement for *TNF-α* exons or introns per se, because pTNF-β(3′UTR-α) contains only *TNF-β* exon/intron junctions. A *cis*-acting element that renders splicing sensitive to inhibition by 2-AP thus is located within the *TNF-α* 3′ UTR.

Splicing of TNF-α *precursor transcripts carrying* TNF-β *3′-UTR sequences is insensitive to 2-AP*

The results obtained above led us to predict that splicing of precursor transcripts encoded by the reciprocal chimeric gene, pTNF-α(3′UTR-β) (Fig. 1H), should proceed unabated in the presence of 2-AP. In pTNF-α(3′UTR-β), the *TNF-α* gene was truncated 219 bp into the 3′ UTR and joined to a 572-bp *TNF-β* gene fragment comprising the 3′-terminal 389 bp of the 628-bp 3′ UTR, the polyadenylation site and downstream sequences. Expression of spliced mRNA (341-nucleotide band) from pTNF-α(3′UTR-β) failed to decline when 2-AP was present, nor did precursor transcripts (700 nucleotides) increase (Fig. 3E). Splicing of *TNF-α* mRNA carrying *TNF-β* 3′-UTR sequences, thus, is insensitive to inhibition by 2-AP, again showing that a 2-APRE resides specifically in the *TNF-α* 3′ UTR.

Activation of PKR by RNA deriving from the TNF-α *3′ UTR*

Because 2-AP is a known inhibitor of PKR, we asked whether the *TNF-α* 3′ UTR harbors an RNA element able to activate this kinase. T7 RNA transcripts were generated from various fragments of *TNF-α* 3′-UTR DNA (Fig. 4A). To eliminate any contaminating dsRNA, these transcripts were purified by gel electrophoresis followed by CF-11 cellulose chromatography, a treatment shown previously to eliminate dsRNA contaminations (Circle et al. 1997). At low RNA concentration (0.05 ng/

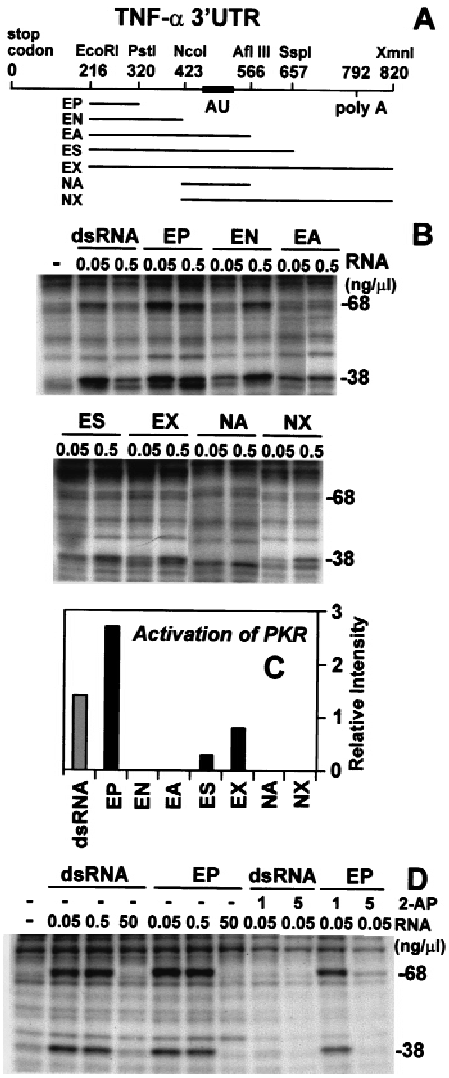

Figure 4. The *TNF-α* 3′ UTR harbors an RNA element that activates PKR. *TNF-α* 3′ UTR DNA constructs (*A*) were used to generate T7 RNA transcripts that at the concentrations shown were incubated with rabbit reticulocyte ribosomal fraction and [γ-^{32}P]ATP; dsRNA [poly(I–C)] served as control (*B*). An autoradiograph of the protein gel (*B*) was quantitated for each *TNF* RNA at 0.05 ng/μl, to yield molar-specific phosphorylation activity for PKR (*C*). In *D*, dsRNA or purified 3′UTR–αEP T7 transcript was incubated and analyzed as in *B*, with 2-AP present at the indicated concentrations (in mM).

μl), RNA derived from the 104-bp *Eco*RI–*Pst*I fragment (3′UTR–αEP) activated PKR even more strongly than dsRNA, as judged by phosphorylation of the PKR (68 kD) and eIF2α (38 kD) bands in the ribosome fraction of rabbit reticulocyte lysate (Fig. 4B,D). Assignment of these bands was verified by Western blot analysis with anti-PKR and anti-eIF2 antibodies (data not shown). Like dsRNA, 3′UTR–αEP RNA induced phosphorylation of PKR and eIF2α at low, but not high, RNA concentration (Fig. 4D). The *Nco*I (EN) transcript required a higher RNA concentration than 3′UTR–αEP for phosphorylation of the 68-kD band, whereas RNAs derived from still longer fragments or other regions of the *TNF-α* 3′ UTR did not induce extensive PKR phosphorylation (Fig. 4B). Apparently, the longer 3′-UTR fragments are less efficient than 3′UTR–αEP RNA in supporting PKR phosphorylation in vitro as a result of hindrance of the activating RNA domain. As an inducer of autophosphorylation of PKR, which is a primary indicator of the activation of this kinase, 3′UTR–αEP RNA stood out with the highest molar specific activity (Fig. 4C). Thus, the EP domain in the *TNF-α* 3′ UTR, located well upstream of the AU-rich element (Fig. 4A), encodes an RNA that, at low concentrations, strongly activates PKR in vitro.

Phosphorylation of PKR and eIF2α elicited by 3′UTR–αEP RNA transcripts in vitro was inhibited progressively by 1 and 5 mM of 2-AP (Fig. 4D). Activation of PKR by 3′UTR–αEP RNA was less sensitive to 1 mM 2-AP when compared with the activation by dsRNA, another indication that the former RNA is also a potent activator of PKR. These results show that 3′UTR–αEP RNA activates PKR in a 2-AP-sensitive manner.

Structure of 3′UTR–αEP RNA

The structure of 3′UTR–αEP RNA transcript was analyzed by T1, U2, and V1 RNase sensitivity mapping (Fig. 5). 3′UTR–αEP RNA forms a stable, 5′-proximal 48-nucleotide stem–loop containing 17 bp (ΔG = –59 kJ at 30°C). As calculated by RNADraw (http://rnadraw.base8.se/) and *mfold* algorithms (Zuker 1989), this stem–loop structure persists in the longer EP-containing RNA fragments shown in Figure 4A.

The 3′UTR-αEP domain is the 2-APRE of the TNF-α *gene*

To test the concept that the EP domain in the *TNF-α* 3′ UTR, able to activate PKR in vitro (Fig. 4), mediates the sensitivity of *TNF-α* mRNA splicing to 2-AP (Fig. 2A,D), we abutted it to the *TNF-α(*Δ3′UTR) and *TNF-β* genes, neither of which show sensitivity to 2-AP at mRNA splicing (Figs. 2 and 3). In the constructs thus generated, pTNF-α(3′UTR–αEP) (Fig. 1I) and pTNF-β(3′UTR–αEP) (Fig. 1J), the EP domain is followed by polyadenylation signal sequences of the *TNF-β* gene.

BHK-21 cells transfected with pTNF-α(3′UTR–αEP) showed transient expression of precursor transcripts and

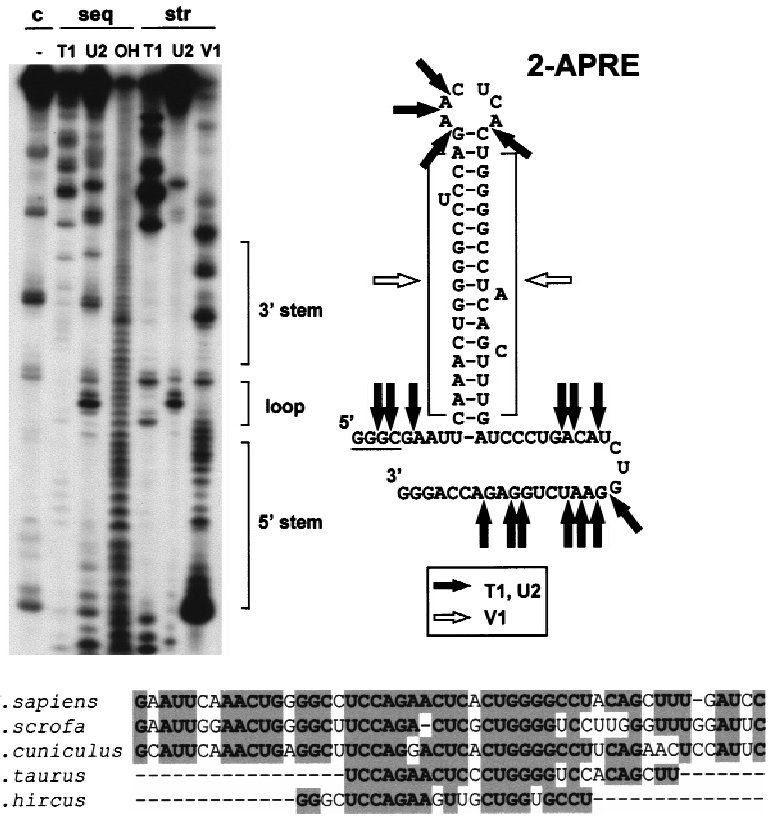

Figure 5. Nuclease sensitivity mapping of *TNF-α* 3′UTR–αEP RNA. 5′-End-labeled 3′UTR–αEP RNA was digested with T1, U2, or V1 nuclease directly to assay structure (str) [(c) without nuclease] and, for T1 and U2, also after denaturation at 50°C in 7 M urea (seq). The nucleotide ladder was generated by alkaline hydrolysis (OH). An autoradiograph of the sequencing gel is shown. Stem and loop regions relate to secondary structure at *right*, showing sites of nuclease attack, on the basis of multiple analyses. GGGC is from plasmid. Phylogenetic conservation of sequences is shown for *Homo sapiens* (human), *Sus scrofa* (wild boar), *Oryctolagus cuniculus* (rabbit), *Bos taurus* (bull), and *Capra hircus* (goat).

spliced mRNA (Fig. 6A, lanes 1–4). Addition of 2-AP at 20 hr elicited a progressive rise in precursor transcripts up to 36-fold at 53 hr, coupled with a decline in mRNA (lanes 5–8). Likewise, in cells transfected with pTNF-β(3′UTR–αEP), addition of 2-AP led to a pronounced shift from spliced mRNA to precursor transcripts, which increased seven-fold (Fig. 7A). In pTNF-α(3′UTR–αEP)-transfected cells, unspliced precursor transcripts accumulating in the presence of 2-AP could be chased into mRNA by washing out 2-AP in the presence of actinomycin D (Fig. 6C). Thus, introduction of the 3′UTR–αEP fragment suffices for a gain of function, rendering splicing of precursor transcripts sensitive to inhibition by 2-AP, whether these arise from *TNF-α* or *TNF–β* gene sequences. We conclude that the 3′UTR–αEP domain is a functional 2-APRE.

The function of the 2-APRE in mRNA splicing involves activation of PKR

On one hand, RNA transcribed from the 3′UTR–αEP domain is able to activate PKR in vitro; on the other hand, this domain renders mRNA splicing sensitive to inhibition by the PKR inhibitor, 2-AP. These results raised the possibility that PKR functions as a *trans*-acting factor that regulates mRNA splicing in precursor transcripts that carry the 2-APRE. To obtain direct evidence for this concept, we cotransfected cells with *TNF* gene constructs and a vector expressing either wild-type PKR or PKRΔ6, a transdominant-negative mutant that prevents the activation of wild-type PKR (Koromilas et al. 1992). If PKRΔ6 were to block the activation of endogenous PKR, it should cause inhibition of mRNA splicing in a manner resembling 2-AP. As seen in Figure 6A for pTNF-α(3′UTR–αEP) and in Figure 7B for pTNF-β(3′UTR–αEP), the effect of *PKRΔ6* cotransfection was strikingly similar to that of addition of 2-AP, shifting the pattern of RNA molecules from spliced to unspliced forms, with precursor transcripts increasing strongly. In Figure 6A, the effect was particularly pronounced at later times when PKRΔ6 expression should become more prominent (lanes 1–4 vs. 9–12), but even at the early times a reduction in spliced RNA was evident. This shift to unspliced RNA forms was not observed for the *TNF-α(Δ3′UTR)* gene (Fig. 6B) nor for the *TNF-β* gene (Fig. 7C), and thus requires the 2-APRE. For the *TNF-β* gene, total expression of RNA was reduced by *PKRΔ6*, reflecting the results with 2-AP (Fig. 3A,B).

Conversely, overexpression of wild-type PKR enhanced mRNA splicing in a 2-APRE-dependent manner (Fig. 6D). In this experiment, precursor transcripts were relatively more abundant than in Figure 6A. Cotransfection with *PKR* led to a shift from unspliced RNA into mRNA encoded by pTNF-α(3′UTR–αEP), the ratio of mRNA over precursor transcripts increasing up to 20-fold by 45 hr. Such enhancement was absent when pTNF-α(Δ3′UTR) was used instead.

Moreover, when *PKR* was cotransfected, the amount of mRNA expressed by pTNF-α(3′UTR–αEP) was significantly greater than that expressed by pTNF-α(Δ3′UTR), despite higher levels of unspliced precursor transcripts in cells transfected by the latter construct (Fig. 6D). This result shows that the ability to respond to PKR at the splicing step is mediated by the 2-APRE.

The 2-APRE renders mRNA splicing dependent on activation of PKR, which can be abrogated by 2-AP or expression of PKRΔ6 and enhanced by expression of wild-

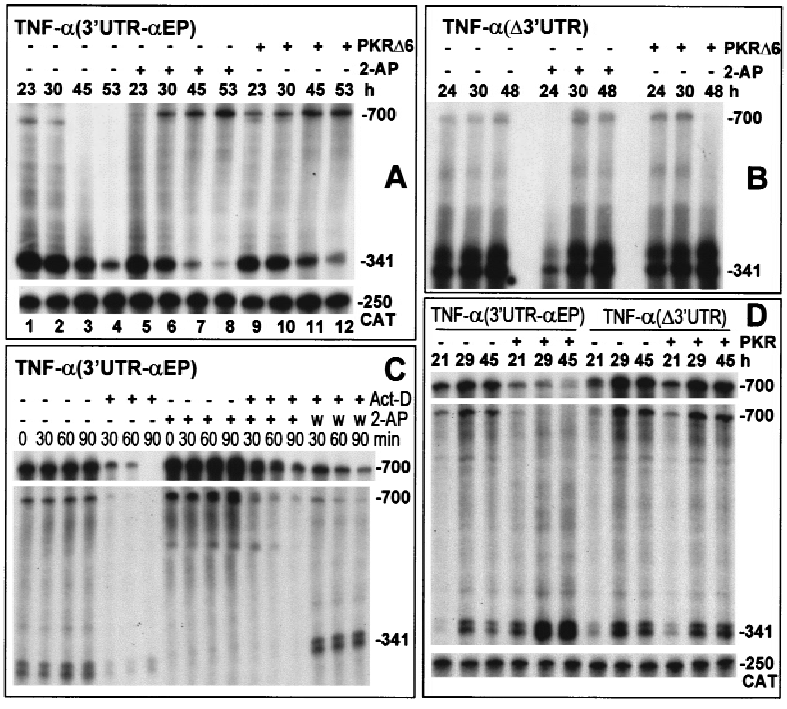

Figure 6. The 2-APRE renders splicing of *TNF-α* mRNA dependent on activation of PKR. BHK-21 cells were transfected with pTNF-α(3′UTR–αEP) or pTNF-α(Δ3′UTR) and where indicated, cotransfected with pPKRΔ6 or pPKR. pSV_2CAT DNA was cotransfected in each case. 2-AP was added at 18 (*C*) or 20 hr (*A*,*B*) after transfection, where shown. In *C*, 2-AP was removed (w) and actinomycin D added as for Fig. 2D. Total RNA was analyzed as for Fig. 2A to quantitate *TNF-α* precursor transcripts (700 nucleotides) and spliced RNA (341 nucleotides); in *C* and *D*, the *top* autoradiograph shows a higher exposure of the 700-nucleotide band. *CAT* mRNA protects 250 nucleotides of probe.

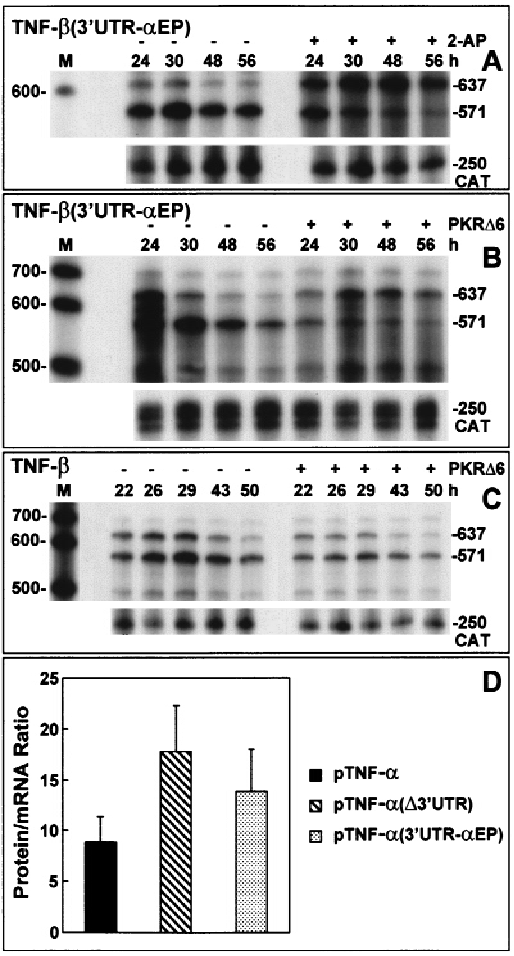

Figure 7. The *TNF-α* 2-APRE renders splicing of *TNF-β* mRNA dependent on activation of PKR and does not affect translation efficiency of *TNF-α* mRNA. BHK-21 cells were transfected with pTNF-β(3′UTR–αEP) (*A*,*B*) or pTNF-β (*C*) and, where indicated, cotransfected with pPKRΔ6. pSV_2CAT DNA was cotransfected in each case. In *A*, 2-AP was added at 20 hr after transfection. Total RNA was analyzed as for Fig. 3A to quantitate *TNF-β* precursor transcripts (637 nucleotides) and spliced RNA (571 nucleotides). (M) Size marker. *CAT* mRNA protects 250 nucleotides of probe. (*D*) BHK-21 cells were transfected with pTNF-α, pTNF-α(Δ3′UTR), or pTNF-α(3′UTR–αEP). pSV_2CAT DNA was cotransfected in each case. The ratios of TNF-α protein secreted into the medium and spliced *TNF-α* RNA normalized to *CAT* mRNA were determined. The bar graph shows mean and S.E. for four independent experiments.

type PKR. Our results reveal that activation of PKR is required for splicing of mRNA when precursor transcripts contain the 2-APRE, and that an increase in the level of PKR enhances their splicing efficiency.

The 2-APRE does not reduce translation efficiency in vivo

The exonic location of the 2-APRE raised the possibility that after enhancing splicing in dependence on PKR in the nucleus, this RNA element might act in the cytoplasm to reduce translation of the resulting mRNA through activation of PKR and eIF2α phosphorylation, as shown for the reticulocyte ribosome fraction in Figure 4. To study this point, we transfected BHK-21 cells with pTNF-α, pTNF-α(Δ3′UTR) and pTNF-α(3′UTR–αEP) DNA. To monitor translation efficiency, transient expression of *TNF-α* mRNA was analyzed by RNase protection analysis and normalized to the expression of *CAT* mRNA transcribed from cotransfected pSV_2CAT DNA, whereas secreted TNF-α protein was quantitated by immunoassay. In four independent experiments, the translation efficiency of the *TNF-α* mRNA forms encoded by the three gene constructs was similar, with mean values differing by no more than twofold (Fig. 7D). In particular, translation efficiency of 2-APRE-containing *TNF-α* mRNA expressed from pTNF-α(3′UTR–αEP) did not deviate significantly from that exhibited by pTNF-α(Δ3′UTR)-encoded mRNA, which differs only in that it lacks the 2-APRE (Fig. 1, cf. D and I). Hence, despite its ability to activate PKR during nuclear splicing, the 2-APRE in the 3′ UTR does not noticeably affect the translation efficiency of the resulting *TNF-α* mRNA in the cytoplasm.

Discussion

A TNF-α *3′-UTR element controls mRNA splicing through the PKR kinase*

We have demonstrated a novel role for the 3′ UTR, in the control of mRNA splicing. A regulatory sequence, 2-APRE, within the human *TNF-α* 3′ UTR is a *cis*-acting element that renders splicing of precursor transcripts dependent on activation of the RNA-activated eIF2α kinase PKR. It is shown that the 2-APRE is a *cis*-acting element by our finding that addition of the kinase inhibitor 2-AP, or cotransfection with transdominant-negative mutant *PKR*, leads to a severe inhibition of splicing of *TNF-α* mRNA but not of *TNF-β* mRNA. Deletion of the 2-APRE, moreover, or its replacement by *TNF-β* 3′ UTR sequences, frees splicing of *TNF-α* precursor transcripts from a dependence on PKR activation, whereas insertion of the 2-APRE into the *TNF-β* 3′ UTR leads to gain of this control. Precursor transcripts encoded by the human *TNF-α* gene harbor the 2-APRE, and, hence, their splicing is dependent on activation of PKR.

Activation of PKR is required for splicing of mRNA when precursor transcripts contain the 2-APRE. Overexpression of PKR leads to a more efficient splicing of *TNF-α* precursor transcripts, provided that they carry the 2-APRE. Thus, PKR responds as *trans*-acting factor to the 2-APRE. We have shown that RNA encoded by the 2-APRE strongly activates PKR in vitro, supporting this functional link.

Our finding that the 2-APRE within the *TNF-α* 3′ UTR is able to activate PKR raised the possibility that subsequent to splicing, such activation could have a negative effect on translation of the resulting mRNA in the cytoplasm, thereby offsetting the stimulatory effect on splicing. However, we have shown that this is not the case,

because relative production of TNF-α protein in transfected cells was similar whether or not the 2-APRE was present. Possibly, binding of cytoplasmic proteins to *TNF-α* mRNA during translation acts, directly or indirectly, to mask the ability of its 2-APRE to activate PKR. Although the downstream AU-rich sequence in the *TNF-α* 3′ UTR inhibits translation when located in *CAT/TNF-α* fusion constructs (Han et al. 1990), there was little, if any, difference in translation efficiency of *TNF-α* mRNA, whether or not it contained the sequences downstream from the 2-APRE.

PKR and the 3′ UTR thus interact during mRNA splicing to confer a novel type of regulation on expression of the *TNF-α* gene.

Physiological role of PKR in splicing

Our results indicate that mRNA splicing follows a novel path for genes containing a 2-APRE, which are thereby placed under the control of PKR, exemplified here by the human *TNF-α* gene, as well as the *TNF* genes in pTNF-α(3′UTR–αEP) and pTNF-β(3′UTR–αEP), as distinct from the canonical path used by genes that lack a 2-APRE, including the human *TNF–β* gene and the mutant *TNF-α* gene in pTNF-α(Δ3′UTR) and pTNF-α(3′UTR-β).

The presence of the 2-APRE not only renders splicing of precursor transcripts dependent on PKR but also allows it to proceed with higher efficiency in response to increased expression of PKR. Thus, even though transfected BHK-21 cells express constitutive levels of PKR that suffice for splicing of 2-APRE-containing mRNA, such levels limit the extent of splicing. For a gene containing a 2-APRE, modulation of splicing efficiency by PKR may thus be expected in conditions in which PKR is induced, as during an inflammatory response.

Our findings lead to the prediction that physiological signals that cause activation of PKR should promote splicing of *TNF-α* precursor transcripts. Conversely, signals causing an inhibition of PKR should act to reduce their splicing. In this context, T-cell activation by anti-CD3 antibodies triggers splicing of pre-existing *TNF-α* precursor transcripts, yielding an immediate production of TNF-α protein (Yang et al. 1998).

TNF-α exhibits antiviral activity (Wong and Goeddel 1986) and induces *PKR* gene expression (Yeung et al. 1996). PKR has a direct role in TNF-α-induced apoptosis (Yeung et al. 1996; Der et al. 1997) through phosphorylation of eIF2α and inhibition of translation (Srivastava et al. 1998). Induction of PKR by TNF-α or other signals may thus create a positive feedback loop for *TNF-α* mRNA expression.

The nature of the splicing control element

Analysis of a 104-nucleotide RNA transcribed from the human *TNF-α* 2-APRE by nuclease sensitivity mapping reveals a stable stem–loop containing 17 bp (including 10 G:C pairs). This structure is conserved in human, porcine, rabbit, bovine, and goat *TNF-α* genes. Phylogenetic conservation is most pronounced in the upper stem and 6-nucleotide loop, which thus are likely to be involved in PKR-mediated regulation of splicing. This folding is preserved in longer, 2-APRE-containing human *TNF-α* 3′-UTR RNA fragments. Moreover, the location of the 2-APRE within the human *TNF-α* 3′ UTR, halfway between the stop codon and the AU-rich motif and ~200 nucleotides upstream of the latter, is conserved in the porcine, rabbit, and bovine genes.

We did not find significant sequence homology of the 2-APRE with adenovirus *VA* RNA, which binds to and inhibits PKR (Clarke and Mathews 1995), nor with *HIV-1* TAR (Maitra et al. 1994) or human *α-tropomyosin* 3′ UTR (Davis and Watson 1996), which reportedly activate PKR.

Moreover, no similar structure is found in the human *TNF-β* 3′ UTR. We have shown that splicing of human *TNF-β* precursor transcripts is inherently sluggish, possibly rendering additional regulation at this step less essential. *TNF-α* and *TNF-β* genes differ also in their transcriptional control and mRNA stability (English et al. 1991). Expression of *TNF-α* mRNA in PBMC occurs earlier and to higher levels but also is more transient than that of *TNF-β* mRNA. Tighter control of *TNF-α* gene expression, through a dependence of mRNA splicing on activation of PKR, may also reflect the wider range of biological activities of TNF-α.

Splicing of *TNF-β* precursor transcripts carrying an inverted *TNF-α* 3′-UTR sequence is as sensitive to inhibition by 2-AP as splicing of *TNF-β*(3′UTR-*α*) transcripts (F. Osman, N. Jarrous, and R. Kaempfer, unpubl.), supporting the concept that structure, rather than sequence, of the 2-APRE is important for its function in splicing control.

Function of the 2-APRE

Although the 2-APRE requires the presence of introns to regulate splicing, there is no requirement for *TNF-α* introns per se. An inhibition of splicing at multiple sites, mediated by the 2-APRE, is also observed when all splice junctions and introns are of *TNF-β* origin.

Our finding that splicing of human *TNF-α* precursor transcripts is regulated by a 3′-UTR element implies that this process takes place only once transcription has progressed well into the last exon, beyond the 2-APRE. Although pre-mRNA splicing is mainly a cotranscriptional event, in which factors supporting transcription, splicing, and 3′-end processing of precursor transcripts interact as functional complexes and colocalize within the nucleus (Neugebauer and Roth 1997), splicing may occur either during transcription or post-transcriptionally, depending on the position of the intron (Bauren and Wieslander 1994; Misteli et al. 1997). The small size of the *TNF* introns and genes may in this case favor a predominantly post-transcriptional splicing mechanism. In resting lymphoid cells, accumulation of unspliced *TNF-α* precursor transcripts has been observed, which

are processed into mRNA only on stimulation (Yang et al. 1998).

When 2-AP was present, precursor transcripts accumulated at the expense of mRNA. This accumulation was pronounced, reaching >10-fold in Figure 2D and >30-fold in Figure 6A. A marked increase in precursor transcripts, coupled with a decline in spliced mRNA, was seen also when transdominant-negative mutant PKRΔ6 was expressed (Figs. 6A and 7B). These reciprocal changes in levels of precursor transcripts and mRNA are indicative of a splicing block and could not be explained by effects on transcription. Precursor transcripts are either processed or degraded and, hence, are unstable intermediates that do not accumulate to the levels of mRNA. The possibility that the 2-APRE would render precursor transcripts more stable in the presence of 2-AP (or of PKRΔ6), yet mRNA less stable, is not supported by our RNA chase experiments. Accumulated precursor transcripts remained unstable in the presence of 2-AP, yet they could be chased into spliced mRNA once 2-AP was removed. In the chase experiments, a pronounced conversion of precursor transcripts into spliced mRNA was observed on the removal of 2-AP (Figs. 2D and 6C). Moreover, *TNF-α* precursor transcripts that accumulated in the nucleus in the presence of 2-AP were not transported to the cytoplasm (Jarrous et al. 1996). Located well upstream of the AU-rich motif, the 2-APRE is in a region not known to be involved in destabilization of *TNF-α* mRNA. Deletion of 3′-UTR sequences, including the 2-APRE as well as the AU-rich motif, did not alter the kinetics of mRNA expression when 2-AP was absent (Fig. 2A,F). In lymphoid cells, 2-AP inhibited splicing of the endogenous *TNF-α* mRNA but did not affect its stability (Jarrous et al. 1996). Thus, the primary action of 2-AP is to block splicing.

A 3′-proximal structure in yeast pre-rRNA initiates processing of upstream rRNA species, with the stem essential for function (Allmang and Tollervey 1998). Removal of transcribed spacers from rRNA and excision of introns from precursor transcripts may be similarly controlled through remote elements. The 2-APRE may represent the first example of a class of ordered *cis*-acting RNA elements involved in mRNA-splicing control.

Involvement of PKR in splicing

Our results show a novel function for the RNA-activated eIF2α kinase, PKR, in the regulation of splicing. Precursor transcripts containing the 2-APRE are not spliced when the function of PKR is inhibited by 2-AP, which blocks the ATP-binding site in the protein, or by expression of transdominant-negative mutant PKR, which blocks *trans*-autophosphorylation of the enzyme obligatory for its activation (Koromilas et al. 1992). The finding that PKR is a *trans*-acting factor required for splicing of *TNF-α* precursor transcripts but not of precursor transcripts encoded by *TNF-β* (Fig. 3), *IL-1α* or *IL-1β* genes (Jarrous et al. 1996) reveals a selective mode of action for this kinase, apparently elicited through local activation by a structure within the 2-APRE. Activation of PKR requires its dimerization on RNA (Bevilacqua et al. 1996; Wu and Kaufman 1997), for which expression of 2-APRE-containing RNA transcripts suffices.

The 2-APRE-containing a 104-nuclotide EP transcript strongly activates PKR in vitro and induces eIF2α phosphorylation more effectively than longer 3′-UTR RNA fragments. Possibly, the latter may bind proteins that reduce their ability to activate PKR. However, it is clear from our transfection experiments that when located in *TNF-α* precursor transcripts, the 2-APRE renders splicing dependent on PKR activation.

Activation and inhibition of PKR by RNA are dependent on length and position of double-helical regions having the A conformation, rather than on their specific sequences. Certain structured RNAs activate or inhibit PKR through imperfectly matched base-paired domains, such as human delta hepatitis agent RNA or *VA* RNA (Robertson and Mathews 1996). The RNA-binding domain in PKR (St-Johnston et al. 1992) requires 11–13 bp of dsRNA for binding (Schmedt et al. 1995; Bevilacqua and Cech 1996; Circle et al. 1997) and can tolerate non-Watson–Crick structures (Bevilacqua et al. 1998). Noncontiguous short helices of RNA can cooperate in binding of PKR and thereby, in its activation (Bevilacqua et al. 1998), properties that characterize the 2-APRE as well. Although the 2-APRE duplex is discontinuous, it complies with conformational constraints typical of highly structured RNAs. Remarkably, EP RNA is a more potent activator of PKR than dsRNA, and unlike human delta hepatitis agent RNA (Robertson et al. 1996), it also induces eIF2α phosphorylation. Consistent with this property, when compared with dsRNA, 2-APRE-mediated activation of PKR and phosphorylation of eIF2α is less sensitive to inhibition by 2-AP.

PKR was detected in the nucleoplasm in an underphosphorylated state (Jeffrey et al. 1995). On induction by interferon, aggregates of PKR are colocalized with interchromatin granule clusters (Besse et al. 1998) known to contain significant amounts of spliceosomal components and to be involved in spliceosome assembly, sorting, and recycling; modified splicing factors are recruited from these clusters into perichromatin fibrils in which gene transcription occurs, facilitating cotranscriptional RNA processing (Misteli et al. 1997).

Our results provide a nuclear role for PKR in splicing of 2-APRE-containing precursor transcripts. Because splicing is regulated by the 2-APRE at multiple splice junctions, and because scission at several 5′ splice sites within the *TNF-α* and *TNF-β* genes fails to occur when activation of PKR is inhibited, we suggest that PKR may act at an early step in splicing, the nature of which will require further study. The involvement of PKR in splicing and the role of the 2-APRE therein are distinct from mechanisms that affect specific splice sites, for example, in alternative mRNA splicing.

Constitutive and alternative splicing is controlled by reversible protein phosphorylation in which activity of kinases and phosphatases is coordinated (Misteli et al. 1997). SR protein kinases and clk/Sty (Gui et al. 1994; Colwill et al. 1996) phosphorylate splicing factors con-

taining an RS domain. SR protein kinases, however, regulate splicing generally rather than selectively as shown here for the human TNF-α gene. ASF/SF2 and other members of the SR protein family are redistributed in the nucleus on their phosphorylation; they regulate alternative splicing for a spectrum of precursor transcripts, usually by promoting the use of 5′-proximal splice sites (for review, see Caceres et al. 1998). Adenovirus protein E4-ORF4 effects dephosphorylation of HeLa cell SR proteins by activating protein phosphatase 2A and thus regulates its alternative pre-mRNA splicing (Kanopka et al. 1998). The broad range of splicing events regulated by the SR kinases differs from the more restricted splicing control described here, which depends on PKR and on an RNA element. Splicing of *IL-1β* (Jarrous et al. 1996) or *TNF-α* transcripts lacking the 2-APRE is insensitive to 2-AP, indicating that 2-AP does not inhibit SR kinases or RNA helicases required for constitutive splicing.

Several models may be considered to account for the observed 2-APRE-mediated dependence of splicing on the activation of PKR, whether this element is located within *TNF-α* or *TNF-β* precursor transcripts, and the finding that on removal of the 2-APRE from the *TNF-α* gene, splicing proceeds without a need for PKR activation (Figs. 2F and 3E) and is no longer stimulated by overexpression of PKR (Fig. 6D). For example, activation of PKR by the 2-APRE may result in local phosphorylation of a substrate that in its unphosphorylated state inhibits splicing by binding to the 2-APRE yet becomes a positive regulator of splicing on phosphorylation, acting not only to allow splicing but also to render it more efficient. This substrate may recognize the conserved loop nucleotides in the 2-APRE structure, presented by the conserved upper stem (Fig. 5). Another possibility is that the 2-APRE induces in the precursor transcript a conformation that prevents splicing unless the element is bound by a splicing regulatory factor that serves as a substrate for phosphorylation by PKR.

Materials and methods

DNA constructs for transfection

p5′CAT was constructed by subcloning a filled-in 821-bp *Eco*RI–*Sau*3AI fragment containing *TNF-α* upstream regulatory sequences, the 151-bp 5′ UTR and the first six codons, into the filled in *Hin*dIII site of pSV_0CAT which lacks the SV40 promoter/enhancer (Gorman et al. 1982). p5′CAT(3′-UTR-α) was constructed by subcloning a 823-bp filled-in *Eco*RI–*Eco*RI *TNF-α* gene fragment comprised of 573 3′-terminal base pairs of the 3′ UTR, the polyadenylation site and downstream sequences, into the *Hpa*I site downstream of the CAT coding region in p5′CAT. pTNF-α, provided by Dr. Arjun Singh (Genentech, San Francisco, CA), contains the entire human *TNF-α* gene, including upstream regulatory sequences (Goldfeld et al. 1990), in pUC13pML. pTNF-α(Δ3′UTR), a construct containing the entire *TNF-α* gene but lacking 573 3′-terminal base pairs of the 3′ UTR, was made by digesting pTNF-α with *Eco*RI and subcloning the 2796-bp 5′-terminal *TNF-α* gene fragment into pBS (Stratagene); a 333-bp *Sph*I–*Sph*I *TNF-β* gene fragment containing 153 terminal base pairs of the 3′ UTR, the polyadenylation site, and downstream sequences was then inserted into the downstream *Sph*I site. pTNF-β, a construct containing the entire human *TNF-β* gene, including 572 bp upstream of the transcription start site and 250 bp downstream from the 3′ UTR, was constructed by digesting cosmid vector 019A containing 35 kb of the *MHC class-II* locus, provided by Dr. Thomas Spies (Harvard University, Cambridge, MA), with *Bam*HI and *Sac*I and subcloning the resulting 2858-bp *TNF-β* genomic fragment into pBS. In pTNF-β(3′UTR-α), the *TNF-β* gene is truncated 160 bp into the 3′ UTR and abutted to a 823-bp *Eco*RI–*Eco*RI *TNF-α* gene fragment comprised of 573 3′-terminal base pairs of the 3′ UTR, the polyadenylation site and downstream sequences; the construct was generated by digesting cosmid vector 019A with *Eco*RI and joining, in pBS, the resulting 2384-bp *TNF-β* gene fragment to the first fragment. In the reciprocal construct, pTNF-α(3′UTR-β), the *TNF-α* gene in pTNF-α was truncated 219 bp into the 3′ UTR by digestion with *Eco*RI and joined to a 572-bp *Eco*RI–*Pst*I *TNF-β* gene fragment comprised of 389 3′-terminal base pairs of the 3′ UTR, the polyadenylation site and downstream sequences.

pTNF-α(3′UTR-αEP) was constructed in pTNF-α by joining a 2894-bp *Sma*I–*Pst*I *TNF-α* gene fragment to a 333-bp *Sph*I–*Sph*I *TNF-β* gene fragment containing 153 3′-terminal base pairs of the 3′ UTR, the polyadenylation site and downstream sequences. To this end, a 3231-bp *Sma*I–*Ssp*I *TNF-α* gene fragment was first inserted into the *Sma*I site of pBS and the 333-bp *Sph*I–*Sph*I *TNF-β* gene fragment inserted into the downstream *Sph*I site. *Pst*I digestion and self-ligation then joined the *TNF-α* gene, truncated at the *Pst*I site in the 3′ UTR, to this *TNF-β* gene fragment, with an intervening 6-bp *Pst*I–*Sph*I sequence from pBS. pTNF-β(3′UTR–αEP) was constructed from pTNF-α(3′UTR–αEP) by digestion with *Eco*RI to remove the *TNF-α* gene up to the *Eco*RI site in the 3′-UTR and its replacement with a 2139-bp *Eco*RI–*Eco*RI *TNF-β* gene fragment from pTNF-β.

pPKR contains the human *PKR* gene and pPKRΔ6, provided by Dr. Nahum Sonenberg (McGill University, Montreal, Canada), the *PKR* gene having a deletion of amino acids 361–366, under the CMV promoter; expression of these proteins was verified by Western blotting with anti-PKR antibody (Santa Cruz).

Cell transfection

Monolayers of BHK-21 cells were seeded at a density of 1×10^6 to 2×10^6 cells/8-cm Petri dish and grown overnight; 4 hr before cotransfection, culture medium was replaced. A mixture of 10 μg of test DNA construct, 4 μg of salmon sperm DNA, and 2 μg pSV_2CAT DNA was permeated into cells by calcium phosphate–DNA coprecipitation (Jarrous et al. 1996). After 12–18 hr, culture medium was replaced with fresh medium prewarmed to 37°C. For Figures 2E, 6, and 7, LipofectAmine (Life Technologies) was used with 1 μg of each DNA for transfection. Efficiency of transfection was >72%, as judged by fluorescence-activated sorting of cells transfected with pCMV–EGFP expressing green fluorescent protein. 2-AP (Sigma) was added at 10 mM unless otherwise indicated.

Hybridization probes

For RNase protection analysis, DNA was subcloned in pBS (Stratagene) under the T3 or T7 promoter and transcribed with [α-^{32}P]UTP to generate labeled antisense RNA transcripts. A *TNF-α* RNA probe (map, Fig. 2A) was generated from a 700-bp *Sau*3AI–*Sau*3AI fragment consisting of 10 bp of intron 2, exon 3, intron 3, and part of exon 4. A *TNF-β* probe was generated from

a 637-bp *Bsu*36I–*Eco*RI DNA fragment, containing part of intron 3 and 571 bp of exon 4 (map, Fig. 3A). A second *TNF-β* RNA probe (map, Fig. 3B) was generated from a 324-bp *Eco*RI–*Bsu*36I DNA fragment, containing part of exon 1, exon 2, exon 3, and a portion of intron 3, derived from pSV–LT, provided by Dr. Rao Movva (Biogen, Geneva, Switzerland). A probe of 250 nucleotides, generated from a *Hin*dIII–*Eco*RI fragment of the coding region of the *CAT* gene (Gorman et al. 1982), was used to quantitate *CAT* mRNA. Intactness of RNA probes was assessed by PAGE (Jarrous et al. 1996).

Ribonuclease protection analysis

Total RNA was isolated with guanidinium isothiocyanate/CsCl. RNase protection analysis with RNases A and T1 was performed with genomic *TNF-α* and *TNF-β* riboprobes and *CAT* or *actin* probes as described (Jarrous et al. 1996). Protected RNA fragments were separated by electrophoresis in 5% polyacrylamide/8 M urea gels. The size marker in Figure 3 was generated by *Msp*I digestion of pGEM-3 DNA and filling in with [α-^{32}P]dCTP (Jarrous and Kaempfer 1994); the size marker in Figure 7 is a 100-bp DNA ladder (Fermentas) that was dephosphorylated and 5′ end labeled with [γ-^{32}P]ATP. These markers were also used to determine fragment sizes in Figures 2, 3, and 6. Autoradiographs were scanned and band intensity was quantitated using NIH Image 1.61 software.

Activation of PKR by 3′-UTR transcripts

An 823-bp *Eco*RI–*Eco*RI *TNF-α* gene fragment comprised of 573 3′-terminal base pairs of the 3′-UTR, the polyadenylation site, and downstream sequences from pTNF-α was inserted into pBS under the T7 promoter. To obtain 3′-UTR sense transcripts, the DNA was digested with *Pst*I (EP), *Nco*I (EN), *Afl*III (EA), *Ssp*I (ES), or *Xmn*I (EX) and transcribed in vitro. A 616-bp *Nco*I–*Eco*RI *TNF-α* gene fragment comprised of 366 3′-terminal base pairs of the 3′ UTR, the polyadenylation site and downstream sequences from pTNF-α was inserted into pBS under the T7 promoter. To obtain 3′-UTR sense transcripts, the DNA was digested with *Afl*III (NA) or *Xmn*I (NX) and transcribed in vitro. The EP RNA transcript was purified twice by gel electrophoresis, followed by chromatography on CF-11 cellulose, washing with ethanol, and eluting with water as described (Circle et al. 1997). Phosphorylation of PKR and the eIF2α chain was assayed by incubating poly(I–C) or 3′-UTR transcript with the ribosome fraction from rabbit reticulocyte lysate in the presence of [γ-^{32}P]ATP for 20 min at 30°C as described (Rosen et al. 1981) and subjecting the reaction mixture to electrophoresis in 10% polyacryamide gels containing sodium dodecyl sulfate. Band intensity was quantitated with NIH Image 1.61 software.

Nuclease sensitivity mapping

RNA (60 pmole), dephosphorylated with calf alkaline phosphatase and 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, was purified on a 6% polyacrylamide gel in 8 M urea before its digestion for 20 min at 30°C with 1 unit of RNase T1 (Worthington) or RNase U2, or 0.15 unit of V1 nuclease (Pharmacia). The mixture was made 9 M in urea, cooled on liquid nitrogen, and separated on an 8% polyacrylamide sequencing gel.

Translation efficiency in transfected BHK-21 cells

BHK-21 cells were cotransfected with pSV$_2$CAT DNA and a *TNF-α* gene construct. Expression of *TNF-α* and *CAT* mRNA in transfected BHK-21 cells was assayed by RNase protection analysis of total RNA isolated at 24 hr and the level of spliced TNF-α RNA (341-nucleotide band) was normalized to that of *CAT* mRNA. Secreted *TNF-α* was assayed by enzyme-linked immunosorbent assay (Bender MedSystems) on culture medium collected at 48 hr after transfection. Relative translation efficiency was expressed in arbitrary units as the ratio of TNF-α protein over spliced RNA.

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