The AU-rich Sequences in the 3′ Untranslated Region Mediate the Increased Turnover of Interferon mRNA Induced by Glucocorticoids

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Summary

Different vectors were constructed that expressed the human interferon-β (IFN-β) mRNA constitutively and contained various deletions in the 3′ untranslated region (UTR). AU-rich sequences in the 3′ UTR were specifically deleted in two vectors. Cell lines secreting human IFN-β were established by transfecting murine L929 cells with the vectors. These cells showed similar levels of IFN-β mRNA and secreted comparable amounts of IFN-β, indicating that the deletion of AU-rich sequences had no effect on the stability and little effect on the efficiency of translation of this mRNA. The synthetic glucocorticoid dexamethasone was previously shown to increase the turnover of IFN-β mRNA. This activity of dexamethasone was clearly observed only in cells expressing IFN-β mRNA with AU-rich sequences in the 3′ UTR. The increased turnover of this mRNA occurred in the presence of cycloheximide; therefore, it did not require synthesis of new proteins. These findings suggest that glucocorticoids may activate a ribonuclease that degrades mRNAs containing AU-rich sequences in the 3′ UTR.

Glucocorticoids regulate gene expression in different ways. When these hormones bind to cytoplasmic receptors, glucocorticoid-receptor complexes translocate into the nucleus where they bind to target DNA sequences with high affinity (see reference 1 for a review). This binding is a primary response that does not require protein synthesis and either stimulates or represses gene transcription (1), as it has been shown for α-fetoprotein (2) and proopiomelanocortin (3). Glucocorticoids can also modulate gene expression post-transcriptionally. For example, the stability of fibronectin (4) and human growth hormone mRNA is enhanced by glucocorticoid treatment (5); concomitantly, the length of the poly(A) tail of this mRNA is increased (6). Moreover, the mRNA for phosphoenolpyruvate carboxykinase contains a a glucocorticoid-responsive stabilizing element (7). In contrast, the glucocorticoids inhibit the synthesis of certain cytokines and of other proteins post-transcriptionally. Treatment with the synthetic glucocorticoid dexamethasone (DEX)[1] has been shown to destabilize the mRNA for IL-1β (8), type 1 procollagen (9) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (10). In addition, DEX inhibits the synthesis of T cell growth factor and the accumulation of IFN-γ mRNA in phytohemoagglutinin-stimulated lymphocytes (11), and decreases the level of nerve growth factor mRNA in L929 cells (12).

Treatment with glucocorticoids inhibits production of IFN and increases the lethality of Coxsackie virus in infected mice (13–14). DEX decreases IFN production (15) and increases the turnover of IFN-β mRNA in human fibroblasts induced with double-stranded RNA and in murine C127 cells carrying an episomal vector for the constitutive expression of human IFN-β mRNA under the control of the herpes simplex virus thymidine kinase (*tk*) promotor (16). This mRNA does not contain the IFN-β 5′ untranslated region (UTR) (17), suggesting that the effect of DEX is presumably mediated by coding or 3′ UTR sequences.

IFNs, lymphokines, proto-oncogenes, and growth factors contain AU-rich sequences in the 3′ UTR of their mRNA. Such sequences were identified by Caput et al. (18) as UUAUUUAU octamers, but shorter AUUUA repeats were described by Shaw and Kamen (19) as determinants of the instability of mRNA for granulocyte-macrophage colony-stimulating factor (GM-CSF). These AU-rich sequences (AU elements) may be involved in regulating the stability and/or the translation (20) of interferon mRNA. Here, we show that the increased turnover of IFN-β mRNA in DEX-treated cells is mediated by AU-elements in the 3′ UTR.

[1] *Abbreviations used in this paper:* AU elements, AU-rich sequences in the 3′ untranslated region; DEX, dexamethasone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GM-CSF, granulocyte-monocyte colony-stimulating factor; TE, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA; *tk*, herpes simplex virus thymidine kinase gene; UTR, untranslated region.

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Materials and Methods

Plasmid Constructions. An expression vector for IFN-β was constructed by subcloning the BamHI-HindIII fragment of a bovine papilloma virus–based vector carrying the human IFN-β gene (17) into the plasmid pGEM-1 (Promega Biotec, Madison, WI). This IFN-β gene is under the control of the *tk* promoter; the 5′ UTR is deleted up to position -3, and additional IFN genomic flanking sequences are present on the 3′ side (17). To obtain deletions of AU elements, this vector was cleaved at a unique NdeI site at position 99 of the 203-bp 3′ UTR and digested with Bal31 exonuclease. After blunting with Klenow enzyme and ligation in the presence of SalI linkers, plasmids with specific deletions were obtained and grown in *Escherichia coli* HB 101. Individual clones were screened by analyzing BglII-SalI or SalI-HincII restriction fragments to estimate the size of the deletions in the 3′ UTR, which was then sequenced. Two clones were selected: in A, there was a deletion from 86 to 121, and in D, from 10 to 173 (Fig. 1). Vector B was constructed by digesting A and D with SalI and HindIII, and ligating the large fragment of A with the small fragment of D. Vector C was constructed by digesting in the same way A and D, and ligating the small fragment of A with the large fragment of D. In this way, we obtained two pairs of vectors differing only in the AU elements deletion (Fig. 1).

Cell Culture and Transfection. L929 cells were grown in DMEM supplemented with 5% FCS and transfected with lipofectin (Bethesda Research Labs, Gaithersburg, MD). 200 ng of *pSV2neo* and 2 μg of vector DNA were mixed with 30 μg of lipofectin in a total volume of 80 μl, incubated at room temperature for 5 min, diluted with 720 μl of DMEM minus serum, and added to a culture of subconfluent L929 cells. After 4 h, 1.2 ml of DMEM supplemented with 3.3% calf serum (HyClone Laboratories, Logan, UT) was added. The next day, the cells were transferred to a 75-cm^2 flask. Selection with 1 mg/ml G418 sulfate (Gibco Laboratories, Grand Island, NY) was initiated after another day. Each transfection yielded ∼2,000–3,000 resistant clones, corresponding to a transfection efficiency of 0.6–1%. These cells were pooled and maintained in selective medium for 1 mo, and then grown in nonselective medium.

RNA Extraction and Analysis. Total RNA was extracted by lysing cells in one well of a six-well cluster plate with 0.5 ml of 2% SDS, 0.2 M Tris, pH 7.4, in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA). After transfer into a microfuge tube, the cellular DNA and protein were precipitated by the addition of 150 μl of 42.9% (wt/vol) potassium acetate and 11.2% (vol/vol) glacial acetic acid. The tube was gently mixed and placed on ice for 2 min. After a 5-min centrifugation, the supernatant was extracted twice with chloroform/*iso*amylalcohol (24:1) and the RNA was precipitated with 650 μl of ice-cold isopropanol on ice for 15 min. The RNA was recovered by centrifugation, washed with 70% ethanol, dried, and resuspended in 100% formamide. Deionized glyoxal (Clontech Laboratories, Palo Alto, CA) was added to 10 μg of RNA to a final concentration of 1 M and the sample was heated to 65°C for 4.5 min. The RNA was analyzed on 1% agarose gels and transferred to Gene Screen (New England Nuclear, Boston, MA) membranes using a vacuum blotting apparatus and 0.2 M NaOH. This alkaline solution increases transfer of high mol wt RNA, reverses glyoxylation, and ensures binding of denatured RNA to the membrane. The blots were briefly rinsed in 100 mM Tris, pH 7.4, and baked for 1 h. Prehybridization, hybridization, and washing conditions were done according to the instructions provided by the manufacturer of Gene Screen membranes (New England Nuclear). The IFN-β probe used for hybridization was a 762-bp HincII fragment of the human IFN-β gene labeled by the random primer method. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin probes used for control hybridizations were obtained from Dr. Riccardo Dalla-Favera (Columbia University, NY), and Dr. Donald Cleveland (Johns Hopkins University, Baltimore, MD).

DNA Analysis. DNA was extracted from confluent cells harvested from a 25-cm^2 flask with PBS containing 1 mM EDTA and lysed in TE containing 1% NP-40. The nuclei were recovered by centrifugation and lysed by the addition of 300 μl of 1% SDS in TE. The aqueous phase was extracted with phenol/chloroform/*iso*amylalcohol (25:24:1), reextracted with chloroform/*iso*amylalcohol (24:1) and precipitated with 0.1 vol of 3 M sodium acetate and 2 vol of ethanol. The DNA was digested with BglII and EcoRI, separated on a 1% agarose gel and transferred to a Gene Screen membrane (New England Nuclear) with 0.2 M NaOH in a vacuum blotting apparatus. The blot was rinsed in 100 mM Tris, pH 7.5, and baked for 1 h at 80°C. Hybridization and washing conditions were identical to those described above.

Nuclear Runoff Assay. This assay was carried out essentially as reported (16), with the exception that the RNA was extracted by the method described above and 40 μg of tRNA were added as carrier. DNA bound to Gene Screen filters (New England Nuclear) was hybridized overnight with 2 × 10^6 cpm of RNA in 2 ml of hybridization buffer. The filters were thoroughly washed before autoradiography.

Interferon Assay. Human IFN produced by transfected L929 cells was titrated by measuring the inhibition of the cytopathic effect (21) caused by Sindbis virus in human osteosarcoma cells (22). The IFN titer was expressed in antiviral units; 1 U/ml of IFN protected 50% of the cells from the cytopathic effect of virus infection.

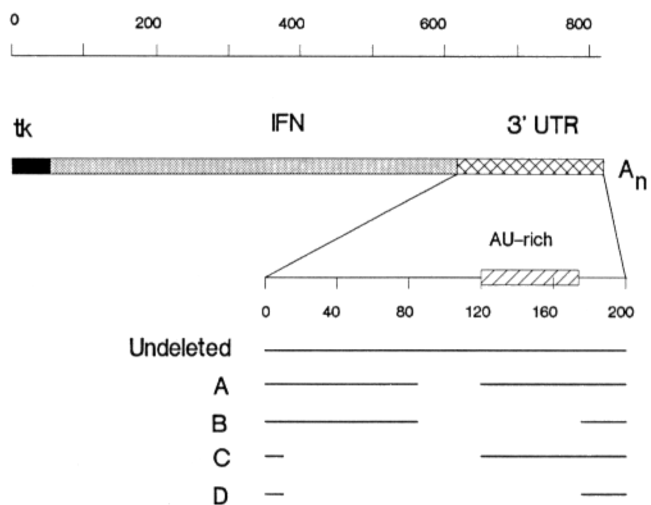

Figure 1. Expression vectors for human IFN-β. The human IFN-β mRNA transcribed from the vector of De Maio et al. (17) is shown. The 5′ UTR is indicated by *tk* and the coding region by IFN. The 3′ UTR is expanded to show in detail the location of the AU-rich sequence. The 3′ UTR of the vectors used is shown: "Undeleted" codes for the full-length 203 bases 3′ UTR; in *A*, there is a deletion (bp 86–121) in the center of the 3′ UTR, but the AU elements are retained; in *B*, there is a deletion of AU elements (bp 86–173), but the remainder of the 3′ UTR is the same as in *A*; in *C*, there is a large deletion of the 3′ UTR (bp 10–121), but the AU elements are retained; and in *D*, there is a deletion including the AU elements (bp 10–173), but the remainder of the 3′ UTR is the same as in *C*. All constructs retain the polyadenylation signal and site of the undeleted gene to insure proper processing of the mRNAs. See Materials and Methods for details of vector construction.

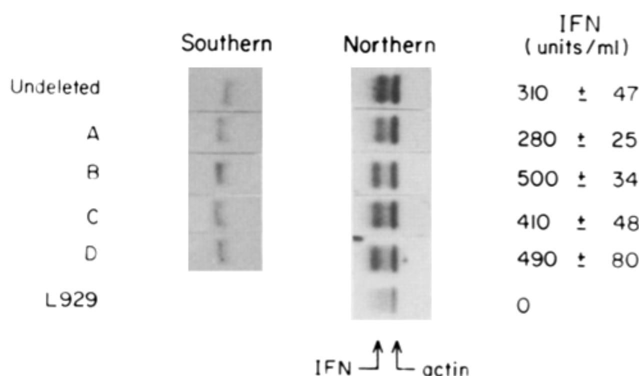

Figure 2. Levels of integrated vector DNA (Southern) and IFN-β mRNA (Northern) in L929 cells transfected with the vectors shown in Fig. 1. For Southern analysis, the DNA was digested with BglII and EcoRI to obtain the 704-bp fragment of the human IFN-β gene detected; the murine IFN-β gene does not yield this restriction fragment. About 10–15 copies of the human IFN-β gene were present per transfected cell, as estimated by comparison with Southern blots of DNA from C127 cells (17). The Northern blot was hybridized simultaneously to the IFN-β and actin probes. The origin of the gels is in each case on the right. The IFN secreted into the medium during 18 h was measured by triplicate assays in two independent experiments; the IFN titer is indicated in antiviral units/ml.

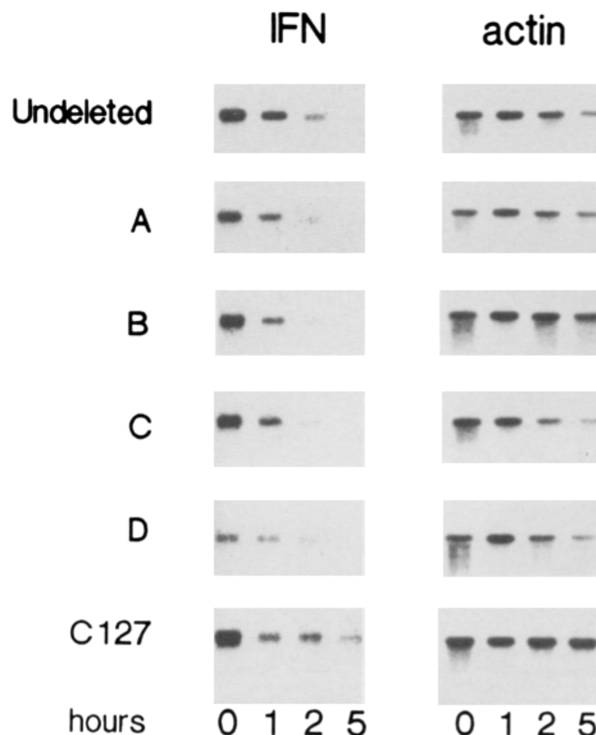

Figure 3. Turnover of IFN mRNA in the L929 cells described in Fig. 2 and in murine C127 cells. Cultures were treated for the indicated time with 2 μg/ml of actinomycin D. The RNA was analyzed by Northern blots. The blots were hybridized to an IFN-β probe, stripped, and rehybridized to an actin probe.

Results

IFN-β mRNA and IFN Secretion in L929 Cells Transfected with Different Vectors. To examine the role of the AU elements in IFN-β mRNA, we cotransfected murine L929 cells with *pSV2neo* and the expression vectors shown in Fig. 1. These vectors express human IFN-β mRNA constitutively from the *tk* promotor (17). Each transfection yielded 2,000–3,000 clones that were pooled to insure random integration of vector DNA in the cell population. Southern blots of genomic DNA showed a comparable copy number of the human IFN-β gene in each pool of transfected cells (Fig. 2). The IFN-β mRNA level was also similar among the five sets of transfected cells (Fig. 2). Cells transfected with the B vector secreted 1.7- and 1.6-fold more IFN than cells transfected by the A or undeleted vector, respectively. However, cells transfected with the C and D vectors secreted similar levels of IFN. These findings indicated that deletion of AU elements in vectors B and D did not lead to accumulation of IFN-β mRNA nor to a significantly greater secretion of IFN.

The steady state level of an mRNA results from the balance between its synthesis and degradation. When we treated transfected L929 cells with actinomycin D to stop transcrip-

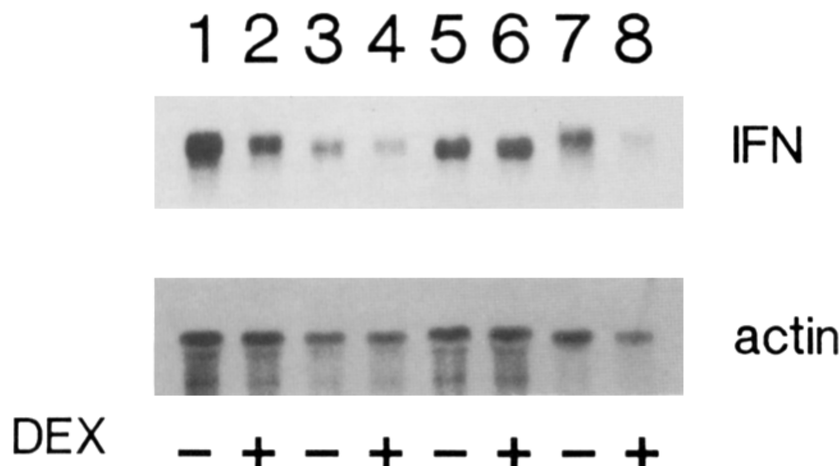

Figure 4. Northern blots of RNA extracted from L929 cells transfected with the "undeleted" (lanes *1–2*), A (lanes *3–4*), and B vectors (lanes *5–6*), and of murine C127 cells expressing IFN-β mRNA from an episomal expression vector (lanes *7–8*). Cell cultures were treated for 20 h with 1 μM DEX or left untreated.

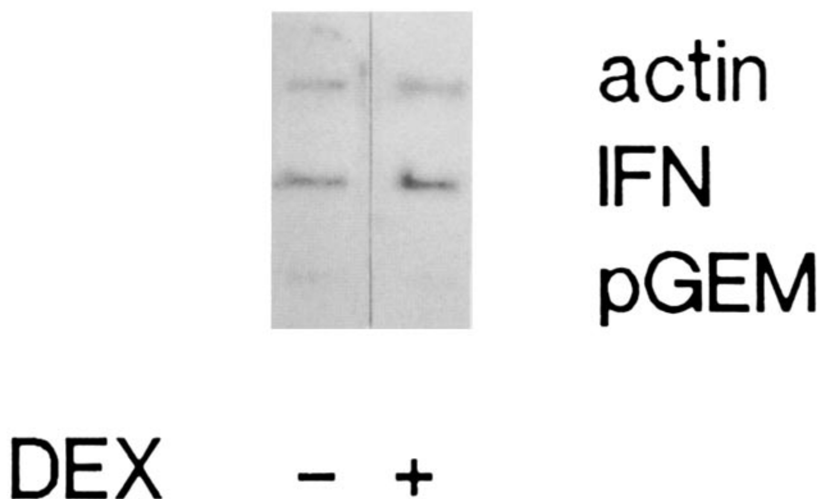

Figure 5. Run-off assays with nuclei of C127 cells expressing IFN-β mRNA from an episomal expression vector. Cultures were treated for 3.5 h with 1 μM DEX or left untreated. DNA from a plasmid containing β-actin cDNA and the pGEM-1 plasmid were used as positive and negative controls.

tion, we observed very similar rates of IFN-β mRNA degradation (Fig. 3). The half-life of this mRNA was ∼45 min when measured by densitometry of the autoradiographs; such half-life was in agreement with previous reports (23). This fast degradation rate was not altered by deletion of the AU elements (Fig. 3, *B* and *D*). Therefore, these elements are not responsible for the fast turnover of IFN-β mRNA.

Decrease of IFN-β mRNA and IFN Secretion After Treatment with Dexamethasone. We treated transfected L929 cells and C127 cells expressing IFN-β mRNA from an episomal vector (17) with 1 μM DEX for 24 h. Total cellular RNA was analyzed by Northern blotting (Fig. 4). DEX specifically decreased the level of IFN-β mRNAs containing AU elements (lanes *1–4*, 7, and *8*) but not that of IFN-β mRNAs lacking AU elements (lanes *5* and *6*). To show that DEX had no effect on the transcription of the IFN-β gene, we performed nuclear runoff transcription assays on C127 cells. No decrease in transcription was observed after 3.5 h of treatment with 1 μM DEX (Fig. 5), in agreement with previous reports (16).

To follow the turnover of IFN-β mRNA, we treated transfected L929 cells for various times with 1 μM DEX and analyzed total RNA by Northern blotting. The IFN-β mRNA containing AU elements decayed rapidly, but that lacking AU elements was much less affected by the DEX treatment (Fig. 6). The mRNA levels in this autoradiograph were quantitated by densitometry (Fig. 7). The mRNAs with AU elements decreased ∼80% after 8 h of DEX treatment whereas mRNAs lacking AU elements decreased only ∼20%. This slight decrease may be due to an AU-rich sequence that is located between the polyadenylation signal (nucleotides 178–182 in the 3′ UTR) and nucleotide 203, where the mRNA is polyadenylated. This sequence was not altered during construction of the various vectors.

We treated L929 cells transfected with the C or D vector for 16 h with 1 μM DEX and measured the titer of IFN secreted in a viral protection assay. Secreted IFN decreased 58% in "C" cells expressing IFN-β mRNA with AU elements, but only 27% in "D" cells expressing mRNA lacking AU elements. Therefore, this decrease was comparable to that in IFN-β mRNA level. These effects of DEX were apparently mediated by its binding to a glucocorticoid receptor, since they were abolished by adding a 100-fold excess of the antagonist cortexolone 15 min before DEX (data not shown).

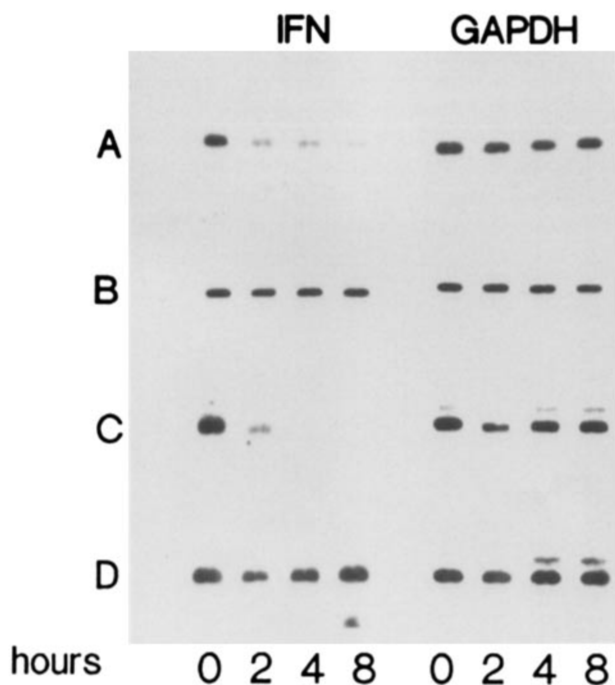

Figure 6. Northern analysis of RNA extracted from L929 cells transfected with vectors A, B, C, and D. Cultures were treated for the indicated time with 1 μM DEX. The blots was hybridized to the IFN-β probe, stripped, and rehybridized to a GAPDH probe.

The Increased Turnover of IFN-β mRNA in DEX-Treated Cells Does Not Require Synthesis of New Proteins. Cycloheximide (CHX) is a protein synthesis inhibitor that superinduces the mRNA for IFN (23). We treated L929 cells transfected with

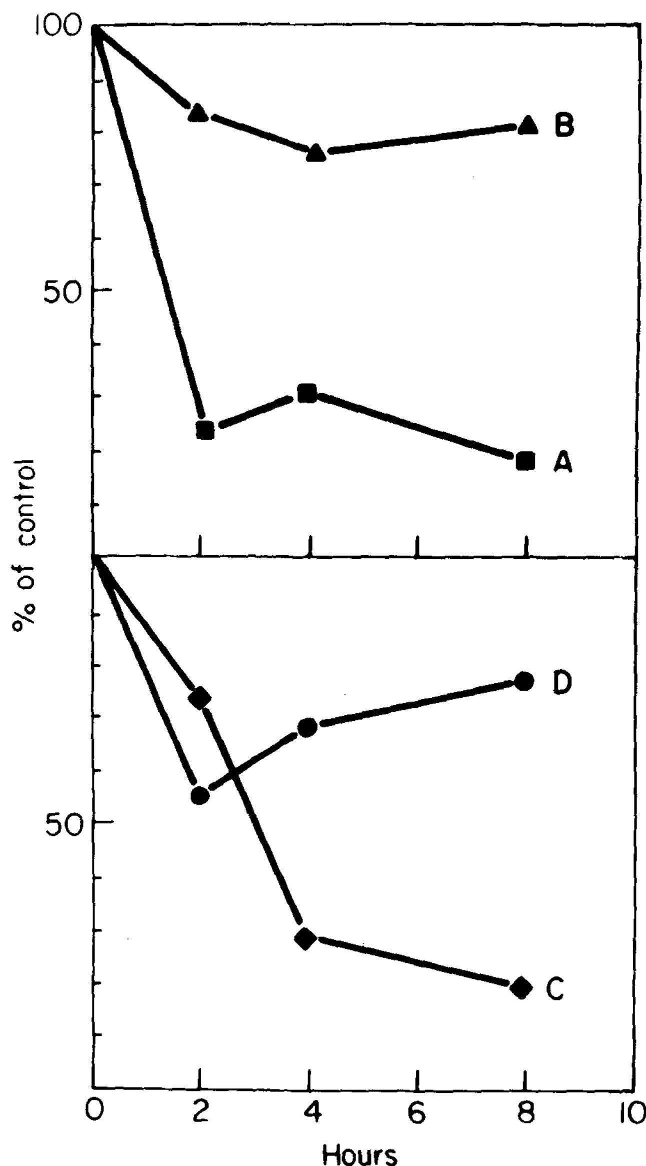

Figure 7. Autoradiographs in Fig. 6 were scanned and the values obtained were normalized to the level of GAPDH mRNA. Densitometric values relative to untreated control cultures are shown.

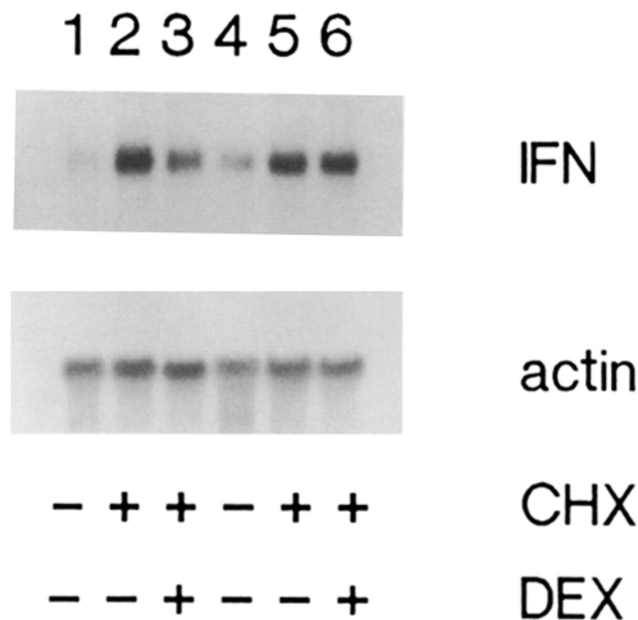

Figure 8. Northern blot analysis of RNA extracted from L929 cells transfected with vectors A (lanes *1–3*) and B (lanes *4–6*). Cultures were treated for 4 h with 100 μg/ml CHX minus or plus 1 μM DEX or left untreated, as indicated. The blot was hybridized to the IFN-β probe, stripped, and rehybridized to the actin probe.

Discussion

Deletion of AU elements does not increase the stability of IFN-β mRNA. This finding is in apparent contrast with the observations of Shaw and Kamen (19), who proposed that AU elements are the recognition signal for a processing pathway that degrades the mRNAs of certain cytokines and proto-oncogenes. However, Shyu et al. (24) reported that at least two distinct pathways are responsible for the turnover of *c-fos* mRNA, since deletion of AU elements does not lead to an increase in its half-life. In agreement with this finding, IFN-β mRNAs lacking AU elements do not show increased stability.

It was previously shown that DEX decreases the level of

the A or B vector with CHX either in the presence or absence of DEX (Fig. 8). The lack of AU elements did not influence the degree of superinduction after CHX treatment. Addition of DEX decreased the level of IFN-β mRNA "A" containing AU elements even in the absence of protein synthesis. However, it did not decrease the level of "B" mRNA lacking AU elements (Fig. 8, compare lanes *2* and *3* to lanes *5* and *6*). By scanning the autoradiographs, we showed that the level of "A" mRNA after CHX + DEX treatment was 52% lower than in cells incubated with CHX alone. The "B" mRNA level was unchanged by the addition of DEX (Fig. 9). This finding suggests that DEX can activate an RNAase activity specific for mRNAs containing AU elements even when protein synthesis is inhibited.

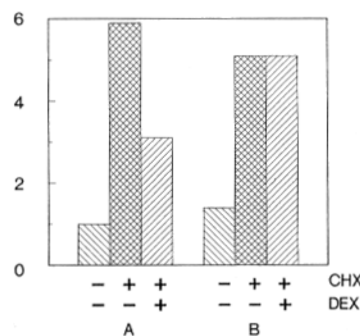

Figure 9. The autoradiograph in Fig. 8 was scanned and the values obtained for IFN-β mRNA were normalized to the levels of actin mRNA. The value for IFN-β mRNA in track 1 of Fig. 8 was arbitrarily set at 1.

human and murine IFN-β mRNA post-transcriptionally and it was suggested that glucocorticoids increase the turnover of this mRNA (16). The present results confirm these findings and extend our understanding of this effect of DEX. The increased turnover of IFN-β mRNA in DEX-treated cells is apparently mediated by AU elements in the 3' UTR. This finding suggests that the degradation of IFN-β mRNA is catalyzed by at least two pathways; the first recognizes unidentified features of its sequence and the second is activated by glucocorticoids and functions through recognition of AU elements. This hypothesis may provide an explanation for the finding that IFN-β mRNA shows a much shorter half-life in some cells, such as macrophages (25), where the first degradative pathway may be most active.

The AU elements are found in the 3' UTR of mRNA of many cytokines. Some of these proteins are encoded by short-lived mRNAs which are degraded even more rapidly after addition of glucocorticoids (26). For example, the synthesis of IFN-γ (11), nerve growth factor (12), and GM-CSF (27) are inhibited by glucocorticoids. It is plausible that an RNAase system recognizes AU elements and is responsible for the increased turnover of this class of mRNAs. A cytosolic protein that binds specifically to AUUUA-containing RNA has recently been described (28). This protein may target susceptible mRNAs for degradation by an RNAase (28). DEX may stimulate the activity of such RNAase but not necessarily its synthesis, as suggested by the results of experiments with CHX (Fig. 8). The superinduction of unstable mRNAs by CHX is thought to be due to the decay of an unstable RNAase or of unstable transcriptional repressors. Our finding that IFN-β mRNA containing AU elements is degraded in the presence of CHX + DEX suggests that the RNAase involved in the glucocorticoid-mediated turnover of this mRNA is not synthesized de novo nor is it highly unstable.

Deletion of AU elements does not appear to increase greatly the efficiency of translation of IFN-β mRNA, as judged by the comparable secretion of IFN from L929 cells transfected with different vectors. This finding is in apparent contrast with reports by Kruys et al. (20, 29) that human IFN-β is poorly translated in a reticulocyte cell-free system and in *Xenopus* oocytes. Translation of IFN-β mRNA in these systems is enhanced by deletion of 3' UTR sequences. However, an effect of AU elements on the efficiency of translation of IFN-β mRNA is not pronounced in intact L929 cells, since secretion of IFN increases at most 1.7-fold in cells transfected with one of the vectors lacking such elements. It should be pointed out that AU elements in concert with essential flanking sequences may be also involved in repressing the translation of tumor necrosis factor mRNA in transformed murine macrophages, as shown by a recent report of Han et al. (30) on the "derepression" of the synthesis of this cytokine by endotoxin in vectors lacking AU-elements. It is thus possible that the translational efficiency of cytokines mRNA may be regulated to a different extent by AU elements in various cells.

The half-life of the unstable GM-CSF mRNA can be greatly increased by treatment of T lymphocytes with phorbol esters (19). Similarly, activators of protein kinase C enhance the accumulation of IFN-β mRNA in some murine cells (31). Therefore, an increased expression of these unstable mRNAs may result from a block in their degradation, but it is not known whether the AU elements may be involved in decreasing their turnover. If this were the case, the half-life of cytokines and oncogenes mRNA may be regulated in opposites ways by agents that increase or decrease the output of specific gene products at the post-transcriptional level. Therefore, the development of an assay specific for a glucocorticoid-activated RNAase would be of great usefulness in establishing the role of AU elements in the regulation of the half-life of specific mRNAs.

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