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Molecular cloning and characterization of an interferon induced human cDNA with sequence homology to a mammalian peptide chain release factor

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Here we report the molecular cloning of several related human cDNAs from which a full-length sequence can be determined. The cDNAs encode a 2.8 kb mRNA that is strongly induced by interferon (IFN) γ and the expression of which is not cell-restricted but observed in fibroblasts, macrophages and epithelial cells. The deduced amino acid sequence predicts a protein of 471 amino acids with high sequence similarity to a previously identified rabbit peptide chain release factor. Functional studies to demonstrate release factor activity showed that the protein encoded by this cDNA inhibited the readthrough activity of a yeast UGA suppressor tRNA in an *in vitro* translation system. The identification of this novel cDNA implies that translational control by IFN induced proteins may not be restricted to the initial steps of protein synthesis but may also act by regulation of peptide chain termination.

Key words: human cDNA/interferon induction/molecular cloning/release factor

Introduction

Interferons (IFNs) constitute a family of polypeptides with pleiotropic biological effects. IFNs are divided into two classes: type I IFNs consisting of IFN-α and IFN-β and type II IFN, or IFN-γ, which shares little structural homology with either IFN-α or IFN-β. IFNs affect a wide variety of cellular functions by altering the expression of specific genes (for review see Revel and Chebath, 1986; Tamm *et al.*, 1987; Vilcek, 1989). Several genes regulated by IFNs are involved in the antiviral response, including oligo 2.5(A) synthetase, eIF-2α kinase and Mx genes (Golgher *et al.*, 1980; Merlin *et al.*, 1983; Samuel *et al.*, 1984; Benech *et al.*, 1985; Saunders *et al.*, 1985; Staeheli *et al.*, 1986; Arnheiter and Haller, 1988; Meurs *et al.*, 1990). Interferons also enhance the expression of genes which may have roles beyond the classical antiviral pathways such as in cell growth and regulation of immune responses. Thus, the enhanced synthesis of class I and class II histocompatibility genes (Böttger *et al.*, 1988; Blanar *et al.*, 1988) as well as of a new family of cytokines related to platelet factor 4 (Luster *et al.*, 1985; Wolpe and Cerami, 1989; Farber, 1990) has been implicated in regulation of immune responses and development of autoimmune reactions (Nakamura *et al.*, 1984; Jacob *et al.*, 1987). The ability of IFNs to inhibit cell growth is observed with many cell types and is particularly effective in the case of tumor cells (Kohase *et al.*, 1986; De Maeyer and De Maeyer-Guignard, 1988).

The termination of protein synthesis is encoded by in-frame nonsense (stop) codons. In contrast to sense codons, which are decoded by specific tRNAs, nonsense codons are decoded by proteins (Caskey, 1980) called release factors (RFs). RFs have been purified from both prokaryotes (Craigen and Caskey, 1987; Craigen *et al.*, 1990) and several eukaryotic sources including rabbit reticulocytes and rat liver (Goldstein *et al.*, 1970; Caskey *et al.*, 1974; Innanen and Nichols, 1974; Konecki *et al.*, 1977). A single RF of a molecular weight of 54 000-56 000 Daltons that recognizes all three nonsense codons (UGA, UAG and UAA) and which has a ribosome-dependent GTPase activity has been described in eukaryotes (Goldstein *et al.*, 1970; Caskey *et al.*, 1974; Konecki *et al.*, 1977).

IFNs have been shown to regulate the initiation of protein synthesis by inducing a kinase which upon activation by ds RNA phosphorylates and thereby inactivates the α-subunit of protein synthesis initiation factor eIF2 resulting in inhibition of protein synthesis (Levin and London, 1978; Samuel *et al.*, 1984). In this report we describe the molecular cloning of an interferon induced human cDNA with sequence homology to a previously identified rabbit peptide chain RF.

Results

A cDNA library was prepared from polyadenylated mRNA extracted from human HeLa cells after treatment with IFN-γ for 48 h. Thirty-six thousand primary recombinants were screened in duplicate by differential hybridization and positive clones were subjected to two further rounds of plaque purification using ^{32}P-labelled first strand cDNA probes from control and IFN-γ treated HeLa cells. Of the resulting clones 40 were found to cross-hybridize and to vary in insert sizes between 0.8 and 1.8 kb. Overlapping clones were identified by restriction enzyme mapping and Southern blot hybridization. An oligonucleotide derived from the 12.1 insert was used as a probe to isolate additional clones from a random-primed cDNA library from IFN-γ treated HeLa cells. A composite map of selected cDNAs is shown in Figure 1 (the gene encoding the cDNAs is named IFP 53 according to the molecular weight).

The steady-state levels of IFP 53 mRNA were assayed by Northern blot analysis in IFN-γ treated HeLa cells. Treatment with 10-1000 U of IFN-γ resulted in a significant increase in the level of an ~2.8 kb mRNA, the maximum being observed with 1000 U/ml IFN-γ (Figure 2B). On treatment with 1000 U/ml IFN-γ, the IFP 53 mRNA was detectable at 6 h and reached a maximum at 48 h (Figure 2A and D). Accumulation of this mRNA therefore continues in the presence of interferon. In a chase experiment using actinomycin D, the half-life of the IFP 53 mRNA was estimated to be 10 h (data not shown). The response of the IFP 53 mRNA was different for class I and class II

interferons. While a detectable increase was seen with as little as 10 U/ml of IFN-γ, none was seen with 250 U/ml of IFN-α or IFN-β (Figure 2C). A more detailed analysis of the effects of IFN-α on IFP 53 mRNA expression using 100, 250, 500 and 1000 U/ml and time courses of 2, 4, 6, 12 and 24 h was performed and confirmed that IFN-α does not affect IFP 53 mRNA levels (data not shown).

To determine the range of cell types expressing the IFP 53 gene we examined primary human fibroblasts, WISH cells (a human epithelial-like amnion tissue derived cell line) and U937 cells (a human histiocytic lymphoma cell line) by Northern blot analysis. The IFP 53 gene was synthesized in response to IFN-γ in all cell types investigated (see Figure 3) including human adherent peripheral mononuclear cells and human keratinocytes (data not shown).

To investigate whether the increase in IFP 53 mRNA in HeLa cells was dependent on protein synthesis, HeLa cells were treated with cycloheximide for various periods in the absence or presence of IFN-γ. As shown in Figure 4A the inducibility of IFP 53 mRNA was abolished by cycloheximide treatment indicating that the effect of IFN-γ on IFP 53 mRNA levels is a secondary response and requires the synthesis of other factors. To determine whether the induction of the IFP 53 mRNA by interferon is regulated at the transcriptional level, nuclear run-on analyses were performed. The transcription of the gene became apparent as early as 2 h of treatment with IFN-γ followed by an increase in transcription rate thereafter (Figure 4B). The effects of interferon on the transcription of the actin gene and the tubulin gene were analysed in parallel and found to be unchanged. The transcription of the IFP 53 gene in HeLa cells is not rapidly down-regulated, in contrast to some interferon induced genes in other cell lines (Friedman *et al.*, 1984). As would have been expected from the Northern blot analysis shown in Figure 4A, inhibition of protein synthesis with cycloheximide prevented the interferon induced level of transcription, although a small increase was still observable after treatment with cycloheximide, which we attribute to some protein synthesis not blocked by cycloheximide. Cycloheximide itself did not induce IFP 53 gene transcription (data not shown).

The 9.2 cDNA was used to hybridize to a genomic Southern blot (Figure 5). A simple pattern of digestion was obtained which is most consistent with IFP 53 representing a single copy gene.

The complete nucleotide sequence of the IFP 53 cDNA was determined by manual sequencing of four overlapping cDNA clones and the predicted protein sequence is shown in Figure 6 (EMBL sequence accession number X62570). Sequence analysis revealed a contiguous open reading frame starting at nucleotide position 119 that is 1413 nucleotides in length. The predicted ATG codon is preceded by a 5′ untranslated region, which contains stop codons in any of the three reading frames. The sequence flanking the putative start codon at position 119 AGCAACATGC shows homology to Kozak's consensus sequence GCCA/GCCATGG for translation initiation sites (Kozak, 1986). Further, albeit less likely (see results of *in vitro* translation) in-frame ATG codons occur at nucleotide positions 242 and 260. The UAG stop codon at position 1532 is followed by 1985 bp of 3′ untranslated sequence including the conventional polyadenylation signal AATAAA at position

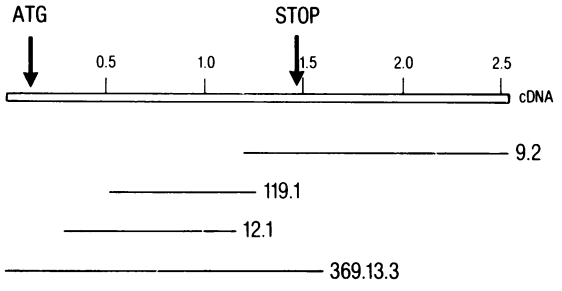

Fig. 1. Composite map of cDNAs encoding the IFP 53 gene. The cDNAs 9.2, 119.1 and 12.1 were isolated from an oligo(dT) primed λgt10 library using differential hybridization. cDNA 369.13.3 was isolated from a random primed λgt11 library using an oligonucleotide probe derived from the sequence of 12.1.

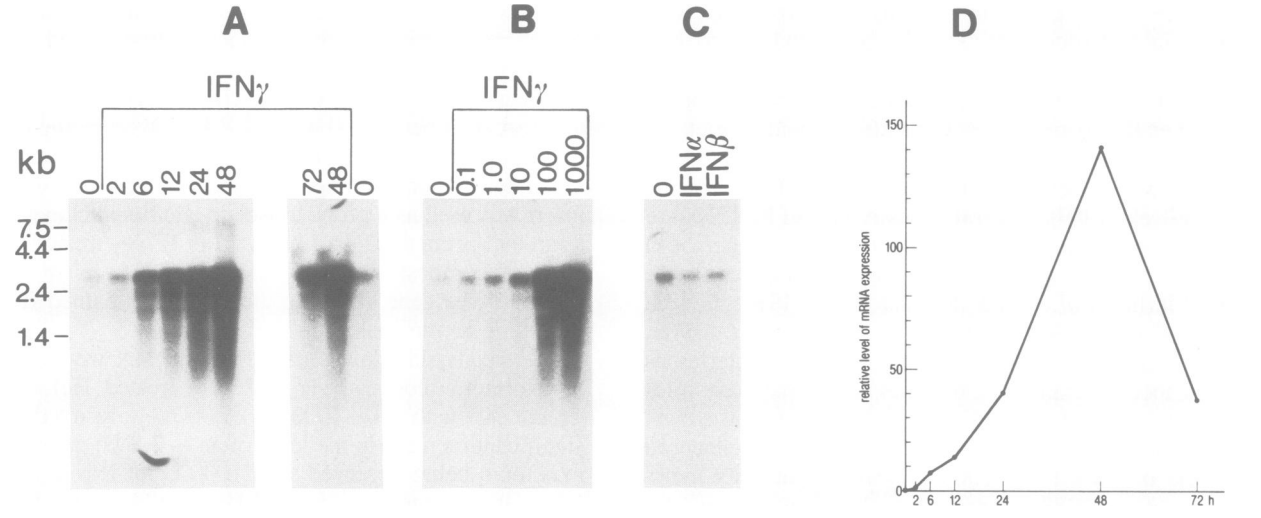

Fig. 2. Dose–response and kinetics of induction of IFP 53 mRNA by IFN-γ. Samples (10 μg) were resolved on a 0.8% agarose gel and hybridized with the 9.2 cDNA probe (the 12.1 cDNA probe gave the same results). (**A**) Total cellular RNA was extracted from HeLa cells after 0, 2, 6, 12, 24, 48 and 72 h with 1000 U/ml IFN-γ. (**B**) Northern blot of total cellular RNA extracted from HeLa cells after 24 h of treatment with increasing doses of IFN-γ (0.1, 1, 10, 10^2 and 10^3 U/ml). (**C**) Total cellular RNA was extracted from HeLa cells after 0 and 24 h with 250 U/ml IFN-α or IFN-β. (**D**) Densitometric analysis of (A). IFP 53 mRNA values were corrected by hybridization to a pyruvate kinase probe to ensure equal loading of mRNA (Böttger *et al.*, 1988).

2598, 19 nucleotides upstream of the poly(A) tail. The deduced polypeptide of 471 amino acids has a calculated isoelectric point of 5.88 and a calculated molecular weight of 53 250 Daltons. A comparison of the predicted protein sequence of the IFP 53 gene to the EMBL database (release 25.0) revealed high sequence similarity with a previously described rabbit peptide chain release factor (Lee *et al.*, 1990). Figure 6 shows an alignment of these two proteins. There is a 86.2% sequence homology of identical amino acids throughout the entire protein (corresponding to a 85.4% sequence identity at the nucleic acid level).

The nearly full-length of the composite cDNA was demonstrated by primer extension analysis. Reverse transcription of IFN-γ stimulated HeLa cell mRNA from an oligonucleotide primer complementary to nucleotides 138−158 of the predicted translated sequence shows that the start site of transcription occurs 280 nucleotides upstream of the primer sequence (Figure 7), indicating that the presumed ATG initiation codon is preceded by 142 bp of 5′ untranslated sequence. In comparison, the cDNA clone with the most 5′ sequences, i.e. 369.13.3, contains 120 bp of 5′ untranslated sequence.

To verify the predicted open reading frame encoding the IFP 53 gene, *in vitro* translation experiments were carried out. Purified recombinant plasmid was used for hybrid selection of the mRNA. The products of translation selected by the 12.1 cDNA and synthesized *in vitro* were analysed by SDS−PAGE and are shown in Figure 8. The mRNA selected directs the synthesis of a 53 kDa polypeptide not present in the control lanes, demonstrating that the initiation codon for the message is at the predicted ATG codon at position 119 and that translation termination occurs at nucleotide 1532.

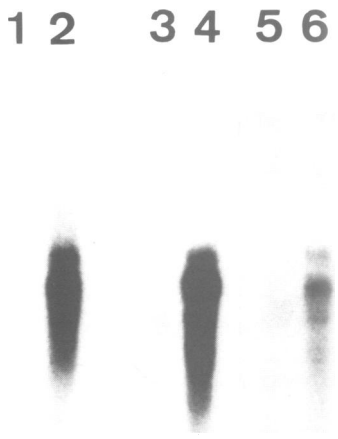

Fig. 3. Interferon induced expression of the IFP 53 gene in different human cell types. Total cellular RNA was extracted after treatment with 1000 U/ml IFN-γ for 24 h or medium alone and hybridized by Northern blot hybridization using the 9.2 cDNA probe. Lane 1, human primary fibroblasts untreated (5 μg total RNA); lane 2, human primary fibroblasts treated with IFN-γ (5 μg total RNA); lane 3, WISH cells untreated (10 μg total RNA); lane 4, WISH cells treated with IFN-γ (10 μg total RNA); lane 5, U937 cells untreated (10 μg total RNA); lane 6, U937 cells treated with IFN-γ (10 μg total RNA).

To address the question of stop codon recognition and release factor activity of IFP 53 functional studies were carried out using an *in vitro* translation system and a mRNA with a known termination codon. The globin mRNA preparation which we used in our experiments is a mixture of α- and β-globin mRNAs. β-globin is terminated by a UGA stop codon (Efstratiadis *et al.*, 1977). In the presence of UGA suppressor tRNA recognition of the UGA termination codon yields a β-globin readthrough protein which differs significantly in size from the major translation product (Hanyu *et al.*, 1986). As can be seen in Figure 9A the synthesis of the readthrough protein in an *in vitro* reticulocyte system is correlated with a suppressor tRNA capable of recognizing the UGA termination codon. The IFP 53 cDNA was tested for its ability to inhibit the UGA suppressor tRNA mediated readthrough of β-globin mRNA.

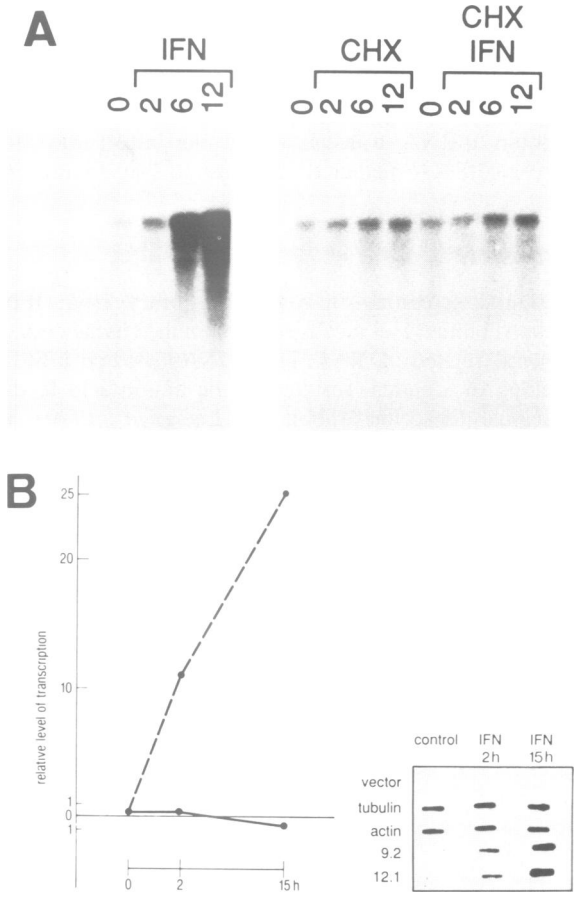

Fig. 4. (**A**) Effects of cycloheximide treatment of HeLa cells on IFP 53 mRNA accumulation. RNA was isolated from HeLa cells treated for various periods with 10 μg/ml cycloheximide (CHX), 1000 U/ml IFN-γ (IFN), 10 μg/ml cycloheximide plus 1000 U/ml IFN-γ (IFN, CHX) or medium alone (○). Total RNA was fractionated, transferred and hybridized to the 9.2 cDNA probe. (**B**) Transcription of the IFP 53 gene in HeLa cells. Assay of nuclear transcripts of the IFP 53 gene after treatment with interferon. HeLa cells were incubated with medium alone (○) or treated for 2 and 15 h with 1000 U/ml IFN-γ. RNAs from isolated nuclei labelled *in vitro* with [α-^{32}P]UTP were analysed by hybridization with different recombinant plasmids (β-tubulin, β-actin, 9.2, 12.1) bound on nitrocellulose membrane filters. Plasmid DNA (Bluescript) was used to measure background levels. Quantitation was by densitometric scanning of the autoradiographs, transcription rates are presented as fold increase compared with the unstimulated transcription rate. Dotted lines represent the IFP 53 gene, solid lines the actin gene.

For this assay the *Eco*RI insert from clone 369.13.1 which contains a full-length IFP 53 cDNA, was cloned into the pGEM4Z vector under control of an SP6 promoter resulting in the recombinant plasmid pGEM13.3. The capped transcript was used for the *in vitro* translation studies. In the presence of preformed IFP 53 protein, which was generated by first translating pGEM13.3 mRNA *in vitro* before adding it to fresh reticulocyte lysates containing globin mRNA and varying amounts of UGA suppressor tRNA, the readthrough of β-globin mRNA was inhibited (see Figure 9B). This inhibition was (i) dependent on the concentration of suppressor tRNA used: complete inhibition was seen when using 30 μg/ml tRNA (only background bands resulting from pGEM13.3 mRNA translation are seen at the respective gel position, compare lane 7 with lane 5, Figure 9B), partial although major inhibition was seen when using 150 μg/ml tRNA. (ii) The inhibition of readthrough activity by IFP 53 was dependent on preformed IFP 53 protein at the initiation of globin translation, i.e. in contrast to the situation where pGEM13.3 was first translated to yield preformed IFP 53 protein in the readthrough assay, the simultaneous addition of pGEM13.3 mRNA and suppressor tRNA at the initiation of globin mRNA translation did not affect the UGA suppressor tRNA mediated readthrough (see Figure 9C).

Discussion

Differential screening of a cDNA library from IFN-γ stimulated human HeLa cells resulted in the characterization of several related cDNAs. The cDNAs showed a 86.2% homology of identical residues at the amino acid level to a previously described rabbit RF (Lee *et al.*, 1990). The structural similarity of RFs to aminoacyl-tRNA synthetases in general and to tryptophanyl-tRNA synthetase in particular as well as the lack of evolutionary conservation between bacterial and eukaryotic RFs has been noted earlier (Lee *et al.*, 1990).

Hybridization selection and *in vitro* translation were performed to confirm the predicted open reading frame and yielded a protein of 53 kDa which is in accordance with the 54–56 kDa estimated for purified mammalian RFs (Goldstein *et al.*, 1970; Caskey *et al.*, 1974; Konecki *et al.*, 1977). Southern blot analysis of human genomic DNA suggested that the IFP 53 gene is encoded by a single gene (see Figure 5), which codes for a 2.8 kb mRNA. Primer extension revealed that the composite cDNA is of full length (Figure 7). The steady-state levels of the mRNA are enhanced several-fold in an interferon dose-dependent manner in a variety of different cells (Figures 2 and 3). Transcription of IFP 53 is induced following exposure to interferon-γ (Figure 4) and is markedly affected by inhibition of protein synthesis. Thus, the induction of the IFP 53 gene by interferon is a secondary response requiring the synthesis of other factors. A similar effect mediated by cycloheximide on the induction of other genes by interferon was reported previously (Böttger *et al.*, 1988; Blanar *et al.*, 1988).

To examine stop codon recognition and RF activity of IFP 53 functional studies using an inhibition assay of suppressor tRNA mediated readthrough were performed. A substantial amount of a UGA readthrough product was produced in the presence of yeast UGA suppressor tRNA when globin mRNA was translated *in vitro* in a reticulocyte cell-free protein synthesizing system (Figure 9A). The IFP 53 protein was assayed for its ability to inhibit the UGA suppressor tRNA mediated readthrough of β-globin mRNA. As shown in Figure 9B the readthrough activity of UGA suppressor tRNA was blocked when IFP 53 protein was present at the initiation of β-globin mRNA translation. These functional studies show that the IFP 53 cDNA encodes a protein which recognizes the UGA stop codon and has peptide chain termination activity.

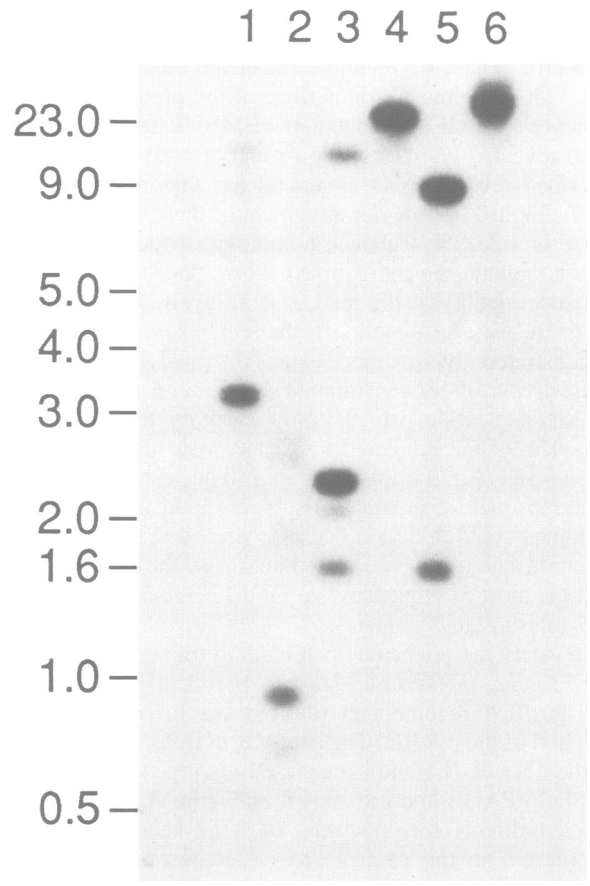

Fig. 5. Genomic Southern blot using DNA from HeLa cells digested with *Xho*I (lane 1), *Pst*I (lane 2), *Hin*dIII (lane 3), *Eco*RI (lane 4), *Bam*HI (lane 5) or undigested (lane 6). The blot was hybridized to the 9.2 cDNA insert.

One well known effect of IFNs on protein synthesis is mediated by a ds RNA-activated protein kinase, also termed p68, which is induced by IFNs (Levin and London, 1978; Samuel *et al.*, 1984). Once induced by IFN the kinase becomes activated by ds RNA in the presence of ATP leading to autophosphorylation (Galabru and Hovanessian, 1977; Levin and London, 1978). The autophosphorylated p68 kinase catalyses the phosphorylation of the α-subunit of the eukaryotic initiation factor 2 (eIF-2α) (Meurs *et al.*, 1990). eIF-2α plays a pivotal role in the earliest regulated steps of translation initiation and phosphorylation of eIF-2α leads to subsequent inhibition of translation initiation (Farrell *et al.*, 1977). Consequently, with respect to translational regulation affected by IFNs much attention was given to the p68 kinase. In addition, the p68 kinase has been suggested to be important in the establishment of the antiviral state mediated by type I IFNs (for review see Hovanessian, 1989).

The termination of protein synthesis is signalled by in-frame termination codons UAA (ochre), UAG (amber)

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IFP 53          MPNSEPASLLELFNSIATQGELVRSLKAGNASKDEIDSAVKMLVSL -46
                      ::  ::: :::.:::::.::::  : :.:::::::::.:: 
rabbit RF   MADVTNGERCASPQELFSSIAAQGELVKSLKARKAPKEEIDSAVKMLLSL -50

IFP 53      KMSYKAAAGEDYKADCPPGNPAPTSNHGPDATEAEEDFVDPWTVQTSSAK -96
            : ::: : :::::::::::: .: :   :.: .  ::::::::: ::::: 
rabbit RF   KTSYKEAMGEDYKADCPPGNSTPDSHGDPEAVDDKEDFVDPWTVRTSSAK -100

IFP 53      GIDYDKLIVRFGSSKIDKELINRIERATGQRPHHFLRRGIFFSHRDMNQV -146
            ::::::::: ::::::::::.:::::::::::: ::::::::::::::::
rabbit RF   GIDYDKLIVQFGSSKIDKELVNRIERATGQRPHRFLRRGIFFSHRDMNQV -150

IFP 53      LDAYENKKPFYLYTGRGPSSEAMHVGHLIPFIFTKWLQDVFNVPLVIQMT -196
            ::::::::::::::::::      :::::::::::::::::: ::::.::.
rabbit RF   LDAYENKKPFYLYTGRGPLLKQCNVGHLIPFIFTKWLQDVFDVPLVVQMS -200

IFP 53      DDEKYLWKDLTLDQAYSYAVENAKDIIACGFDINKTFIFSDLDYMGMSSG -246
            ::::::::::::.: : :..:::::::. ::::.::::::::::::::: :
rabbit RF   DDEKYLWKDLTLEQVYGYTLENAKDIMPCGFDVNKTFIFSDLDYMGMSPG -250

IFP 53      FYKNVVKIQKHVTFNQVKGIFGFTDSDCIGKISFPAIQAAPSFSNSFPQI -296
            ::::::::::::::::::::::::::::::::::::::::::::::::::
rabbit RF   FYKNVVKIQKHVTFNQVKGIFGFTDSDCIGKISFPAIQAAPSFSNSFPQI -300

IFP 53      FRDRTDIQCLIPCAIDQDPYFRMTRDVAPRIGYPKPALLHSTFFPALQGA -346
            :   .::::::::::::::::::::::::::::::::::::::::::::::
rabbit RF   FHGQADIQCLIPCAIDQDPYFRMTRDVAPRIGYPKPALLHSTFFPALQGA -350

IFP 53      QTKMSASDPNSSIFLTDTAKQIKTKVNKHAFSGGRDTIEEHRQFGGNCDV -396
            ::::::::::::::::::::::::::::::::::::::::::::::::::
rabbit RF   QTKMSASDPNSSIFLTDTAKQIKTKVNKHAFSGGRDTIEEHRQFGGNCDV -400

IFP 53      DVSFMYLTFFLEDDDKLEQIRKDYTSGRMLTGELKKALIEVLQPLIAEHQ -446
            :::::::::::::::::::::::.:: :::::::: ::.::::::.::::
rabbit RF   DVSFMYLTFFLEDDDKLEQIRKDYSSGAMLTGELKKELIDVLQPLVAEHQ -450

IFP 53      ARRKEVTDEIVKEFMTPRKLSFDFQ -471
            :::::::::.:::::::: : : .:
rabbit RF   ARRKEVTDEMVKEFMTPRQLCFHYQ -475
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Fig. 6. Deduced amino acid sequence (top strand) of the IFP 53 gene aligned with the rabbit release factor (bottom strand). Identical amino acids are indicated by colons (:), similar amino acids by dots (·). The predicted protein sequence of the IFP 53 gene is composed of 471 amino acids. The polypeptide has a calculated isoelectric point of 5.88 and a calculated molecular weight of 53 250 Daltons. Potential *N*-glycosylation sites (Asn-Xaa-Ser/Thr) occur at positions 30, 230 and 356. The IFP 53 gene shows a homology of 86.2% identical amino acids with the rabbit release factor (Lee *et al.*, 1990), if conserved amino acid substitutions are included in this calculation the homology increases to 90.7%.

or UGA (opal). Unlike sense codons which are decoded by specific tRNAs via RNA−RNA interactions, stop codons are decoded by proteins termed release factors (Caskey, 1980).

Termination codons are likely to contribute to the regulation of a variety of genes. Studies in prokaryotes have shown that RFs and suppressor tRNAs compete for translation of termination codons *in vitro* (for review see Craigen *et al.*, 1990). Normal sense-coding tRNAs can translate termination codons (resulting in readthrough proteins), albeit at low efficiency in a wide range of organisms (for review see Valle and Morch, 1988). Examples of such natural suppressor tRNAs have been described in calf liver (Valle *et al.*, 1987) and in mouse liver (Kuchino *et al.*, 1987). Recognition of termination codons might also serve to insert modified amino acids into proteins. Se-Cys-specific tRNAs specifically recognize the UGA codons and lead to cotranslational incorporation of Se-Cys into proteins (Zinoni *et al.*, 1986; for review see Stadtman, 1990; Böck *et al.*, 1991).

Retroviruses synthesize gag−pol fusion proteins, which are later cleaved by a virus-encoded protease to yield the mature pol protein responsible for reverse transcription and integration (for review see Weiss *et al.*, 1982). The genetic structure of the gag−pol domain of the Moloney murine leukemia virus (Mo-MuLV) precludes synthesis of this fusion protein as gag and pol are separated by an in-frame termination codon (Yoshinaka *et al.*, 1985). *In vitro* translation analysis was used to demonstrate that yeast suppressor tRNA can efficiently suppress the termination codon between the *gag* and *pol* genes of Mo-MuLV (Philipson *et al.*, 1978). *In vivo*, as shown by protein analysis, a stop codon readthrough resulting in the insertion of a Gln residue in response to the UAG codon located at the end of the Mo-MuLV *gag* gene results in expression of the gag−pol fusion protein (Yoshinaka *et al.*, 1985).

For type I IFNs translational control has been shown to act at the level of initiation of protein synthesis by the p68 kinase (Meurs *et al.*, 1990). Here we characterize a human cDNA, named IFP 53, which is induced in a variety of different cells in response to IFN-γ. IFP 53 shows near identity to a rabbit RF and has peptide chain termination activity for the UGA stop codon. As eukaryotes have a single RF recognizing all three termination codons UGA, UAG and UAA (Caskey *et al.*, 1974) these results suggest that IFP 53 may represent the human peptide chain release factor

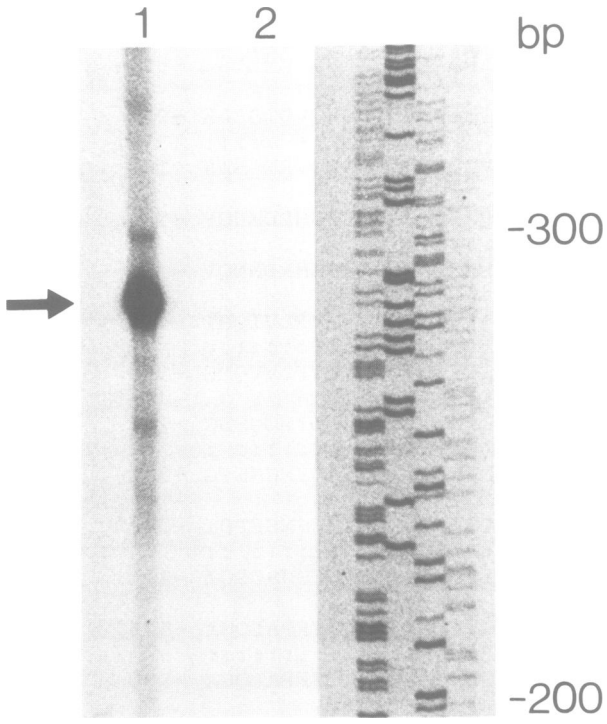

Fig. 7. Transcription initiation site of IFP 53 mRNA. The start site for initiation of IFP 53 mRNA was determined by primer extension using a synthetic oligonucleotide complementary to nucleotides 138–158 of the predicted translated sequence. Total RNA from HeLa cells stimulated with 1000 U/ml of IFN-γ or medium alone was hybridized to the primer and extended using reverse transcriptase. The extended products were electrophoresed adjacent to dideoxy sequencing reactions as size markers. The extended product (280 bp) is indicated; lane 1, interferon treated HeLa cells; lane 2, HeLa cells treated with medium alone.

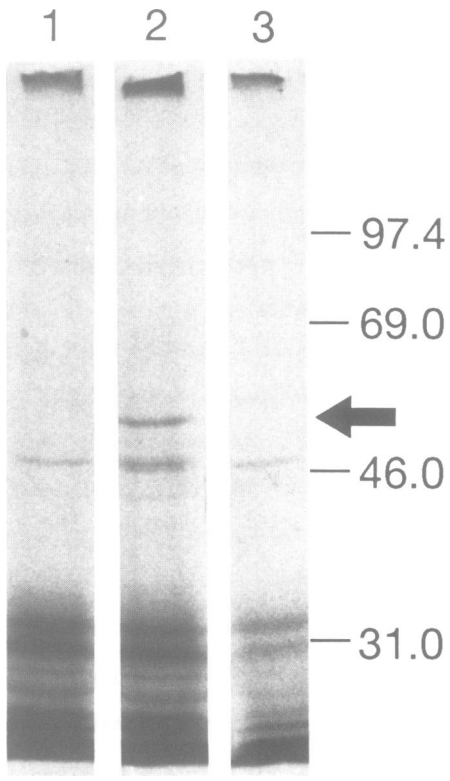

Fig. 8. SDS–PAGE of an *in vitro* translation of hybrid selected poly(A)$^+$ mRNA from HeLa cells treated with 1000 U IFN-γ/ml for 48 h. The 12.1 cDNA selects for a mRNA encoding a protein of 53 kDa which is indicated by an arrow. Lane 1, Bluescript vector was used for hybrid selection; lane 2, purified recombinant plasmid 12.1 was used for hybrid selection; lane 3, *in vitro* translation without added hybrid selected poly(A)$^+$ mRNA. Molecular weight markers were obtained from Bio-Rad [rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (69.0 kDa), hen ovalbumin (46.0 kDa), bovine carbonic anhydrase (31.0 kDa)].

or a protein of related function. Our findings indicate that besides IFN-α and IFN-β influencing the initiation of protein synthesis IFN-γ may exert translational control by affecting the termination of protein synthesis.

Materials and methods

Cells

The human epithelial adenocarcinoma cell line, HeLa, was obtained from A.Baldwin and P.Sharp (Massachusetts Institute of Technology Cancer Center) and has been described previously (Blanar *et al.*, 1988). HeLa cells were grown as monolayer cultures in DMEM supplemented with 10% fetal calf serum. The human amnion tissue derived epithelial cell line, WISH, was received from P.v.Wussow (Medical School, Hannover) and was grown in monolayer cultures in RPMI supplemented with 10% fetal calf serum. U937 cells, a human histiocytic lymphoma cell line, were provided by M.Goppelt-Strübe (Medical School, Hannover) and grown in suspension in RPMI supplemented with 10% fetal calf serum. Human primary fibroblasts (16th passage) were a gift from B.Meyer (Veterinary School, Hannover) and were grown as monolayers in DMEM supplemented with 10% fetal calf serum. Circulating adherent human peripheral mononuclear cells were obtained from a healthy volunteer and isolated by Ficoll Hypaque gradient centrifugation followed by 1 h adherence on plastic Petri dishes. Non-adherent cells were washed off and the adherent cells subsequently incubated for the indicated periods in RPMI supplemented with 10% AB serum. Recombinant human IFN-γ (Bioferon, 2×10^7 U/mg protein) and IFNα-2b (Intron A, 1×10^8 U/mg protein, Schering) were kindly provided by P.v.Wussow (Medical School, Hannover), recombinant human IFN-β was a gift from H.-J.Hauser (Gesellschaft für Biotechnologische Forschung, Braunschweig). Cycloheximide was used at a final concentration of 10 μg/ml. This concentration has previously been shown to reduce protein synthesis by 95% in the HeLa cells used (Blanar *et al.*, 1988).

cDNA libraries, differential screening and isolation of cDNA clones

Cytoplasmic RNA was extracted from HeLa cells treated for 48 h with 1000 U/ml of human IFN-γ, polyadenylated RNA prepared and purified twice by oligo(dT)–cellulose chromatography and a cDNA library was then constructed in λgt10 according to standard protocols using oligo(dT)-primed cDNA from 10 μg of poly(A)$^+$ mRNA (Sambrook *et al.*, 1989). The complexity of the library was 2×10^6 independent recombinants and for further analysis the library was amplified once. Duplicate plaque lifts were performed according to standard protocols and 36 000 plaques of the interferon treated HeLa cell cDNA library were screened by differential hybridization using ^{32}P-labelled first strand cDNA probes derived from interferon stimulated or untreated cells. For synthesis of the cDNA probes, 1–2 μg of poly(A)$^+$ RNA were primed with oligo(dT) in 30 μl [50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 2 mM dATP, 2 mM dGTP, 50 μCi [α-^{32}P]dCTP (3000 Ci/mmol, Amersham), 1.5 μg oligo(dT), 32 U RNasin (Promega), 200 U M-MLV reverse transcriptase (Gibco)]. After 3 min at 37°C dTTP to 2 mM was added and after an additional 30 min at 37°C the reaction was chased by adding dCTP to 2 mM. After a further 30 min at 37°C the reaction was stopped by adding SDS to 0.05% and EDTA to 0.05 M. Free nucleotides were separated by spun columns. The RNA was hydrolysed by incubation in 0.1 M NaOH at 56°C for 2 min, then quickly chilled and neutralized by adding acetic acid. The resulting cDNA probe had an activity of ~1×10^8 c.p.m./μg poly(A)$^+$ RNA. Filters were hybridized for 16 h at 65°C in 5 × SSC, 5 × Denhardt's solution, 1% SDS, 5 mg/ml yeast tRNA, 5 μg/ml poly(A) (Sigma) and 1×10^6 c.p.m./ml cDNA probe, thereafter washed four times for 5 min each at room temperature in 1 × SSC, 0.5% SDS, then washed with three changes at 65°C for 30 min each in 0.38 × SSC and 0.5% SDS and autoradiographed. Positive plaques were subjected to two further rounds of plaque purification using the differential screening procedure.

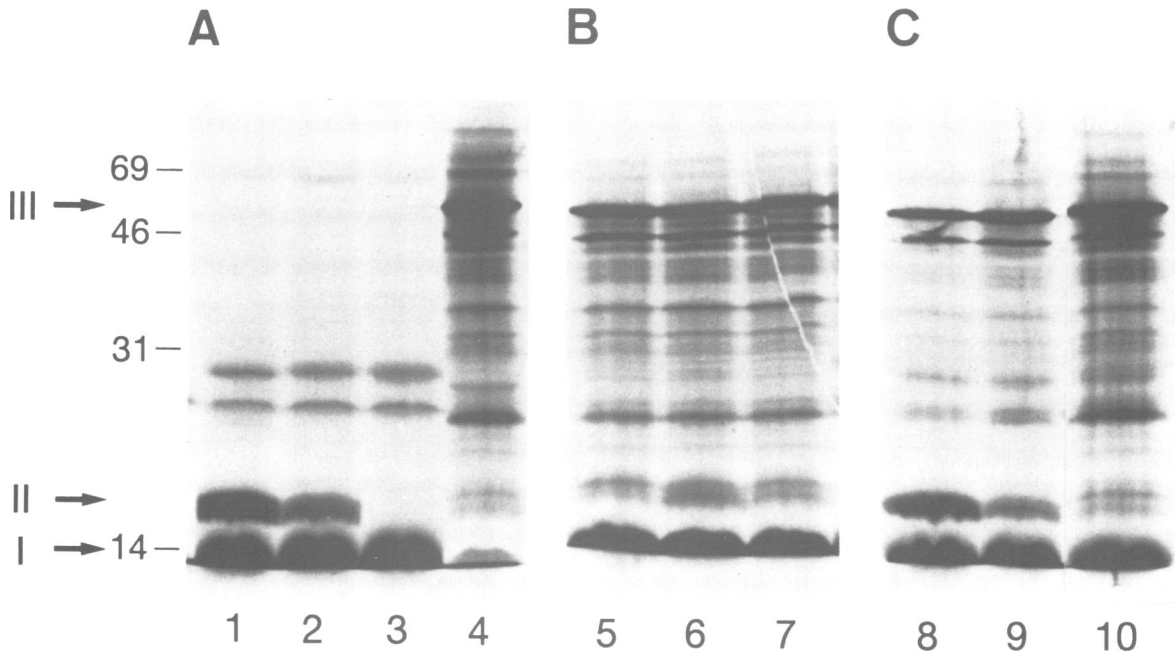

Fig. 9. *In vitro* inhibition of yeast UGA suppressor tRNA readthrough activity by IFP 53 in a reticulocyte lysate. (**A**) Translation of globin mRNA *in vitro* in the presence of yeast UGA suppressor tRNA: (1) 150 μg/ml suppressor tRNA, (2) 30 μg/ml suppressor tRNA, (3) no suppressor tRNA; (4) pGEM13.3 mRNA. (**B**) Translation of globin mRNA when pGEM13.3. mRNA was first translated *in vitro* to yield IFP 53 protein and then added to fresh reticulocyte lysates containing globin mRNA and yeast UGA suppressor tRNA: (5) no suppressor tRNA, (6) 150 μg/ml suppressor tRNA, (7) 30 μg/ml suppressor tRNA. (**C**) *In vitro* translation of globin mRNA in the presence of pGEM13.3 mRNA, and yeast UGA suppressor tRNA: (8) 150 μg/ml suppressor tRNA, (9) 30 μg/ml suppressor tRNA, (10) no suppressor tRNA. In experiments (B) and (C) longer exposure times of the dried polyacrylamide gels were necessary to compensate for the decreased translation of globin mRNA. Arrow I indicates the translation products of α- and β-globin mRNA, arrow II indicates the readthrough product of β-globin mRNA, arrow III indicates the translation product of pGEM13.3 mRNA.

An oligonucleotide derived from the sequence of cDNA clone 12.1 was used as a probe to isolate cDNA clone 369.13.3 from a random primed λgt11 cDNA library prepared from HeLa cells treated with IFN-γ. The λgt11 library was a kind gift from Dr Michael A.Blanar, University of California at San Francisco.

For further analysis (sequence determination, preparation as probes, *in vitro* translation, run-on analysis) phage inserts 12.1, 9.2 and 119.1 were subcloned into Bluescript II KS (Stratagene) according to standard techniques. Plasmid pGEM13.3 was derived from the recombinant λgt11 clone 369.13.3 by inserting the *Eco*RI insert, which contains a full-length IFP 53 cDNA, in the *Eco*RI site of pGEM4Z (Promega, Heidelberg), such that expression of IFP 53 mRNA is under control of the SP6 promoter. The resulting plasmid was transformed into *Escherichia coli* DH5α by electroporation (Böttger, 1988).

DNA and RNA analysis

Human genomic DNA was prepared from HeLa cells as described (Sambrook *et al.*, 1989) and digested with the indicated enzymes. Conditions for Southern blotting (Sambrook *et al.*, 1989) were as follows: prehybridization for 30 min at 65°C in Plaque Screen buffer (0.2% polyvinylpyrolidone, 0.2% Ficoll-400, 0.2% bovine serum albumin, 50 mM Tris, pH 7.5, 1 M NaCl, 2.2 mM sodium-pyrophosphate, 1% SDS) containing 100 μg/ml salmon sperm DNA. Hybridization was performed for 12 h at 65°C in Plaque Screen buffer containing 100 μg/ml salmon sperm DNA, 10% dextran sulphate and 1×10^6 c.p.m./ml probe. Filters were washed twice in Plaque Screen buffer and then three times for 30 min each in 0.5 × SSC and 1% SDS at 65°C.

Total cellular RNA was prepared using the guanidium isothiocyanate procedure (Sambrook *et al.*, 1989), purified by centrifugation through caesium chloride, electrophoresed on formaldehyde agarose gels and transferred to nitrocellulose. Conditions for Northern blotting were as follows: prehybridization for 60 min at 65°C in 2 × SSC, 0.2% Ficoll-400, 0.2% polyvinylpyrolidone, 0.2% bovine serum albumin, 5% dextran sulphate, 0.1% SDS, 2.2 mM sodium pyrophosphate, 2 mM EDTA, 0.5 mg/ml yeast tRNA and 5 μg/ml poly(A). Hybridization was performed for 16 h at 65°C in the same buffer containing 1×10^6 c.p.m./ml probe. Washings were performed once with 4 × SSC, 0.1% SDS, 2.2 mM sodium pyrophosphate, 2 mM EDTA for 20 min at 65°C, twice with 2 × SSC, 0.1% SDS, 2.2 mM sodium pyrophosphate, 2 mM EDTA for 10 min at 65°C, twice with 1 × SSC, 0.1% SDS, 2 mM EDTA for 10 min at 65°C and once with 0.4 × SSC, 0.1% SDS, 2 mM EDTA for 10 min at 65°C. Quantitative loading of RNA was determined by staining with ethidium bromide and by hybridization to a pyruvate kinase probe (Böttger *et al.*, 1988).

Transcription rate measurement (nuclear run-on assay)

Nuclei were isolated from untreated or IFN-pretreated HeLa cells, incubated in the presence of radiolabelled UTP and nuclear RNA was extracted as described (Larner *et al.*, 1984). The labelled RNA was hybridized to immobilized DNA on nitrocellulose and the resulting autoradiograph was evaluated by densitometry as previously reported (Decker *et al.*, 1989). Specific signals of IFP 53 and β-actin transcription were normalized to the tubulin signal on the same filter. DNA probes were as follows: chicken β-actin was a genomic *Pst*I fragment (Cleveland *et al.*, 1980) cloned into pGem-1 (Promega), β_2-tubulin was a genomic *Sst*I fragment cloned into pSP64 (Blanar *et al.*, 1988), 9.2 and 12.1 were *Eco*RI cDNA fragments cloned into Bluescript II KS (Stratagene).

Primer extension

A synthetic oligonucleotide (2 pmol) complementary to nucleotides 138–158 of the predicted translated sequence was hybridized to 10 μg total RNA in 30 μl 50 mM Tris, pH 8.0, 5 mM $MgCl_2$, 50 mM KCl, 0.05 mg/ml bovine serum albumin, 5 mM DTT, 34 U RNasin (Promega), 2.7 mM each dNTP, 10 μCi [α-^{32}P]dATP (3000 Ci/mmol, Amersham) for 15 min at 37°C. Subsequent reverse transcription was carried out for 60 min at 42°C by adding 30 U AMV reverse transcriptase (Seigakaku). The reaction was stopped by supplementing EDTA to 20 mM, nucleic acids were phenol extracted, ethanol precipitated and analysed by electrophoresis on a 6% polyacrylamide–6 M urea sequencing gel.

Hybrid selection, in vitro translation and transcription of mRNA

Purified recombinant plasmid (10 μg) was denatured and bound to nitrocellulose (Manifold II, Schleicher and Schuell) for hybrid selection of mRNA. Slots containing the bound nucleic acids were cut out and hybridized in a 50 μl volume at 50°C for 3 h. The hybridization mix contained 65% formamide, 30 mM PIPES (piperazine-*N*,*N*′-bis[2-ethansulphonic acid]) at

pH 6.5, 0.4 M NaCl, 0.2% SDS, 50 μg yeast tRNA and 10 μg poly(A)$^+$ mRNA from HeLa cells treated with 1000 U/ml IFN-γ for 48 h. Filters were rinsed 10 times with 10 mM Tris, pH 7.6, 0.15 M NaCl, 1 mM EDTA, 0.5% SDS prewarmed to 65°C and twice with the same buffer without SDS. mRNA was eluted by boiling the filter in 300 μl of water with 20 μg yeast tRNA as carrier for 1 min. Samples were quickly frozen on dry ice, thawed on ice, the filters removed, nucleic acids phenol extracted, precipitated and resuspended in 6 μl water. 3 μl were translated in a rabbit reticulocyte lysate system (final volume 20 μl) using [^{35}S]methionine (20 μCi; >1000 Ci/mmol at 10 mCi/ml) according to the manufacturer's instructions (Stratagene).

After linearization with *XbaI* plasmid pGEM13.3 was transcribed essentially as described by Melton *et al.* (1984) using SP6 polymerase (Promega, Heidelberg) and ^{7}mGpppG (Pharmacia, Freiburg) to generate capped mRNA. Following transcription, mRNA was extracted once with TE-saturated phenol:chloroform:isoamylalcohol (49:49:2), once with chloroform:isoamylalcohol (24:1), ethanol-precipitated in the presence of 2.5 M ammonium acetate and checked for integrity by electrophoresis on 1.5% agarose–formaldehyde gels (Sambrook *et al.*, 1989). Translations in a nuclease-treated and supplemented reticulocyte lysate (Promega) were performed using [^{35}S]methionine (10 μCi) and globin mRNA (Gibco/BRL) at a concentration of 7.7 μg/ml. Purified yeast UGA suppressor tRNA was a generous gift from Professor H.Beier (Biozentrum, Universität Würzburg, Germany) and added at the concentrations indicated. In the pretranslation experiments 100 ng of capped pGEM13.3 were first translated *in vitro* in a total reaction mixture of 3 μl and then added to a fresh reticulocyte mixture (6.5 μl) containing 50 ng globin mRNA and the indicated amounts of UGA suppressor tRNA. In the cotranslation experiments 100 ng of capped pGEM13.3, 50 ng globin mRNA and the indicated amounts of UGA suppressor tRNA were added simultaneously to reticulocyte lysates (final volume 6.5 μl). Reaction mixtures were incubated at 30°C for 60 min. The translated products were analysed on 9% and 15% SDS–polyacrylamide gels. Following electrophoresis, the gels were fixed and dried and labelled proteins detected by autoradiography.

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