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Human fibroblast interferon RNA transcripts of different sizes in poly(I).poly(C) induced cells

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ABSTRACT

Northern blot analysis reveals that total RNA from human fibroblastoid cells (MG 63) induced with poly(I).poly(C) under conditions of IFN-β production, contains predominantly a ±1,200 nucleotide long poly $(A)^+$ mRNA (mRNA.M) which hybridizes with a Hu IFN-β cDNA specific probe. But hybridization with this probe also enabled the detection of a polyadenylated RNA (RNA.I) with a length of between 3.5 kb-3.8 kb, representing 0.6% of the total hybridizable cellular RNA in superinduced cells. Mapping shows that the RNA.I contains all the sequence information present in mRNA.M. Furthermore, it also hybridizes to sequences, located downstream from the IFN-β gene up to 2.5 kb beyond its poly A attachment site, while no hybridization to fragments located upstream of the IFN-β mRNA cap site was observable. Hence this RNA.I corresponds to a transcript that starts at the same position as the major mRNA.M but which extends up to 2.5 kb beyond the 3'-end of mRNA.M where another polyadenylation signal is located.

INTRODUCTION

Expression of the human IFN-β gene in fibroblastic cells is dependent upon a complex control system. The possibility of quantifying the end product using very sensitive biological assays based on the high specific activity of IFN has allowed detailed studies (see reviews in 1 and 2). Several phenomena such as priming, poly(I).poly(C) induction, shut-off, refractory period, and superinduction are known to govern expression and are mainly (or exclusively) operative at the transcriptional level (3-5). This system of IFN-β gene expression can now be studied at the molecular level because of the recent advances made in the cloning and sequencing of the corresponding cDNA (6-9). The gene is present as a single copy per haploid genome (10-12) and is located on chromosome 9 (13). The sequence of a 285 nucleotide long 5'-flanking zone and of 714 nucleotides corresponding to the 3'-flanking zone have been determined (14-16) confirming that the IFN-β contains no intervening sequences [as is also true for Hu IFN-α genes, some histone genes, heat shock protein genes in Drosophila and protein IX from adenovirus, reviewed

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by Darnell (17)]. Furthermore, the 5' end of the mRNA (or cap site) has been mapped accurately and shows the major Hu-IFN-β mRNA (mRNA.M) to have a length of 836 nucleotides (excluding the poly A tail).

The previous evaluations of the size of Hu IFN-β mRNA were based on sucrose gradient centrifugation or agarose gel electrophoresis followed by translation of the mRNA into biologically active material in Xenopus laevis oocytes (3, 5, 18-20) or rabbit reticulocyte lysate (21). In total poly A^+ mRNA from induced mouse cells, Montagnier et al. (22) detected a large mRNA species of about 30 S. We have also observed a large RNA (>18S) by formamide sucrose gradient analysis of RNA from poly(I). poly(C) induced human fibroblasts (Figure 4 in ref. 20). Using a different approach based on UV targetting, Sehgal and Tamm (23) suggested that the size of the (nuclear) primary transcript of human IFN mRNA could be 3-10 times larger than the ~1,000 nucleotides proposed by several other groups (23).

Direct sizing by Northern blotting and hybridization with a Hu IFN-β cDNA probe confirmed the existence of a major Hu IFN-β mRNA with a length of ~1,100 nucleotides in total poly A^+ RNA from poly(I).poly(C) induced fibroblasts but provided no evidence for any larger transcript (5, 24). Furthermore, initiation and termination of transcription determined by S1 nuclease mapping (24) were as predicted from genomic DNA sequencing data (14).

In view of these apparently conflicting data, and our interest in analysing the process of synthesis of an unspliced polyadenylated mRNA, and also as a prerequisite to more detailed studies on the mechanism of its transcriptional regulation, we have reinvestigated the size of Hu IFN-β mRNA. In poly(I).poly(C) induced human fibroblasts, we found, in addition to the major mature mRNA, at least one large RNA transcript (3.5 kb-3.8 kb) of the IFN-β gene comprising, in addition to its 836 nucleotides long mRNA sequence, a 3' extension of at least 1,350 nucleotides (and presumably more) downstream from the main polyadenylation site.

MATERIALS AND METHODS

1. Cells

MG 63, a human osteosarcoma fibroblastoid cell line, grown as previously described (20), was used for most of the experiments.

2. RNA extraction

RNA was extracted from whole cells by SDS-proteinase K treatment and was then precipitated twice in 2M LiCl, extracted with phenol, and further chromatographed on oligo (dT) cellulose (21).

3. Northern blotting and dot hybridization

These procedures were as described by Thomas (25). Glyoxalated RNAs were run on 1.1% agarose gel. After transfer, the nitrocellulose filters were baked for 2 h at 80°C under vacuum, prehybridized in a buffer containing 5X SSC, 50% formamide, for 3 h at 42°C and then hybridized in the same buffer containing 0.5-1.0 x 10^6 cpm/ml of ^{32}P-labelled probe and 10% dextran sulfate 500, for 17-20 h at 42°C. The filters were washed 4 times, 5 min at 20°C in 2X SSC containing 0.1% SDS and 2 times, 20 min at 50°C in 0.1X SSC containing 0.1% SDS. The dry filters were exposed to Kodak XAR films, with a Cawo intensifying screen for 1-6 days at -70°C. Escherichia coli ribosomal RNA (16 S and 23 S) (Boehringer, Mannheim) were separately stained with ethidium bromide. In other experiments, 10,000 cpm of ^{32}P-labelled chick embryo fibroblast rRNA prepared as described by Verhaegen et al., (26) was subjected to agarose gel electrophoresis and transferred by blotting. 18 S RNA transferred to the nitrocellulose filter with greater efficiency (80%) than 28 S RNA (40%).

4. DNA probes

As a reference probe for the cDNA region of the Hu IFN-β, we used the total plasmid DNA from construction pPLc-HFIF 67-11 (27). All the other probes were prepared from excised DNA fragments that had been purified by two cycles of low melting point agarose gel electrophoresis. Purity of the probes was checked by Southern blotting of restriction- digested plasmid DNA from which the probe in question had been isolated. The various probes and their locations are shown in Figure 1. All the probes

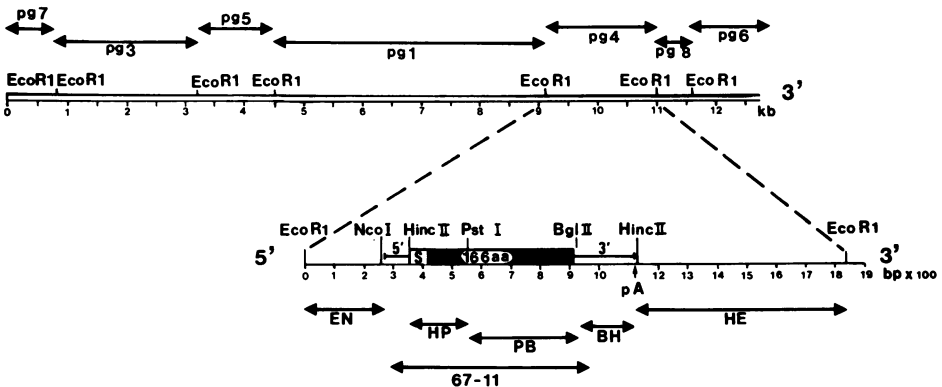


Fig. 1. Partial map of 13 Kbp of Hu IFN-β genomic DNA region
The human IFN-β coding region is represented as a black box with S indicating the signal sequence. The 5' and 3' untranslated regions and the polyadenylation site of the Hu IFN-β mRNA.M are also shown. Arrows indicate the various probes used in this study. The restriction sites used to isolate these probes are also given.

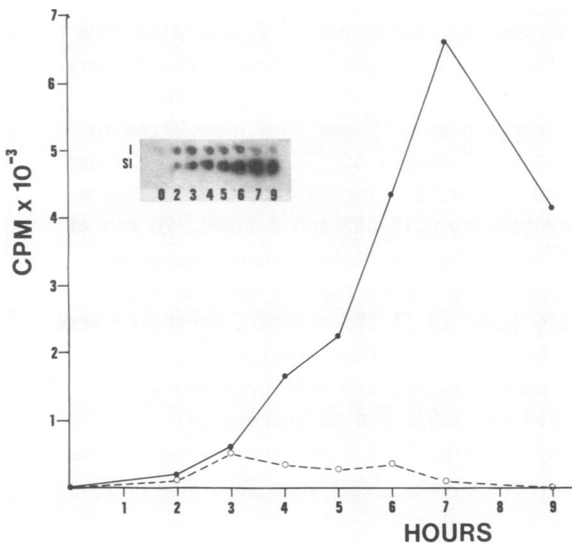


Fig. 2. Kinetics of IFN-β mRNA synthesis in induced (I) and superinduced (SI) MG 63 cells by dot hybridization with DNA probe 67-11 (see Fig 1.) 1 μg samples of poly (A)$^+$ RNA extracted at the indicated times (see Methods) were applied to a nitrocellulose filter that was treated and processed for hybridization as described by Thomas (25). After autoradiography (see insert) the dots were cut out and counted in a liquid scintillation counter. Open circles: RNA from poly(I).poly(C) induced cells; closed circles: RNA from superinduced cells.The protocols for poly(I).poly(C) induction and superinduction were as described in Materials and Methods.

were ^{32}P-labelled by nick translation (28) to a specific radioactivity of 1-5 x 10^8 cpm/μg.

RESULTS

1. Effect of superinduction by cycloheximide

Total Hu IFN-β hybridizable mRNA content as a function of time after poly(I).poly(C) induction was first evaluated by dot hybridization of total cellular poly A$^+$ RNA (Figure 2). As expected, the addition of cycloheximide prolongs and considerably increases the amount of this mRNA; thus the difference between normally induced and superinduced cells becomes even more pronounced at later times [6-9 hr after addition of poly(I).poly(C)].

The results from Northern blot analysis on the kinetics of IFN-β mRNA production in cycloheximide-treated cells are presented in Figure 3. Most of the hybridizable material present in the poly A$^+$ fraction (after one passage on oligo (dT) cellulose) corresponds to IFN-β mRNA.M although a weaker signal corresponding to an RNA of 3.5-3.8 kb is also detectable at 5 hr and later on. As described by Raj and Pitha (5), the

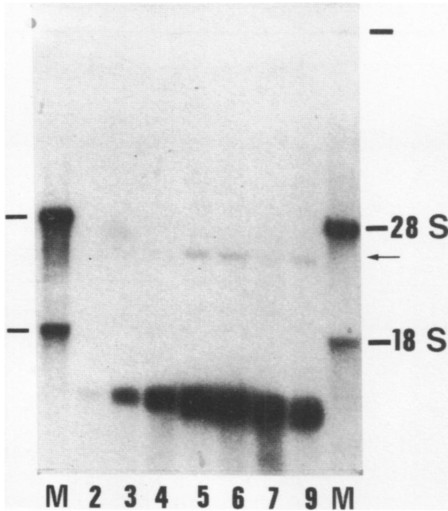


FIG. 3. Kinetics of IFN-β mRNA synthesis in poly(I).poly(C) superinduced MG 63 cells by Northern blot hybridization Various samples of RNA were denatured with glyoxal and analysed by 1.1% agarose gel electrophoresis followed by blotting and hybridization with the IFN-β cDNA probe derived from pPLc-HFIF 67-11 as described under methods. Each lane represents the pattern obtained with 2 g poly A^+ RNA. The numbers (2-9) correspond to time (in hours) after induction. Lane M indicates the migration of 28S and 18S ^{32}P rRNA markers. The arrow indicates the position of RNA.I.

mRNA.M appears heterogeneous on this kind of gel and this heterogeneity increases with incubation time, possibly corresponding to progressive degradation of IFN mRNA.M with increasing time.

These observations led us to make a more detailed examination of this large molecular weight RNA, (the 3.5-3.8 kb IFN-β transcript), referred to as RNA.I.

2. Size and poly A content of the various RNA transcripts

The size of RNA.I was estimated on a 1.1% agarose gel by comparison with four molecular weight markers (16 S, 18 S, 23 S and 28 S rRNAs) to be about 3.5 kb-3.8 kb. The standard IFN-β mRNA.M was estimated to have a mean value of about 1,200 bases but, as mentioned above, it gives a very broad heterogenous band.

It is clear from the data presented in Figure 4 that RNA.I is exclusively present in the poly A^+ fraction, even after a second run through oligo (dT) cellulose. Since we do not know the length of its poly(A) tail, the size of its transcribed region might be a few hundred nucleotides less than the value indicated above. Another positive signal comigrating with 28S rRNA is present in both the poly A^+ and poly A^- fractions after a first round of oligo (dT) cellulose chromatography; possibly this might be due to artifactual binding of the probe to the ribosomal RNA.

RNA.I represents 0.6% of the total IFN-β cDNA hybridizable RNA. This low value may indicate either that it is produced in very minute quantities or that it represents an intermediate with a rapid turnover.

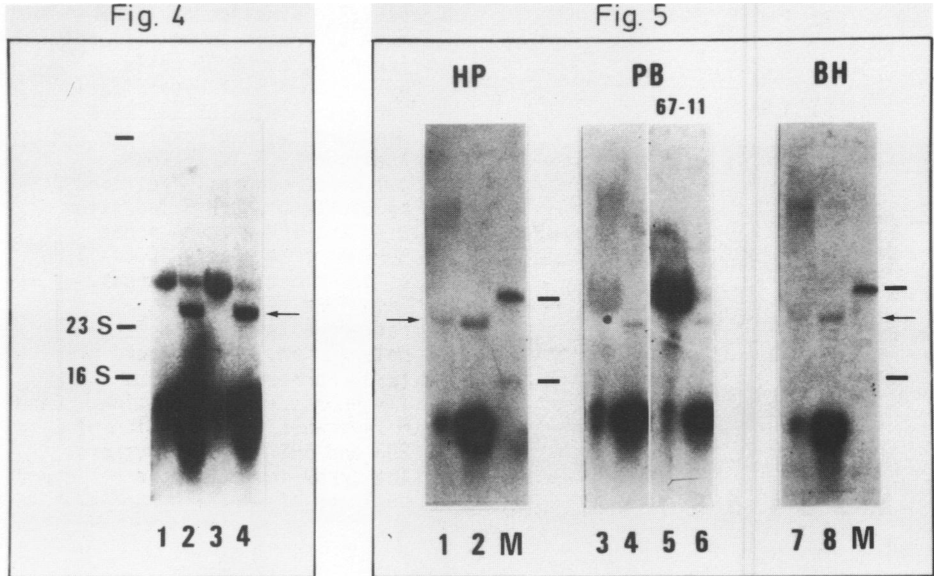


Fig. 4. Size analysis of IFN-β mRNA from poly(I).poly(C) superinduced human fibroblasts by Northern blot hybridization with DNA probe 67-11.

Distribution of the different RNAs after one cycle of oligo (dT) cellulose chromatography: lanes 1 and 3; 14 and 18 μg of the run-through RNA, lanes 2 and 4; 14 μg of the retained material after one and two consecutive cycles of oligo (dT) cellulose chromatography respectively. RNA samples from lanes 1 + 2, 3 and 4 were obtained from three different batches of superinduced MG 63 cells. Arrow indicates the position of RNA.I.

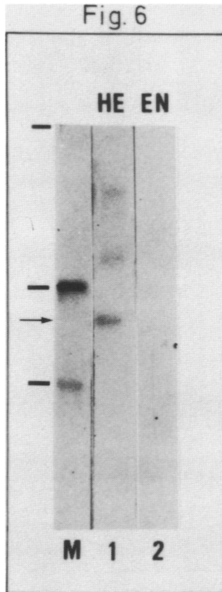
Fig. 5. Northern blot hybridization of IFN-β mRNA samples with four different DNA probes

HP, PB, BH DNA probes are defined in the map (Figure 1). RNAs were extracted from MG 63 superinduced cells 6 hr after starting the induction by the procedure described in the Methods. Each panel represents the blot hybridization of 30 μg of poly $(A)^-$ and 4 μg of poly $(A)^+$ RNA with the indicated DNA probe (or 10 μg poly $(A)^+$ RNA for the probe PB).

For panel PB, the same nitrocellulose filter was dehybridized by incubation for 2 hr in 5 mM Tris HCl, pH 8.0, 0.2 mM EDTA, 0.05% pyrophosphate, 0.002% BSA, 0.002% Ficoll, 0.002% polyvinyl pyrrolidone at 65°C. After drying, the filter was autoradiographed to check the absence of residual radioactivity. It was then hybridized to probe 67-11. Lane M indicates the migration of 28S and 18S ^{32}P rRNA markers. The arrow indicates the position of RNA.I.

3. Genomic mapping analysis of the RNA transcript I with specific DNA probes

Three probes derived from the IFN-β gene, encompassing the mRNA.M region, respectively corresponding to the coding region [210 bp,



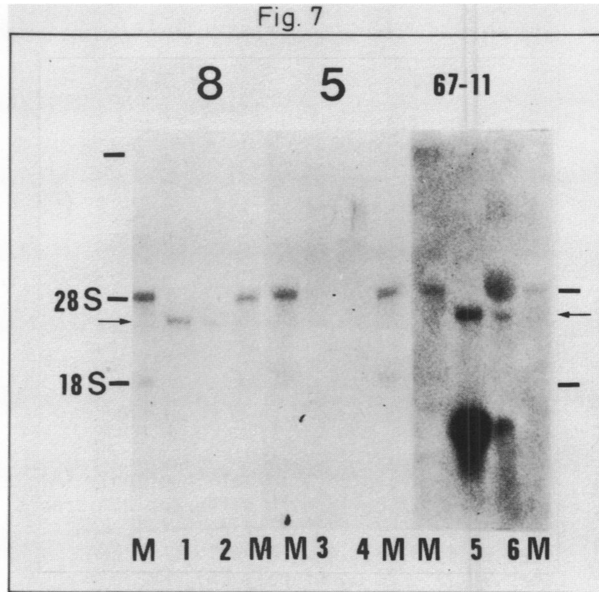


Fig. 6. Northern blot hybridization of poly $(A)^+$ RNA from MG 63 cells with different DNA probes

Each lane contains 4 μg of poly $(A)^+$ RNA that was prepared and analysed as described in legend to Figure 4. Lane 1: hybridization with a 3' flanking genomic fragment (Hinc II-Eco R1) (Figure 1). Lane 2: hybridization with a 5' flanking genomic fragment (Eco R1-Nco I) (see Figure 1). Lane M indicates the migration of 28S and 18S ^{32}P rRNA markers. The arrow indicates the position of RNA.I.

Fig. 7. Northern blot hybridization of poly $(A)^-$ and poly$(A)^+$ from superinduced MG 63 cells with different DNA probes

Lanes 1, 3, 5 : 2 μg poly $(A)^+$ RNA

Lanes 2, 4, 6 : 20 μg of poly $(A)^-$ RNA

RNAs were prepared and analysed as descibed in legends to Figures 1-4. Hybridizations were with probe pg 8 (lanes 1-2), pg 5 (lanes 3-4), pPLc 67.11 (lanes 5-6). Localization of the various probes on the genomic map is presented in Figure 1. Lane M indicates the migration of 28S and 18S ^{32}P rRNA markers. The arrow indicates the position of RNA.I.

Hinc II-Pst I (HP), and 360 bp Pst I-Bgl II (PB)], and the 3'-untranslated region [200 bp Bgl II-Hinc II (BH)] were first used to check whether the entire major mRNA region of IFN-β was present in the large transcript I. This is apparently the case since all these probes hybridized as well to mRNA.M as to RNA.I (Figure 5).

In contrast, none of the DNA fragments located upstream from the cap site (EN, pg1, pg5, pg3, pg7) hybridized with either RNA.M or RNA.I (Figures 6-8). Conversely, three DNA fragments (HE, pg8 and pg6) located

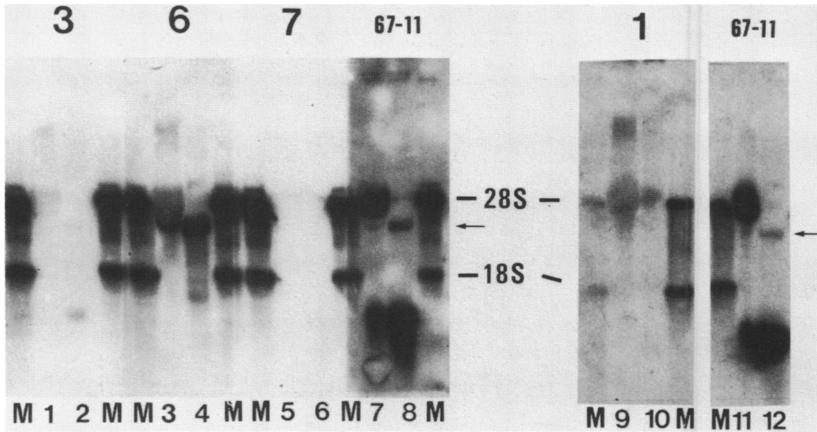


Fig. 8. Northern blot hybridization of poly $(A)^-$ and poly $(A)^+$ from superinduced MG 63 cells with different DNA probes
Lanes 1, 3, 5, 7: 30 μg of poly $(A)^-$ RNA
Lanes 2, 4, 6, 8: 4 μg of poly $(A)^+$ RNA
Lanes 9, 11: 20 μg of poly $(A)^-$ RNA
Lanes 10, 12: 4 μg of poly $(A)^+$ RNA
RNAs were prepared and analysed as described in Figure 6. Hybridizations were with probe pg 3 (lane 1 and 2), pg 6 (lane 3 and 4), pg7 (lane 5 and 6), 67-11 (lane 7 and 8), pg 1 (lane 9 and 10), 67-11 (lane 11 and 12). Locations of the various probes on the genomic map are presented in Figure 1. Lane M indicates the migration of 28S and 18S ^{32}P rRNA markers. The arrow indicates the position of RNA.I.

downstream from the poly A attachment site of the IFN-β mRNA gave a positive hybridization signal with RNA.I but not with IFN-β mRNA.M (Figures 6-8). The results, summarized in Table 1, indicate that RNA.I extends at least 1,350 nucleotides downstream from the poly A attachment site and presumably further.

DISCUSSION

We demonstrate here by hybridization the occurrence of a new component related to IFN-β mRNA but with a higher molecular weight than the major 1,200 bp component in poly A^+ RNA from poly(I).poly(C) induced MG 63 cells. This RNA of 3.5-3.8 kb (RNA.I) has been characterized as a 3' extended transcript. Using total poly A^+ RNA extracted from superinduced human fibroblasts, Raj and Pitha (5), Gross et al., (24) and Zinn et al., (29) did not detect such RNAs even in MG 63 cells (29) either by Northern blotting hybridization or by S1-mapping (24). Such differences could be due to the method used to extract the RNA or to a different stability of the large RNAs during the experimental procedures.

Table I Summary of the hybridization results obtained with the various Hu IFN-β gene derived DNA probes (see Fig. 1 for a map).

		size (bp)	mRNA.M	RNA.I
'upstream'	pg 7	800	-	-
flanking region	pg 3	2400	-	-
	pg 5	1300	-	-
	pg 1	4600	-	-
	E N	270	-	-
coding region	H P	200	+	+
	P B	360	+	+
	B H	200	+	+
'downstream'	H E	750	-	+
flanking region	pg 8	600	-	+
	pg 6	1200	-	+

The higher molecular weight hybridization signals do not belong to the group of non-IFN-β_1 mRNAs described by Sehgal and coworkers (18, 30) since in the latter case no cross-hybridization between β_2 and β_1 mRNA was observed. There is also no reason to believe that they are transcribed from the 26K protein gene (a fibroblast secreted protein co-induced with Hu IFN-β) since the latter has no nucleotide sequence homology with the IFN-β gene (20, 31). Specificity of the hybridization data also indicated that this signal does not represent other poly(I).poly(C) induced fibroblast mRNAs corresponding to an IFN-β adjacent gene as previously described (16, 32).

We have shown that RNA.I is polyadenylated, contains most probably the entire IFN-β mRNA coding region and, by virtue of its ability to hybridize to a region beyond the 3'-end of mRNA.M and not to 5'-adjacent DNA probes (Table I), represents a downstream extension of the major mRNA beyond the poly (A) termination site of at least 1,350 base pairs and probably more since its apparent molecular weight in agarose gel is close to 3.5-3.8 kb.

Recently, mouse cells have been transformed by the introduction of either Hu IFN-α (33) or the IFN-β cloned gene (29, 34-35). S1 mapping analysis of RNA from non-induced transformed mouse cells showed that in all cases some of the Hu IFN mRNA transcripts are longer than the major mRNA observed in induced human cells, and that this is due to 'incorrect' initiation, upstream from the cap site. This mechanism does not explain

our findings since RNA.I did not hybridize with DNA probes derived from the 5' flanking region (up to 9 kb upstream of the cap site) (Table I) and this phenomenon of 'upstream extension' of the RNA transcript seems more related to the introduction of a 'foreign' gene into mouse cells as similar observations have been made for the chicken ovalbumin gene (36) and for the rabbit β-globin gene system (37).

As yet we have not determined the physiological significance (if any) of this RNA.I but there are two major possibilities worth considering: 1) mRNA.M could be the primary transcription product of the Hu IFN-β gene. As a rare event this transcript might be further elongated and polyadenylated at a second site, roughly 1,350-2,600 nucleotides further downstream. The attachment of poly (A) to multiple polyadenylation sites has already been described for late gene transcription of adenovirus (17) bovine prolactin mRNA (38) and Hu IFN-α mRNA in mouse transformed cells (33). There are then three possible outcomes. This rare RNA.I could be either: i) degraded, and could therefore be considered as a minor 'discard product' (17, 39); ii) it might be effectively translated on the polysomes, or iii) it could be further cleaved down to the size of mature mRNA.M and then readenylated.

2) The other possibility is that the synthesis of RNA.I (or any other large transcripts) could conceivably represent (an) obligatory intermediate step(s) in the production of mRNA.M. A somewhat analogous situation has been described for the transcription of β -globin mRNA in nuclei from cells of mice with erythroleukaemia (40); however, the 3' extended transcripts were produced at high frequency but not polyadenylated. A precursor/product relationship although implied (17) has not actually been demonstrated. In the case of IFN-β , the successful expression of the human gene in heterologous cells after transformation with segments of DNA extending only 750 bp beyond the poly (A) site (29, 34-35) does not argue in favour of an obligatory precursor.

Finally, if additional results would strengthen the latter hypothesis, our results could be viewed as evidence for the importance of the untranscribed 3' flanking region in the transcriptional control of the IFN-β gene whereas the 5'-flanking region seems to be essential for triggering the induction process (29, 35, 41-43).

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