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Secretory proteins induced in human fibroblasts under conditions used for the production of interferon β

[human interferon β heterogeneity/26-kilodalton protein/ppp(A2′p5′)$_n$A synthetase/dog pancreas microsomes/superinduction]

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ABSTRACT **Human fibroblast cells treated with a combination of inhibitors of protein and RNA synthesis [cycloheximide and actinomycin D as used to superinduce interferon β (IFN-β)] secrete two proteins with molecular masses of 22000 and 27000 kilodaltons (called 22-kDal and 27-kDal) that are precipitable with an antiserum raised against impure IFN-β but are antigenically distinct from IFN-β_1. Translation *in vitro* of mRNA extracted from human fibroblast cells induced for the production of IFN-β leads to the synthesis of a 26-kDal protein that is structurally closely related to the 22- and 27-kDal proteins. This 26-kDal protein mRNA is relatively abundant and also appears in human fibroblasts induced only with cycloheximide. It has been partially purified by sucrose gradient centrifugation and more extensively by diazobenzyloxymethyl-cellulose hybridization to plasmid DNA from a bacterial cDNA clone. When translated in an *in vitro* reticulocyte system supplemented with dog pancreas microsomes, the 26-kDal protein and two other intermediates corresponding presumably to its signal-cleaved (19-kDal) and partially glycosylated (24-kDal) forms were observed. Crude, partially purified, and highly purified 26-kDal mRNA failed to program the synthesis of antiviral or ppp(A2′p5′)$_n$A synthetase-inducing activity when translated in *Xenopus laevis* oocytes. Moreover, partially purified 22-kDal and 27-kDal (i.e., the *in vivo* equivalents of the 26-kDal protein) are also devoid of antiviral or ppp(A2′p5′)$_n$A synthetase-inducing activity. Hence, this 26-kDal mRNA, although presumably identical to the human IFN-β_2 mRNA described by Weissenbach *et al.* [Weissenbach, J., Chernajovsky, Y., Zeevi, M., Shulman, L., Soreq, H., Nir, U., Wallach, D., Perricaudet, M., Tiollais, P. & Revel, M. (1980) *Proc. Natl. Acad. Sci. USA* 77, 7152–7156], cannot be considered to be a fibroblast interferon mRNA.**

The superinduction of interferon β (IFN-β), a complex procedure consisting of at least four different treatments [IFN, poly(I)·poly(C), cycloheximide, and actinomycin D] (1) results in the cellular synthesis of additional proteins (2, 3). Moreover, Sehgal and Sagar (4) have described a second IFN-β mRNA (IFN-β_2 mRNA), which is larger than the predominant IFN-β_1 mRNA (5–8) and which does not cross-hybridize with an IFN-β_1 cDNA probe. However, there is as yet no direct evidence that IFN-β_2 is expressed in fibroblasts or other human cells. Weissenbach *et al.* (9, 10) cloned from poly(I)·poly(C)-induced human fibroblasts an mRNA species (14S, 1,300 nucleotides long) that is translated in a reticulocyte cell-free system into a 23- to 26-kilodalton (kDal) protein‖ immunoprecipitable with anti-IFN-β antiserum. The cloned cDNA, however, does not cross-hybridize with IFN-β_1 mRNA, and when the mRNA was purified by hybridization to a corresponding cloned cDNA probe it could be translated into a 23- to 26-kDal protein with antiviral and 2-5A synthetase-inducing activity (10). [2-5A represents a series of oligoadenylates, ppp(A2′p5′)$_n$A.] This activity was also named IFN-β_2, but there is no evidence that it is identical to the species characterized by Sehgal and Sagar (4).

During the course of our work on the cloning of IFN-β_1 from poly(I)·poly(C)-induced human fibroblast mRNA, we also observed such a 26-kDal protein mRNA. This 26-kDal protein is undoubtedly identical to that studied by Weissenbach *et al.* (9, 10) because both are inducible by cycloheximide alone and have the same apparent molecular mass and the same plastic- and antibody-binding properties. Sequence analysis of the DNA for the 26-kDal protein showed that the distance between the two putative polyadenylylation sites A-A-T-A-A-A is 72 nucleotides and that the length of the 3′ untranslated region is over 400 nucleotides (unpublished results), which is in agreement with the results quoted by Soreq *et al.* (11). Here we show that the *in vivo* equivalent of the 26-kDal protein can be induced in the absence of interferon production in human fibroblasts and is devoid of antiviral and 2-5A synthetase-inducing activities; thus it is distinct from IFN-β_2.

MATERIALS AND METHODS

Confluent cell cultures were induced by various combinations of the four steps used for superinduction of IFN-β; the timing and sequence of the treatments were never altered. (*i*) Cells were primed with 100 units of IFN-β (10^6 units/mg) for 24 hr. (*ii*) Cycloheximide (50 μg/ml) was added for 6 hr. (*iii*) Poly(I)·poly(C) (100 μg/ml) was added during the first hour of cycloheximide treatment. (*iv*) Actinomycin D (2 μg/ml) was added during the last 2 hr of cycloheximide treatment.

Thirty hours after initial treatment with IFN-β, or 6 hr after initial treatment with cycloheximide, the cells were washed twice with Eagle's medium without methionine and labeled for 1–4 hr with [^{35}S]methionine (≈1,000 Ci/mol; Amersham, England; 1 Ci = 3.7×10^{10} becquerels) at 30 μCi/ml. After labeling the medium was collected and the proteins were ana-

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Abbreviations: IFN, interferon; kDal, kilodalton(s); DBM, diazobenzyloxymethyl; 2-5A, a series of oligoadenylates, ppp(A2′p5′)$_n$A; CPG, controlled-pore glass.

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‖ Proteins (19-, 22-, 24-, 26-, and 27-kDal) are designated according to their apparent molecular mass as observed on NaDodSO$_4$/polyacrylamide gel electrophoresis. [This may not reflect the true molecular mass; for instance, although IFN-β_1 moves as 16.5 kDal it has a real molecular mass of 20,030 daltons (6).] As suggested in this paper, the biosynthesis of these proteins proceeds as follows:

$$\text{mRNA} \rightarrow \text{26-kDal} \xrightarrow{\text{Processing}} \begin{matrix} \nearrow \text{24-kDal} \ldots \rightarrow \text{27-kDal} \\ \searrow \text{19-kDal} \ldots \rightarrow \text{22-kDal} \end{matrix} \quad \text{(Secretion)}$$

lyzed by immunoprecipitation and electrophoresis.

Two goat antisera were used. The first, raised against human IFN-β (2×10^6 units/mg) purified by passage through a controlled-pore glass (CPG) column, had a titer of 200,000 units/ml. The second antiserum, raised against human IFN-β (1.7×10^9 units/mg) purified with CPG and zinc chelate, had a titer of $1–3 \times 10^5$ units/ml (12) and was provided by J. Heine and A. Billiau (Rega Institute).

RESULTS

Induction and Secretion of 26-kDal-Related Proteins. Translation *in vitro* of mRNA isolated from induced fibroblast cells revealed a prominent 26-kDal protein band that seems identical to the protein studied by Weissenbach *et al.* (9, 10). We therefore screened by immunoprecipitation the culture medium of the [^{35}S]methionine-labeled fibroblasts after induction in various conditions. Polyacrylamide gel electrophoresis of such immunoprecipitates indeed indicated the presence of two polypeptides, of 22 and 27 kDal (Fig. 1). The amounts of these polypeptides were slightly increased by treatment with actinomycin D (1 μg/ml for 2 hr), or cycloheximide (10 μg/ml for 4 hr) and dramatically increased when the two inhibitors were used in sequence. However, this treatment without poly(I)·poly(C) was not sufficient for induction of interferon.

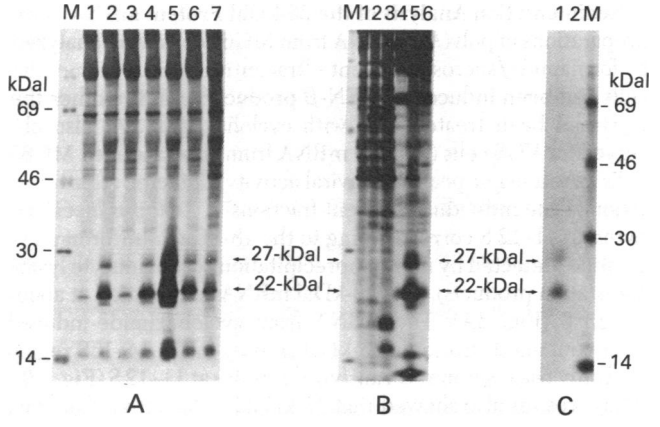

FIG. 1. Immunoprecipitable proteins secreted by human fibroblast cells under various conditions. (*A*) Confluent monolayers of T_{21} human diploid cells were grown in 4-cm^2 square wells (Sterilin). The cells were induced as described below and labeled with [^{35}S]methionine for 1 hr. Culture medium (0.5 ml) was immunoprecipitated with 5 μl of goat serum directed against partially purified IFN-β, analyzed by electrophoresis on a 13% polyacrylamide gel, and fluorographed for 20 hr at −70°C (13). Lane 1, untreated cells; lane 2, priming with IFN-β (0–24 hr) at 100 units/ml followed by treatment with actinomycin D (2 μg/ml; 28th to 30th hr); lane 3, priming alone; lane 4, cycloheximide alone (50 μg/ml; 24th to 30th hr); lane 5, cycloheximide and actinomycin D; lane 6, priming and cycloheximide; lane 7, actinomycin D alone; M, molecular mass markers. (*B*) Confluent monolayers of VGS cells were prepared in 25-cm^2 flasks and after induction the cultures were labeled and culture fluid were processed as for *A*. The cells were washed three times in phosphate-buffered saline, lysed in a small volume of 0.5% Nonidet P40 in 20 mM Hepes, pH 7.4/10 mM KCl/0.5 mM dithiothreitol/1.5 mM magnesium acetate/1 mM phenylmethylsulfonyl fluoride, and immunoprecipitated as above. Lanes 1–3 are cytoplasmic extracts and lanes 4–6 are culture medium. Lanes 1 and 4, untreated cells; lanes 2 and 5, priming followed by cycloheximide and actinomycin D; lanes 3 and 6, priming and cycloheximide. (*C*) A 0.2-ml sample of culture medium from VGS cells induced by cycloheximide and actinomycin D (same conditions as *A*, lane 5) but labeled for 4 hr with [^{35}S]methionine, purified with CPG, and concentrated (cf. Fig. 5) was immunoprecipitated with antiserum raised against impure IFN-β (lane 1) or against pure IFN-β_1 (lane 2). Electrophoresis was on a 15% polyacrylamide gel and was followed by fluorography for 16 hr at −70°C.

Priming with IFN-β at 100 units/ml for 24 hr before the actinomycin D or cycloheximide treatment, either alone or combined with the two inhibitors, did not seem to influence the amount of 22-kDal and 27-kDal in the medium (compare lane 7 to lane 2 and lane 4 to lane 6 in Fig. 1*A*). As shown again in Fig. 1*B* (lanes 4–6) for another cell line, it is clear that the combined use of actinomycin D and cycloheximide was the main determinant for the enhanced production of 22-kDal and 27-kDal. There was no significant difference between the T_{21} and VGS cells, and we have also observed the same proteins in MG63 cells. In general, early cell passages gave higher yields of 22-kDal and 27-kDal. The 22- and 27-kDal proteins were not detected in cytoplasm (Fig. 1*B*, lanes 2 and 5), indicating that they are probably rapidly and efficiently secreted during the labeling period and that they do not represent material released from lysed cells. The same two products were labeled and secreted when the full induction procedure for IFN-β production was applied [i.e., with poly(I)·poly(C), actinomycin D, and cycloheximide; results not shown]. This explains why serum directed against partially purified IFN-β contains antibodies

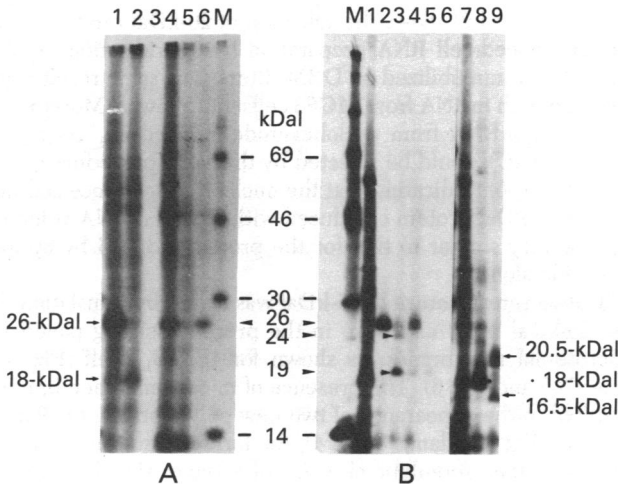

FIG. 2. Cell-free translation of human IFN-β mRNA and 26-kDal protein mRNA after hybridization with different plasmid cDNAs. RNA samples were incubated for 45 min at 31°C in 25-μl reaction mixtures containing 1×10^7 cpm of [^{35}S]methionine (≈1,000 Ci/mmol) and calf liver tRNA at 150 μg/ml in a micrococcal nuclease-treated rabbit reticulocyte lysate and immunoprecipitated with antiserum raised against impure IFN-β (*A*, lanes 1 and 3–6, and *B*) or with antiserum against pure IFN-β_1 (*A*, lane 2). Other experimental procedures have been described (6). (*A*) Lanes 1 and 2, 2 μg of poly(A)$^+$ mRNA from MG63 superinduced cells [MG63 cells primed for 24 hr with IFN-β at 100 units/ml and then induced with poly(I)·poly(C) at 100 μg/ml for 1 hr and cycloheximide for 4 hr in the presence of 5 mM $CaCl_2$] (14). Lane 3, no mRNA added. Lane 4, 2 μg of total mRNA from T_{21} human fibroblasts induced by 4-hr treatment with cycloheximide alone. Lane 5, 5 μg of the same mRNA not hybridizing to diazobenzyloxymethyl (DBM)-cellulose filters (0.5 cm^2) containing 5 μg of 26-kDal cloned cDNA. Lane 6, hybridized mRNA under the same conditions. Lane M, molecular mass markers. (*B*) Samples of total cytoplasmic RNA (60 μg) from superinduced VGS cells were hybridized to DBM-cellulose filters containing 5 μg of DNA from pHFIF-1 (IFN cDNA) (lanes 7–9) or 5 μg of cloned cDNA from plasmid 26k-1 (lanes 1–6). Lanes 1 and 7, nonhybridized mRNA; lanes 2–6, mRNA hybridized with 26-kDal cDNA. Lanes 8 and 9, mRNA hybridized with IFN-β_1 cDNA. Lanes 3 and 9, translation in the rabbit reticulocyte lysate supplemented with dog pancreas microsomes at 5 A_{280} units ml (3); lane 4, same as lane 3, but microsomes were added 20 min after initiation of translation. Lane 5, same as lane 3, but after translation and processing the cell-free products were digested for 90 min with trypsin (0.3 mg/ml) and chymotrypsin (0.3 mg/ml) at 4°C; Trasylol (3,000 units/ml) was then added to arrest the digestion. Lane 6, same as in lane 5 except that the cell-free system was first treated with 0.5% sodium deoxycholate.

against 22-kDal and 27-kDal. An antiserum prepared against a pure preparation of human IFN-β_1, however, failed to precipitate the same two labeled proteins (Fig. 1*C*, lane 2), clearly showing that they are not antigenically related to IFN-β_1.

Cell-Free Translation of the 26-kDal Protein mRNA. VGS diploid cells and MG63 cells induced for the production of IFN-β by treatment with poly(I)·poly(C) and cycloheximide after 24-hr priming (6) contain a mRNA that codes for a 26-kDal protein that is immunoprecipitable with antiserum directed against crude interferon but not with preimmune antiserum or antiserum against pure human IFN-β (Fig. 2*A*, lane 2). No 26-kDal mRNA was found in untreated cells. In addition, T_{21} cells that were treated for only 4 hr with cycloheximide (for mRNA production actinomycin D was always omitted) also contained 26-kDal protein mRNA (Fig. 2*A*, lane 4). Hence the induction process for the 22- and 27-kDal proteins correlates well with the appearance of the 26-kDal protein mRNA.

During the cloning of human IFN-β_1 cDNA corresponding to mRNA from poly(I)·poly(C) plus cycloheximide-induced VGS cells (6), cDNA clones that contained the nucleotide sequence coding for the 26-kDal protein were also selected. Fig. 2*B* (lanes 1 and 2) shows the selection of 26-kDal mRNA from a total induced-cell RNA preparation by hybridization to 26-kDal cDNA immobilized on DBM filters. The same result was obtained with mRNA from MG63 cells (not shown). Moreover, a 26-kDal mRNA from cycloheximide-induced T_{21} cells [no poly(I)·poly(C)] could be selected by the same procedure (Fig. 2*A*, lanes 4–6), indicating that the nucleotide sequence coding for the 26-kDal protein coinduced with IFN-β mRNA is identical or very similar to that for the protein induced by cycloheximide alone.

The secretory nature of 26-kDal was tested by translation of the 26-kDal mRNA *in vitro* in the presence of dog pancreas microsomal membranes, as shown for IFN-β_1 itself (Fig. 2, lanes 7–9 and ref. 6). The presence of microsomal membranes resulted in the appearance of two new polypeptides, of 19 and 24 kDal (Fig. 2*B*, lanes 3 and 4), the former resulting presumably from the proteolytic cleavage of a longer signal sequence than the one described for human IFN-β_1 (i.e., 21 amino acids), whereas 24-kDal may represent a glycosylated form of 19-kDal. The 19- and 24-kDal proteins, however, have electrophoretic mobilities which differ from those of the 22- and 27-kDal proteins secreted into the culture medium *in vivo*; this may be due to incomplete or incorrect processing or modification. Processing of the 26-kDal precursor by the microsomal system requires a synchronous coupling with cell-free translation (15). When the microsomes were added 20 min after initiation of translation, the extent of processing was considerably reduced, as judged by the decrease of the 19-kDal/26-kDal ratio, whereas the 24-kDal peptide was no longer detectable (lane 4). An indication that processing occurs inside microsomal vesicles was provided by the resistance of the processed proteins (19-kDal and 24-kDal) to proteolysis, whereas the nonprocessed 26-kDal was destroyed under these conditions. After detergent disruption of the microsomes, all three proteins were protease sensitive (Fig. 2*B*, lanes 5 and 6).

Structural Comparison Between the 26-kDal Protein Translated *in Vitro* and the 22- and 27-kDal Proteins Secreted *in Vivo*. The 22- and 27-kDal proteins were labeled with [^{35}S]methionine, immunoprecipitated, and isolated from a preparative polyacrylamide gel, and their digests were examined by electrophoresis after partial proteolysis with *Staphylococcus aureus* V8 protease (16). The two protein digests were similar (results not shown).

Two-dimensional tryptic peptide analysis of 22-, 27-, and 26-kDal proteins translated *in vitro* in the presence of [^{35}S]-methionine showed that the three are very similar (Fig. 3).

Sedimentation Analysis of the 26-kDal Protein mRNA. Two preparations of poly(A)$^+$ mRNA from MG63 cells were analyzed by formamide/sucrose gradient ultracentrifugation. In one, the cells had been induced for IFN-β production; in the other the cells had been treated only with cycloheximide. As also observed for VGS cells (18), the mRNA from superinduced MG63 cells gave a major peak of antiviral activity (detected after translation of the individual gradient fractions in *Xenopus laevis* oocytes) at 11–12 S corresponding to the 18-kDal interferon polypeptide (detected by immunoprecipitation of reticulocyte lysate translation products). The 26-kDal mRNA sedimented at about 14–15 S (Fig. 4*A*). The mRNA from cycloheximide-induced cells contained also some 26-kDal activity in the 14–15S region and only trace amounts of antiviral activity at 11–12 S (Fig. 4*B*). These results also showed that 26-kDal has no antiviral activity

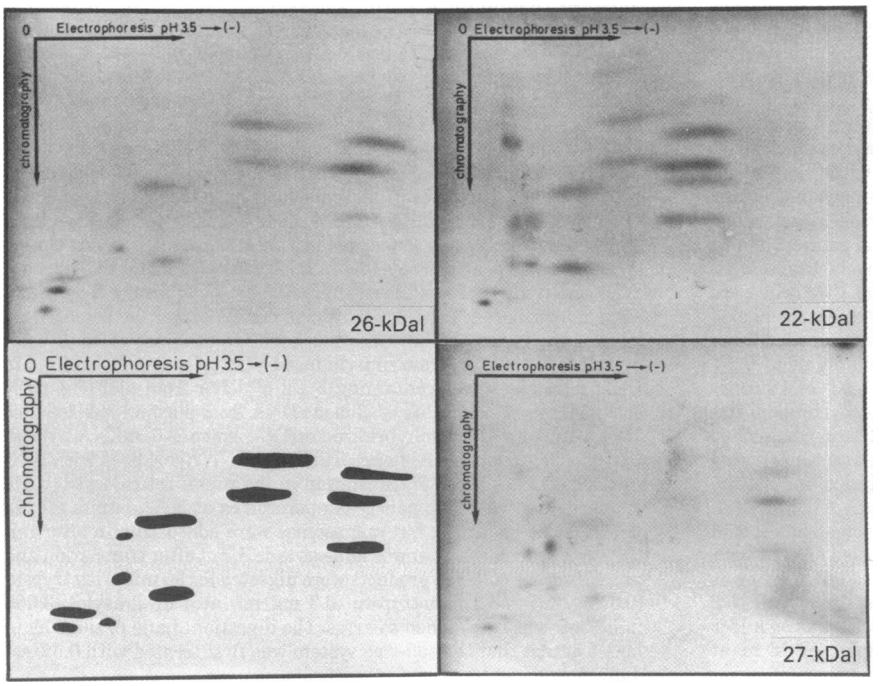

FIG. 3. Tryptic peptide analysis of [^{35}S]-methionine-labeled 22-, 27-, and 26-kDal proteins. Immunoprecipitates of labeled 22- and 27-kDal proteins from cycloheximide and actinomycin D-induced VGS cells (Fig. 1*A*, lane 5) or from a 260-μl rabbit reticulocyte translation reaction mixture programmed with 20 μg of total cytoplasmic mRNA from superinduced VGS cells were electrophoresed on a 13% preparative polyacrylamide gel. After autoradiography for 24 hr, the three protein bands were cut from the dry, stained gel and digested twice for 16 hr at 37°C with tosylamidophenylethyl chloromethyl ketone-treated trypsin at 100 μg/ml in 1% NH_4HCO_3. The digests were lyophilized twice, and they contained 20,000 cpm (22-kDal), 8,960 cpm (27-kDal), and 11,000 cpm (26-kDal). Thin-layer electrophoresis on silica gel-coated plates (Merck) at pH 3.5 was followed by ascending chromatography and fluorography at −70°C for 14 days (17). O, origin. The schematic drawing indicates the 12 spots that are common to the three maps. These spots represent the entire content of 26-kDal except for the four spots that did not migrate during electrophoresis or migrated slightly to the cathode.

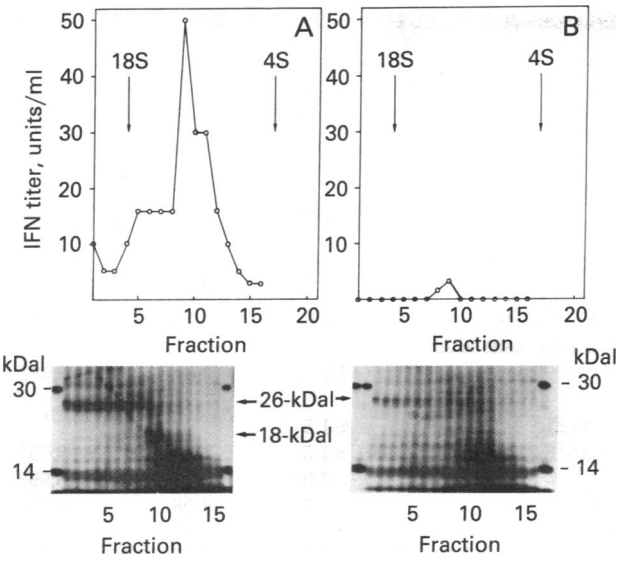

FIG. 4. Formamide/sucrose gradient analysis of poly(A)$^+$ mRNA from superinduced (*A*) and from cycloheximide-induced (*B*) MG63 cells. Samples (100 μg) of each RNA were heated for 2 min at 37°C in 50% (vol/vol) formamide and sedimented on a 5–20% (wt/vol) sucrose gradient in 50% formamide for 16.5 hr at 20°C and 40,000 rpm in a Beckman SW 60 Ti rotor. Fractions were translated in *Xenopus* oocytes and assayed for antiviral activity (*Upper*) (6, 19) or translated in a reticulocyte lysate and analyzed as described for Fig. 2 (*Lower*).

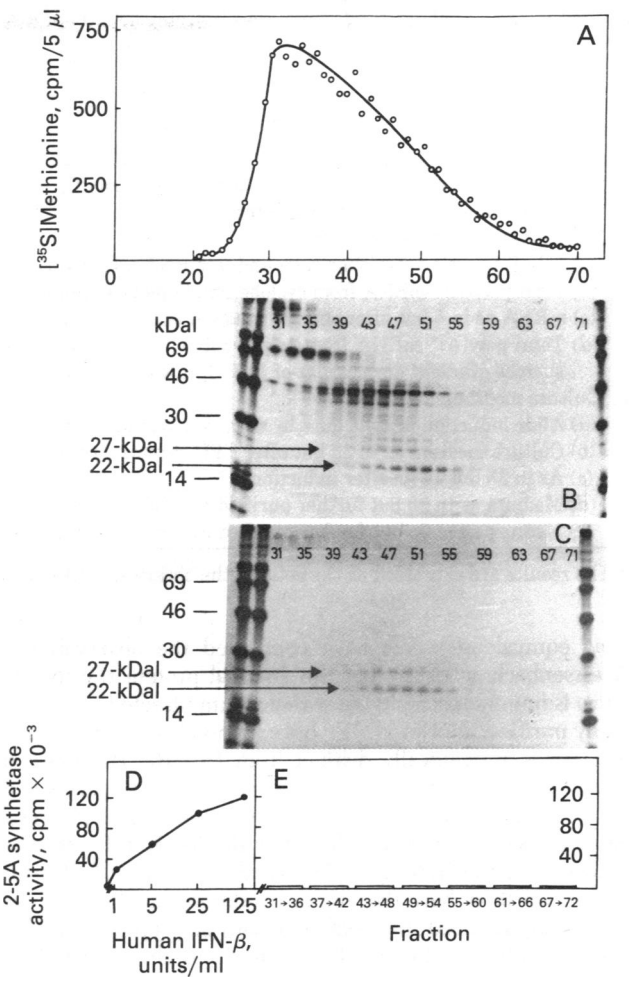

FIG. 5. Sephacryl S-200 chromatography of the 22- to 27-kDal proteins. (*A*) Two 670-cm^2 roller bottles of confluent VGS cells were induced with cycloheximide and actinomycin D (cf. Fig. 1). After the actinomycin D treatment the cultures were washed twice with phosphate-buffered saline and incubated with minimal essential medium containing 1.5% fetal calf serum (15 ml per bottle) for 4 hr at 37°C. Three milliliters of [^{35}S]methionine-labeled material (Fig. 3) was added to the collected medium. After clearing, CPG chromatography, and concentration by dialysis against polyethylene glycol 20,000, the sample was applied on a silicone (SERVA) -treated 0.7 × 30 cm column containing 10 ml of Sephacryl S-200 superfine (Pharmacia); the column was developed at 4°C, 2 ml/hr, with 0.5 M NaCl/0.1 M Tris·HCl, pH 8.0. The radioactivities of 5-μl samples of each fraction were measured in 4 ml of Aqualuma (LUMA). (*B*) Samples of alternate fractions were analyzed by electrophoresis on a 15% polyacrylamide gel followed by staining and fluorography. (*C*) Another series of 20-μl samples were immunoprecipitated with goat antiserum against impure IFN-β prior to electrophoresis and fluorography as above. (*D*) Calibration curve of the 2-5A synthetase-inducing activity on RSa cells (20–22). (*E*) Seven pools (six fractions each) were desalted by centrifugation over small Sephadex G-25 fine columns and assayed for antiviral activity (by inhibition of vesicular stomatitis virus growth on human diploid cells trisomic for chromosome 21; data not shown) and 2-5A synthetase-inducing activity at a final dilution of 1:10. The activity is expressed in cpm of [^{32}P]2-5A synthesized per 7-μl reaction mixture obtained with each of these seven regions of the column.

when its mRNA has been translated in *Xenopus* oocytes and should therefore not be considered to be another human IFN-β.

Partial Purification of the 22- and 27-kDal Proteins Synthesized *in Vivo*. The culture medium from cycloheximide/actinomycin D-induced VGS cells was mixed with a small amount of [^{35}S]methionine-labeled 22- and 27-kDal protein and first purified by CPG column chromatography. The 22- and 27-kDal material was entirely retained and could be recovered by elution with 50% (vol/vol) ethylene glycol in 1.5 M NaCl. After Sephacryl S-200 column chromatography (Fig. 5*A*) most of the 22- and 27-kDal material was in fractions 43–49, whereas 22-kDal in a rather pure form was recovered in fractions 53–57. Although not completely separated, the two proteins in the peak fractions were at least 20–50% pure, as indicated by fluorography (Fig. 5 *B* and *C*) and staining (not shown) of the polyacrylamide gels. Neither antiviral activity (<1.5 international units/ml) nor 2-5A synthetase-inducing activity (<1 unit/ml) (Fig. 5 *D* and *E*) was observed in any of these fractions. This is corroborated by the absence of these biological activities from the 26-kDal mRNA translation product (Table 1).

DISCUSSION

Several reports have described the properties of a human IFN-β mRNA that differs from the mRNA for IFN-β_1 in size (1.3 vs. 0.9 kilobases) and nucleotide sequence, although both protein species are neutralized by the same anti-IFN-β antiserum (4, 11). However, it is not clear whether this serum was anti-β_1 or anti-(β_1 + β_2). Other investigators have characterized and cloned a species of mRNA from induced human fibroblasts that also differs from IFN-β_1 mRNA in its size (1.3 kilobases) and nucleotide sequence; the *in vitro* translation product of this mRNA is a 26-kDal protein immunoprecipitable with antisera raised against crude IFN-β but not by antiserum raised against pure IFN-β_1 (9–10). After purification of this mRNA by sucrose gradient centrifugation or hybridization on cloned cDNA, it was found to code for a biologically active product having both antiviral and 2-5A synthetase-inducing activity. For this reason this mRNA was also named IFN-β_2 (10). More recently, Soreq *et al.* (11) compared the metabolic stabilities of IFN-β_1 and IFN-β_2 mRNAs and clearly implied that their IFN-β_2 mRNA species was the same as the one described by Weissenbach *et al.* (10). We attempted to clarify the situation by defining the function of the 26-kDal protein and the secreted proteins that are its *in*

Table 1. Antiviral and 2-5A synthetase-inducing activity of the *in vitro* synthesized 26-kDal protein and the *in vivo* 22- and 27-kDal proteins of different purity

Source of protein	Antiviral activity, IFN units/ml	2-5A synthetase-inducing activity*	
		Ratio	Control, cpm
1. Translation in a rabbit reticulocyte system of poly(I)·poly(C) + cycloheximide-induced RNA after hybridization on a 26-kDal cDNA filter	Not done	0.78	1,700
2. Translation in *Xenopus* oocytes of			
(*a*) Poly(I)·poly(C) + cycloheximide-induced RNA after hybridization on a 26-kDal cDNA filter	≤1.5	1.26	13,754
(*b*) Total poly(A)$^+$ mRNA from cycloheximide-induced fibroblast cells	≤1.5	0.70	15,000
(*c*) mRNA as in *b* but after sucrose gradient enrichment of the 26-kDal mRNA activity	≤1.5	1.11	2,288
(*d*) Total poly(A)$^+$ mRNA from poly(I)·poly(C) + cycloheximide-induced fibroblast cells after sucrose gradient enrichment of IFN-β_1 mRNA activity	50	5.9	2,387
3. Culture medium			
(*a*) After induction of cells for 4 hr with cycloheximide and actinomycin D (see Fig. 1)	≤1.5	Toxic	
(*b*) Culture medium as in 3*a* but after CPG chromatography and concentration (Fig. 5)	≤1.5	1.24	3,700
(*c*) As in 3*b* but 18 hr after induction	3.0	3.2	6,992
(*d*) Medium as in 3*b* but further purified by Sephacryl S-200 chromatography	≤1.5	1.08	4,182
(*e*) Medium as in 3*c* but further purified by Sephacryl S-200 chromatography	≤1.5	1.17	6,992

* The results are expressed as the ratio of the observed 2-5A oligomer to the control cpm.

vivo equivalent(s). We have confirmed the observations of Weissenbach *et al.* (9) that the 26-kDal protein translated *in vitro* is immunoprecipitable with a serum directed against partially purified interferon. We have found also that the 26-kDal protein is antigenically distinct from IFN-β_1. However, the translation product after injection into *Xenopus* oocytes is devoid of antiviral and 2-5A synthetase-inducing activity (results summarized in Table 1). The latter finding is difficult to reconcile with the findings of Weissenbach *et al.* (10). Furthermore, we show here that under conditions in which IFN-β is minimally induced (by treatment with only cycloheximide), a mRNA coding for the 26-kDal protein was also clearly detectable and it could be hybridized with 26-kDal cDNA cloned from poly(I)·poly(C)-induced human fibroblasts (Fig. 2). Again, after translation in *Xenopus* oocytes it produced neither interferon nor 2-5A synthetase-inducing activity (Table 1). The 26-kDal protein could be further processed in a reticulocyte system in the presence of dog pancreas microsomes. Presumably, the 19-kDal protein has lost a relatively long signal sequence (≈7 kDal) and is glycosylated to form the 24-kDal derivative.

Fibroblasts induced by cycloheximide and actinomycin D secrete two proteins, 22-kDal and 27-kDal, that are immunoprecipitable with the same serum that precipitated the 26-kDal protein (and its processed forms). These two proteins are structurally similar to 26-kDal as demonstrated by two-dimensional tryptic peptide analysis, but it is not known by which additional structural features they differ from the 19- and 24-kDal polypeptides (e.g., additional glycosylation, phosphorylation, etc.). The 22- and 27-kDal proteins have been purified to 20–50% by a rather mild two-step procedure and, either before or after this purification, both proteins lack antiviral or 2-5A synthetase-inducing activity. According to our data, neither the 26-kDal protein nor its *in vivo* equivalents (22-kDal and 27-kDal) can be considered as interferons (i.e., IFN-β_2).

Further studies on the kinetics of induction of 26-kDal mRNA may help to determine whether the 26-kDal protein shares some control mechanisms with those regulating the synthesis of IFN-β_1. It would also be of interest to know what the biological function of this protein is—e.g., whether it is unique to human fibroblast cells—and whether it can also be produced concomitantly with other types of interferon.

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