Species-Specific Posttranscriptional Regulation of Interferon Synthesis†

James J. Greene, Carl W. Dieffenbach, Limei C. Yang, and Paul O. P. Ts'o*

ABSTRACT: Human fibroblast and Syrian hamster embryo cells were induced to synthesize interferon (IF) with $rI_n \cdot rC_n$ and $rI_n \cdot rC_n$ + DEAE-dextran, respectively. Following induction, these cells synthesized IF for only a short time before entering into a *repressed* state and shutting off the synthesis of IF. Homologous and heterologous whole cell translational systems were developed to investigate the molecular basis for the shut-off of IF synthesis. These systems allowed for the introduction of exogenous hamster and human IF-mRNAs into intact normal and *repressed* hamster and human cells via an improved $CaCl_2$ precipitation technique. Human IF-mRNA was translated in normal human and hamster cells and in *repressed* hamster cells but not in *repressed* human cells. In contrast, the hamster IF-mRNA was translated in normal human, normal hamster, and *repressed* human cells but not in *repressed* hamster cells. These results indicate that a species-specific mechanism inhibiting translation of IF-mRNA is directly responsible for the shut-off of IF synthesis in human fibroblasts and Syrian hamster embryo cells.

The synthesis of interferon (IF) is a highly regulated model of gene expression. While mammalian cells do not synthesize IF constitutively, exposure to viruses or to double-stranded polyribonucleotides such as poly(inosinic acid)·poly(cytidylic acid) ($rI_n \cdot rC_n$) results in the de novo synthesis of IF-mRNA and the subsequent production of IF (Colby & Morgan, 1971; Stewart, 1979). Upon exposure to $rI_n \cdot rC_n$, human fibroblasts rapidly initiate IF synthesis. IF synthesis persists only for 3 to 4 h and abruptly shuts off (Vilček & Hayell, 1973; Kohase & Vilček, 1977). This cessation of IF synthesis represents a biological control of exceptional stringency. Consequently, increasing emphasis has been placed on understanding the mechanism of the shut-off of IF synthesis.

The initial studies of the regulatory steps involved in IF synthesis shut-off have been predominantly in terms of the susceptibility of IF production to metabolic inhibitors. Inhibitors of transcription such as actinomycin D (Tan et al., 1970; Vilček & Ng, 1971; Sehgal et al., 1976a) or 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (Sehgal et al., 1976b,c) and inhibitors of protein synthesis such as cycloheximide or puromycin (Lab & Kochran, 1972; Sehgal et al., 1976a) can delay the onset of IF shut-off when given to cells after the initiation of IF synthesis. These metabolic studies suggested the involvement of a posttranscriptional mechanism in the shut-off of IF synthesis.

Recently, the levels of IF-mRNA in human cells following induction by $rI_n \cdot rC_n$ have been measured in our laboratory (Greene et al., 1978) and in others (Cavalieri et al., 1977; Sehgal et al., 1977) as a direct evaluation of posttranscriptional elements in the IF shut-off process. The level of IF-mRNA was quantitatively measured in our laboratory by translation in a heterologous whole cell system employing the Syrian hamster embryo (SHE) cells. This system is similar to that formerly developed by De Maeyer-Guignard et al. (1972) for avian cells. Measurements of IF-mRNA in the other laboratories were done by translation in *Xenopus* oocytes. These studies have established an association between the shut-off of IF synthesis and the inactivation of functional IF-mRNA. However, the molecular nature of the controls exerted by the cell to regulate the synthesis of IF in this way is unknown.

In this paper, we present a further characterization of the IF shut-off mechanism. The whole cell translational system was used to demonstrate that the IF shut-off machinery operates in a species-specific manner, inhibiting the translation of homologous IF-mRNA but not heterologous IF-mRNA.

Experimental Procedures

$rI_n \cdot rC_n$ Induction of Human Fibroblasts. Human foreskin fibroblasts (HF926) grown in 850-cm^2 Corning roller bottles were treated for 1 h at 37 °C with 100 μg/mL $rI_n \cdot rC_n$ in buffer A (0.15 M NaCl, 0.01 M phosphate, and 0.001 M $MgCl_2$, pH 7.0–7.4). After incubation, the $rI_n \cdot rC_n$ solution was removed and the cells were washed at least 4 times with phosphate-buffered saline (PBS) before being refed with Dulbecco's modified minimum essential medium (ME medium) supplemented with 2% fetal bovine serum (maintenance medium).

$rI_n \cdot rC_n$ + DEAE-dextran Induction of Syrian Hamster Embryo (SHE) Cells. This procedure was used to induce SHE cells (Barrett et al., 1977) into the *repressed* state. Confluent cultures of SHE cells were treated as described for the $rI_n \cdot rC_n$ induction of human cells except that the induction solution contained 100 μg/mL $rI_n \cdot rC_n$ + 100 μg/mL DEAE-dextran in buffer A. The $rI_n \cdot rC_n$ formed a precipitate at this concentration of DEAE-dextran.

Newcastle Disease Virus (NDV) Induction of SHE Cells. NDV induction was used only to induce SHE cells to synthesize IF for the purpose of extracting IF-mRNA. Since induction of SHE cells with NDV yielded between 10- and 100-fold more IF than did induction with $rI_n \cdot rC_n$ + DEAE-dextran, this procedure was preferred for the preparation of hamster IF-mRNA. NDV was grown in embryonated chick eggs and was UV inactivated prior to induction. The inactivated virus was then diluted 1:10 in PBS and 15 mL of the diluted solution applied to PBS-washed SHE cells in 850-cm^2 roller bottles (approximate moi = 10 pfu/cell). After incubation for 1 h at 37 °C, the excess NDV inoculum was removed and the cells were washed with PBS, refed with maintenance medium, and reincubated at 37 °C.

Extraction of Human and Hamster Cytoplasmic RNA. Human cells were lysed 3 h after $rI_n \cdot rC_n$ induction and SHE cells were lysed 18–20 h after NDV induction, with a solution of 0.5% NP-40, 0.1% deoxycholate, 0.1% Tween-40, 0.5 M KCl, 0.01 M Tris-HCl, and 0.005 M $MgCl_2$, pH 8.0, saturated

† From the Division of Biophysics, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland 21205. *Received October 26, 1979.* This work was supported in part by a grant from the National Institutes of Health (GM-16066). C.W.D. was supported in part by Training Grant CA-09110 from the National Cancer Institute.

0006-2960/80/0419-2485$01.00/0

with dextran sulfate. The extraction and purification of the RNA were done at 5 °C. After 10–15 min was allowed for lysis, the solution was transferred from roller bottles to 30-mL Corex tubes. The lysate was centrifuged at 10000*g* for 10 min to pellet the nuclei. The supernatant was then transferred to 250-mL Corning centrifuge tubes, and EDTA was added to 5 mM. The cytosol was extracted 3 times, with equal volumes of a solution containing a mixture of phenol–chloroform–isoamyl alcohol in a ratio of 25:24:1 (v/v/v). Following extraction, the RNA was precipitated with 2 volumes of ethanol at −20 °C.

Chromatography on Oligo(dT)–Cellulose Columns. The precipitated RNA was dissolved in 0.4 M NaCl and 10 mM Tris, pH 7.6, and applied to an oligo(dT)–cellulose (Collaborative Research T-4 or Boehringer Mannheim T-1) column. After extensive washing of the column with 0.4 M NaCl and mM Tris, pH 4.6, the bound RNA was eluted with 10 mM Tris, pH 7.6, and precipitated with ethanol at −20 °C.

Translation in Intact Whole Cells. The precipitated RNA was washed extensively with cold ethanol to remove trace amounts of phenol and then was diluted in Hepes–PBS buffer (0.5% w/v Hepes in calcium-free PBS) to a concentration of 50 μg/mL as determined by optical absorbance using an extinction coefficient of 7400 at 260 nm. For the species-specificity experiments, the pH of the Hepes–PBS buffer was 6.6. Five milliliters of the RNA solution was used for each translation. The RNA was precipitated by the addition of 0.15 mL of 1 M $CaCl_2$ to each RNA sample and applied immediately to confluent cultures of human fibroblasts or SHE cells in 100-mm dishes which had previously been depleted of calcium by incubating in calcium-free Hepes–PBS for 10 min. The cultures were incubated with the RNA precipitate for 45 min at 37 °C, after which the Hepes–PBS buffer was removed and replaced with 5 mL of maintenance medium and the mixture was incubated for an additional 3 to 4 h. At the end of the incubation, the media were collected for titering.

Interferon Assay. The media collected from the translations were dialyzed overnight in ME medium at 5 °C. After dialysis, 1:2 serial dilutions of the media were applied to either confluent cultures of trisomic 21-GM-258 cells (for human IF activity) or confluent cultures of SHE cells (for hamster IF activity). The assays were then conducted as previously described by Finter (1969) using vesicular stomatitis virus (VSV). The data for human IF activity are expressed as GM-258 reference units, except for data in Figure 3 which are expressed in Medical Research Council (MRC) 69/19 reference units. The ratio of GM-258 units to MRC 69/19 units is 12:1. The data for the hamster IF activity are all expressed in laboratory reference units.

Results

Strategy for Studying the Species-Specific Nature of the IF Synthesis Shut-off Mechanism. The species specificity of the mechanism shutting off the synthesis of IF was investigated by translation of human and hamster IF-mRNA in normal and *repressed* cells of both the human and hamster species. *Repressed* cells are cells that have been preinduced with $rI_n{\cdot}rC_n$ to synthesize IF and are in the shut-off phase of IF synthesis. Cells in the *repressed* state are also refractory to further induction of IF synthesis. Since the IF shut-off mechanism is in operation during the *repressed* state, comparison of the translational efficiencies of exogenous human IF-mRNA in *repressed* cells vis-à-vis normal cells of human and hamster origins provides an indication of the extent to which the IF shut-off mechanism of both species operates on exogenous human IF-mRNA.

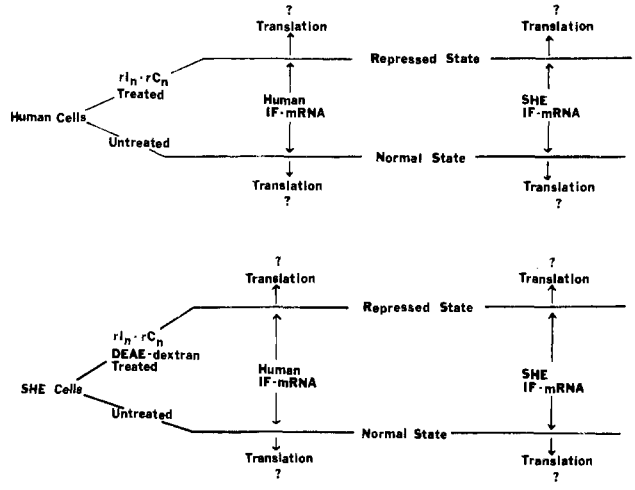

FIGURE 1: Experimental design in the investigation of the species specificity of the interferon synthesis shut-off mechanism.

These experiments were repeated with hamster IF-mRNA to verify the results obtained with human IF-mRNA. Figure 1 describes the experimental design.

The Whole Cell Translational System. DEAE-dextran and $CaCl_2$, which enhance the infectivity of viral nucleic acids, were used to facilitate the uptake of exogenous mRNA by intact cells. Since the formation of a physical precipitate was found necessary for $CaCl_2$-mediated transfection with adenovirus DNA (Graham & Van der Eb, 1973), the conditions for achieving the precipitation of mammalian cytoplasmic RNA were determined. Precipitation of SHE [^{3}H]RNA in Hepes–PBS buffer by $CaCl_2$ is nearly complete at 30 mM $CaCl_2$. This concentration (30 mM) of $CaCl_2$ was therefore selected to facilitate the uptake of IF-mRNA by intact cells. DEAE-dextran was used at a concentration of 50 μg/mL, the same as that known to facilitate the uptake of viral DNA (Graham et al., 1973).

IF-mRNA dissolved in Hepes–PBS buffer was incorporated and translated by either human fibroblasts or SHE cells when applied together with DEAE-dextran or $CaCl_2$. However, $CaCl_2$ was a much more effective facilitator than DEAE-dextran in mediating translation (Greene et al., 1978). The applied RNA was extracted from $rI_n{\cdot}rC_n$-induced human cells or NDV-induced SHE cells, using the total cytoplasmic RNA fraction or the poly(A)-containing RNA fraction isolated from cytoplasmic RNA. Translation of IF-mRNA was quantitated by a bioassay which is based on the species-specific antiviral properties of IF. When exogenous IF-mRNA was translated by the recipient cells, the IF produced was active only in cells of the same species of origin as the IF-mRNA. The titer of the antiviral activity produced in the recipient cell culture medium is a measure of the extent to which IF-mRNA was translated. This translational system is similar to the avian cell system originally described by De Maeyer-Guignard et al. (1972).

Our initial studies on the translation of exogenous IF-mRNA in intact cells have concentrated on the $CaCl_2$-mediated translation of human IF-mRNA in heterologous SHE cells. The amount of human antiviral activity produced in SHE cells was directly proportional to the amount of IF-mRNA applied when subsaturating amounts of RNA were introduced. At a dosage of 10 μg/mL RNA (50 μg/dish), the SHE translation system becomes saturated and the human IF yield becomes independent of RNA dose above this level. Under saturating conditions, the SHE translation system responded proportionately not to the absolute amount of human

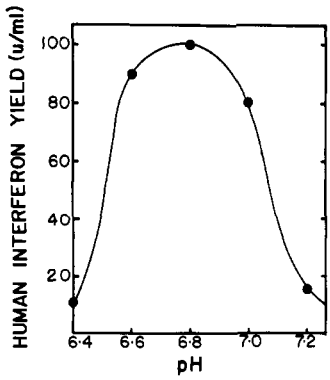

FIGURE 2: Dependence of the translation of human IF-mRNA in SHE cells on the pH of the application buffer. RNA from induced human cells was dissolved in PBS–Hepes buffered to various pH values and translated in SHE cells as described under Experimental Procedures.

Table I: Characterization of the Interference Activity Produced in SHE Cells

(1) formation of the product is the result of a metabolic process; pretreatment of SHE cells with actinomycin D increases yield of the product
(2) formation of the product is associated only with the application of poly(A)-containing RNA to SHE cells
(3) interference activity is sensitive to trypsin
(4) interference activity is resistant to ribonuclease
(5) interference activity is species specific in its action
(6) interference activity is neutralized by monospecific antibody to human fibroblast IF
(7) Chromatographic properties of the interference activity on blue dextran–Sepharose columns are very similar to those of human fibroblast IF

IF-mRNA but rather to the relative concentration of IF-mRNA within the preparation (Greene et al., 1978). The translations reported below were done under saturating conditions which yielded more reproducible results. The production of human antiviral activity by SHE cells following the application of human IF-mRNA was highly dependent upon the pH of the application buffer (Figure 2). The pH range 6.6–7.0 was optimal. Outside of this range, the translational efficiency was greatly reduced.

The antiviral activity resulting from the presumed translation of exogenous human mRNA in SHE cells was subjected to a variety of biological and physical characterizations. On the basis of the results summarized in Table I, it was concluded that this activity is characteristic of human fibroblast IF. These results are briefly described below.

The production of the antiviral activity was influenced by the following treatments. (1) Pretreatment of the recipient cells with actinomycin D showed that the production of the antiviral activity following the application of human IF-mRNA is the result of a metabolic process (Greene et al., 1978). (2) The association of the production of antiviral activity by the recipient cells to the application of poly(A)-containing RNA was demonstrated in the following experiment. Poly(A)-containing RNA was isolated from cytoplasmic RNA prepared from induced human cells on an oligo(dT)–cellulose column. Both the oligo(dT)–cellulose-bound RNA and the "flow-through" RNA (RNA which has a low affinity for the column) were applied to SHE cells. Antiviral activity was produced only by SHE cells treated with oligo(dT)–cellulose-bound RNA and not by SHE cells treated with the flow-through RNA. These two observations indicate that the production of the antiviral activity in SHE cells resulted from the translation of human mRNA.

The nature of the antiviral activity produced in the SHE cells has been characterized by the following procedures. (1) Trypsin sensitivity was determined by incubating the translation product in 100 μg/mL trypsin for 90 min at 37 °C. This treatment destroyed all IF activity as determined by titering on GM-258 cells. The antiviral activity was unaffected when the translation product was incubated in the presence of 40 μg/mL ribonuclease A for 30 min at 37 °C. This ribonuclease treatment was sufficient to completely destroy the IF inducing ability of a 100 μg/mL solution of $rI_n \cdot rC_n$. The antiviral activity was tested for species specificity by titering the material on cultures of SHE cells and human GM-258 cells. When challenged with VSV, the translation product protected the human cells but not the hamster cells. These observations indicate that the antiviral activity is species specific and most likely mediated by a protein(s). (2) In the antibody neutralization experiment the translation product was incubated with monospecific antibody to human fibroblast IF obtained from the National Institute of Allergy and Infectious Diseases. Incubation for 15 min at room temperature abolished the ability of the translation product to protect GM-258 cells against VSV challenge. (3) Chromatography of the translation product on blue dextran–Sepharose columns was done in accordance with the procedure of Jankowski et al. (1976) and Bollin et al. (1978). Blue dextran–Sepharose, a sulfonated polyaromatic blue dye covalently linked to Sepharose, was chosen for chromatographic characterization since human fibroblast IF binds very strongly to this ligand by means of both ionic and hydrophobic interactions. Moreover, human IF and hamster IF chromatograph differently. Although both human and hamster IF bind to blue dextran–Sepharose, hamster IF can be eluted with high concentrations of sodium chloride (0.85 M NaCl) while human IF must be eluted with a mixture of sodium chloride and ethylene glycol (1.0 M NaCl and 50% ethylene glycol). This difference in binding probably reflects a greater hydrophobicity of human IF as compared to hamster IF. Culture media from several translation experiments were pooled and applied directly to a blue dextran–Sepharose 4B column (Sigma). The column was washed with PBS and then eluted with 0.85 M NaCl and with 1.0 M NaCl and 50% ethylene glycol. Human-specific antiviral activity was found to be eluted completely with 1.0 M NaCl and 50% ethylene glycol. No activity was eluted with either PBS or 0.85 M NaCl. These combined observations provide convincing evidence that the antiviral activity produced by the SHE cell culture was mediated by human fibroblast IF, which was produced from the translation of exogenous human IF-mRNA.

The homologous translation of human IF-mRNA in uninduced human fibroblasts showed characteristics similar to those of the heterologous translation in SHE cells except for the dependence on pH. The optimal pH for translation in human fibroblasts was pH 6.6, while no significant translation was observed at pH 7.0. This strong dependency of translation on pH may be related to the adherence of the RNA–calcium phosphate coprecipitate to the cells. Adherence of the coprecipitate to the human cells at pH 6.6 was much stronger than that at pH 7.0. In contrast, the coprecipitate adhered strongly to SHE cells at both pH 6.6 and pH 7.0.

For the homologous translation of human IF-mRNA in human cells, particular care was given to ensure that there was no carry-over of $rI_n \cdot rC_n$ used in the induction of IF to the IF-mRNA sample. This was done by extensive washing of the induced cells prior to the extraction of RNA. Precipitation of the RNA extracted from the induced cells was obligatory for the production of IF in the noninduced recipient cells. No

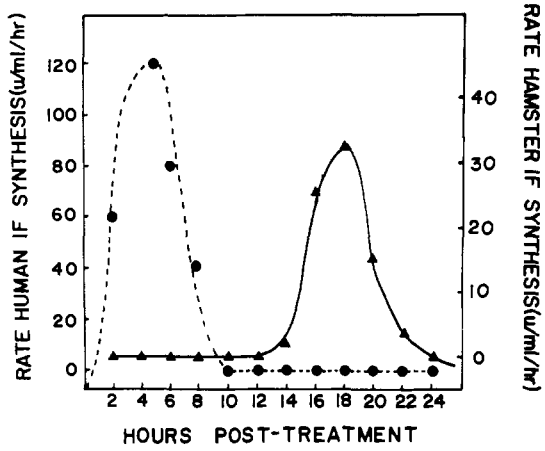

FIGURE 3: Kinetics of human and hamster IF synthesis. SHE cells were induced in a solution of buffer A containing 100 μg/mL $rI_n \cdot rC_n$ and 100 μg/mL DEAE-dextran for 1 h. After 1 h the cultures were washed extensively and refed with ME medium containing 2% fetal calf serum. Conditions for the induction of human cells were the same as those for the SHE cells except that the induction solution only contained $rI_n \cdot rC_n$. The culture media were removed at various times for titering on either GM-258 (human IF) or SHE cells (hamster IF): (—) hamster IF; (---) human IF.

IF activity was detected when RNA from noninduced cells was used. Moreover, the "translation" showed the strong pH dependency described above. These observations are consistent with the IF production from the recipient cells resulting from the translation of exogenously supplied human IF-mRNA rather than from the induction of endogenous IF synthesis due to contaminating amounts of $rI_n \cdot rC_n$ in the applied RNA sample.

Hamster IF-mRNA was extracted from NDV-induced SHE cells. The translation of hamster IF-mRNA demonstrated the same characteristics as the translation of human IF-mRNA, suggesting that the whole cell translational system is more dependent on recipient cell type than on the type of RNA applied. Additionally, translation of hamster IF-mRNA could only be observed for application of poly(A) RNA collected from oligo(dT)–cellulose columns (see below).

Translation of Human IF-mRNA in Homologous Cells. Figure 3 shows the kinetics of IF synthesis in human fibroblasts following exposure to $rI_n \cdot rC_n$. The rate of IF synthesis begins to decline sharply after 3–6 h, indicating the start of the *repressed* state at this time.

For translation experiments in *repressed* human cells, cultures of cells were incubated for 45 min with $rI_n \cdot rC_n$, after which the cells were washed 4 or 5 times with ME medium and then incubated in fresh ME medium containing 2% fetal bovine serum for an additional 3–6 h before the application of exogenous human IF-mRNA. Before the application of exogenous IF-mRNA, the cells were again washed extensively to remove any residual $rI_n \cdot rC_n$. The translation of exogenous IF-mRNA in *repressed* human cells can be obscured by simultaneous translation of endogenous IF-mRNA. Endogenous IF synthesis is the result of incomplete shut-off of IF synthesis in the $rI_n \cdot rC_n$-induced translational cells. This background synthesis was accounted for by measuring the amount of human IF activity produced from a "background control" in which RNA extracted from uninduced human cells was applied to the *repressed* translational cells instead of RNA extracted from induced human cells.

When exogenous human IF-mRNA was introduced into normal (untreated) and *repressed* human cells, the amount of IF produced by the *repressed* cells was less than 10% of that produced by normal cells (Table II). These results

Table II: Translation of Human IF-mRNA in Normal and *Repressed* Human Fibroblasts[a]

	expt 1	expt 2	expt 3	expt 4
translation in normal state	110	195	235	135
gross translation in *repressed* state	80	145	0	25
background (human IF synthesis not shut off)	75	135	5	15
net translation in *repressed* state	5	10	−5	10
% of normal translation	5	5	0	7

[a] Cytoplasmic RNA extracted from $rI_n \cdot rC_n$-induced HF926 human fibroblasts was translated in untreated HF926 cells and in HF926 cells treated with $rI_n \cdot rC_n$ 4 h (experiments 1 and 2) or 7 h (experiments 3 and 4) prior to translation with $rI_n \cdot rC_n$. Interferon data are expressed as GM-258 reference units (see Experimental Procedures).

suggest the presence of a mechanism in *repressed* human cells which inhibits the translation of exogenous human IF-mRNA. There are, however, other interpretations for this result, such as a reduced translational capacity in the *repressed* human cells making them unable to translate all of the exogenous mRNA. Human IF-mRNA was translated in normal and *repressed* SHE cells to resolve this question and also that of the species specificity of the shut-off mechanism.

Translation of Human IF-mRNA in Hamster Cells. Syrian hamster cells are normally refractory to $rI_n \cdot rC_n$ for IF induction, and attempts to induce by $rI_n \cdot rC_n$ alone or by $rI_n \cdot rC_n$ in combination with various facilitators have so far been unsuccessful (Grossberg et al., 1975). In the experiments where DEAE-dextran was used as a facilitator, the DEAE-dextran was always present in a nonequivalent amount to $rI_n \cdot rC_n$ (w/w greater than or less than 1:1).

The $rI_n \cdot rC_n$ + DEAE-dextran induction of IF in mouse L cells was the precedent for the similar attempts in hamster cells. The addition of almost any amount of DEAE-dextran to $rI_n \cdot rC_n$ enhanced IF induction in mouse L cells (Dianzani et al., 1970). Whereas the DEAE-dextran/$rI_n \cdot rC_n$ ratio was not critical in inducing mouse IF, it may be critical in inducing hamster IF. Therefore, cultures of SHE cells were exposed to 100 μg/mL $rI_n \cdot rC_n$ in buffer A (0.15 M NaCl, 0.01 M phosphate, and 0.001 M $MgCl_2$, pH 7.0–7.4) containing various amounts of DEAE-dextran. Induction was seen only in the solution which contained a 1:1 ratio of DEAE-dextran to $rI_n \cdot rC_n$. Pitha & Carter (1971) have reported that DEAE-dextan precipitates $rI_n \cdot rC_n$ when mixed together in a 1:1 ratio. The formation of the precipitate only occurs at this ratio. This suggests that induction of IF in SHE cells requires the physical precipitation of $rI_n \cdot rC_n$. Greater reproducibility in inducing SHE IF was obtained when the higher molecular weight DEAE-dextran from Pharmacia (M_r 2×10^6) was used instead of DEAE-dextran from Sigma (M_r 5×10^5). Also, more consistent results were obtained when concentrated DEAE-dextran was added to the $rI_n \cdot rC_n$ solution after the $rI_n \cdot rC_n$ was applied to the cells instead of premixing the DEAE-dextran and $rI_n \cdot rC_n$ before application to the cells.

When SHE cells were induced with a mixture of 100 μg/mL $rI_n \cdot rC_n$ and 100 μg/mL DEAE-dextran, hamster IF was produced according to the kinetics shown in Figure 3. No IF was produced until 14–16 h postinduction. After this time, IF synthesis persisted for ~4 h and abruptly shut off, indicating the existence of a regulatory system and the likelihood of an induced *repressed* state similar to that known to exist in human cells.

Table III: Translation of Human IF-mRNA in Normal and *Repressed* Syrian Hamster Embryo Fibroblasts[a]

	expt 1	expt 2	expt 3	expt 4
translation in normal state	90	115	210	150
gross translation in *repressed* state	255	410	470	295
background ($rI_n \cdot rC_n$–DEAE-dextran carry-over)	170	275	195	95
net translation in *repressed* state	85	135	275	200
% of normal translation	94	117	130	133

[a] Cytoplasmic RNA extracted from $rI_n \cdot rC_n$-induced HF926 human fibroblasts was translated in untreated Syrian hamster embryo (SHE) cells and in SHE cells treated 22–24 h prior to translation with $rI_n \cdot rC_n$–DEAE-dextran. Interferon data are expressed as GM-258 reference units (see Experimental Procedures).

Human IF-mRNA was translated in untreated SHE cells as well as in *repressed* SHE cells that had been treated with $rI_n \cdot rC_n$–DEAE-dextran 22–24 h prior to translation. In this experiment, there was no background interference due to endogenous human IF synthesis since the translation occurred in hamster cells. Endogenous translation results in the synthesis of hamster IF which does not interfere with the titering of human IF translated from exogenous human IF-mRNA. There was, however, a background problem that resulted from the carry-over of residual $rI_n \cdot rC_n$–DEAE-dextran used to induce the SHE cells into the *repressed* state. During induction, the $rI_n \cdot rC_n$–DEAE-dextran precipitate adheres tightly to the SHE cells and cannot be totally removed even after extensive washing. Some of the residual $rI_n \cdot rC_n$–DEAE-dextran on the treated *repressed* SHE cells leaks out into the medium during translation of the human IF-mRNA. The small amount of $rI_n \cdot rC_n$–DEAE-dextran carried over into the translation media contributes to an artificially high titer when the media are titered on human GM-258 cells for human IF. The amount of this background was measured by a background control experiment in which RNA extracted from uninduced human cells was used for the translation. The $rI_n \cdot rC_n$–DEAE-dextran carry-over was minimized by centrifuging the translation media in a Sorval centrifuge at 20000*g* for 20 min before titering.

The results of these experiments are shown in Table III. Experiments 1–4 correspond to the same numbered experiments in Table II in which human IF-mRNA was translated in *repressed* and normal human cells. Similarly numbered experiments in Tables II and III were done simultaneously with the same preparation of human IF-mRNA. In contrast to translation in human cells, when exogenous human IF-mRNA was introduced into untreated SHE cells and *repressed* SHE cells, the amounts of human IF translated by these two hamster systems were equivalent. This indicates that the regulatory machinery of hamster cells in the *repressed* state is inoperative on human IF-mRNA translation.

Translation of Hamster IF-mRNA in Human and Hamster Cells. The observations on the translation of human IF-mRNA in normal and in *repressed* human and SHE cells are consistent with a species-specific translational inhibitory mechanism. Extension of these studies to the translation of hamster IF-mRNA in normal and in *repressed* cells of both the human and hamster species may provide verification of the species-specific mechanism.

When cytoplasmic RNA extracted from NDV-induced hamster cells was applied directly to normal human or hamster cells, little detectable translation occurred. This was assumed to be the consequence of a low concentration of IF-mRNA in the population of RNAs and the relatively low sensitivity of hamster indicator cells to the hamster IF produced during translation. The concentration of IF-mRNA was increased over 50-fold by chromatographing the cytoplasmic RNA on oligo(dT)–cellulose columns. The large quantity of RNA required for these translation experiments necessitated diluting the poly(A)-containing RNA with some carrier RNA. The enrichment of IF-mRNA in the applied RNA mixture was at least 20-fold over its original concentration in cytoplasmic RNA. Translation was detected under these conditions.

Table IV: Translation of Hamster IF-mRNA in Normal and *Repressed* Human and Syrian Hamster Embryo Fibroblasts[a]

	expt 1	expt 2
Translation in Hamster Cells		
translation in normal	105	145
gross translation in *repressed* state	75	75
background (hamster IF synthesis not shut off and DEAE-dextran carry-over	90	65
net translation	−15	10
% normal translation	0	7
Translation in Human Cells		
translation in normal state	110	140
translation in *repressed* state	90	120
% normal translation	82	86

[a] Poly(A)-containing RNA was isolated on oligo(dT)–cellulose from cytoplasmic RNA extracted from NDV-induced SHE cells. The poly(A) RNA was mixed with carrier SHE RNA and translated in untreated HF926 and SHE cells, in HF926 cells treated 4–7 h prior to translation with $rI_n \cdot rC_n$, and in SHE cells treated 22–24 h prior to translation with $rI_n \cdot rC_n$–DEAE-dextran. The total enrichment of poly(A) mRNA in the applied RNA mixture was 20 times its concentration in cytoplasmic RNA. Interferon data are expressed as SHE laboratory reference units (see Experimental Procedures).

Hamster IF-mRNA was introduced into normal and *repressed* human and hamster cells. Carrier RNA from uninduced SHE cells was applied to the treated (*repressed*) human and hamster cells to measure the background level of IF activity. Table IV shows the results of these experiments. Significant background was detected for translation in treated hamster cells which was due to endogenous hamster IF synthesis and $rI_n \cdot rC_n$–DEAE-dextran carry-over. No background activity was detected for translation in treated human cells since (1) endogenous human IF synthesis could not interfere with the assay of hamster IF synthesized during translation and (2) $rI_n \cdot rC_n$ used in inducing the human cells into the *repressed* state was completely removed. Even if some carry-over of $rI_n \cdot rC_n$ occurred, this contamination still could not interfere with the titering of the hamser IF produced from translation, since $rI_n \cdot rC_n$ alone cannot induce hamster IF and, therefore, cannot increase the protection of hamster cells during titering.

As shown in Table IV, SHE cells in the *repressed* state have essentially lost their ability to translate exogenously introduced hamster IF-mRNA. However, *repressed* human cells and untreated human cells were able to translate hamster IF-mRNA equally well. These results obtained with hamster IF-mRNA are the reverse of those obtained with human IF-mRNA and confirm the species-specific nature of the IF translational inhibitory mechanism.

Discussion

Translation of human and hamster IF-mRNAs in *repressed* and in normal homologous and heterologous cells has indicated the existence of a species-specific translational inhibitory

mechanism during the shut-off of IF synthesis. These experiments have also directly demonstrated that the shut-off of IF synthesis is regulated at the translational level. This shut-off mechanism does not appear to inhibit translation randomly since the $rI_n \cdot rC_n$-induced *repressed* state (4–8 h after induction) does not result in the suppression of endogenous protein synthesis as measured by [^{3}H]leucine and [^{14}C]-methionine incorporation (data not shown).

It is conceivable that these observations are the fortuitous results of the artificialities inherent in our $CaCl_2$-mediated translation technique. Therefore, we are attempting to corroborate the species specificity of the IF shut-off mechanism by studying the coexpression of human and hamster IFs induced by $rI_n \cdot rC_n$ as well as with NDV in human–hamster hybrid cells. Preliminary results indicate that the IF shut-off mechanisms of hamster and human cells operate independently. Slate & Ruddle (1979) have examined the coexpression of human and mouse IFs in human–mouse hybrids. They reported that mouse and human IF synthesis shuts off at different times following induction by NDV, suggesting species-specific regulation of IF synthesis. However, extrapolation of results obtained with viral induction to the shut-off process following $rI_n \cdot rC_n$ induction must be done with caution since viral induction of IF is more complex and less understood than $rI_n \cdot rC_n$ induction of IF.

The enhancement of IF synthesis by antimetabolites is similar to that observed for the enzyme tyrosine aminotransferase in induced rat hepatoma cells. Both IF and tyrosine aminotransferase can be "superinduced" when inhibitors of protein synthesis or RNA transcription are added alone or in combination to the cells following induction. To explain this phenomenon, Tomkins et al. (1969) postulated the existence of a posttranscriptional control mechanism. This mechanism would involve the synthesis of a labile regulatory protein or repressor. The synthesis of this repressor is controlled by a specific gene and acts by inhibiting the translation of tyrosine aminotransferase mRNA. Several investigators have suggested such a mechanism for the regulation of IF synthesis (Vilček & Ng, 1971; Kohase & Vilček, 1977; Sehgal et al., 1978).

However, the repressor for IF-mRNA translation need not exclusively be a protein. The inhibition of IF synthesis shut-off by RNA transcription inhibitors can argue equally well for an RNA or a protein repressor. The strongest evidence for a protein repressor is based on the paradoxical enhancement of $rI_n \cdot rC_n$-induced IF synthesis by inhibitors of protein synthesis [see Stewart (1979) for a review]. This enhancement may be due to effects other than the inhibition of the synthesis of a putative repressor protein. A variety of secondary effects have been associated with inhibitors of protein synthesis, some of which may produce suppression or inhibition of RNA transcription. Therefore, on the basis of available evidence, the contribution of an RNA species to the "repression" of IF-mRNA translation cannot be excluded.

The participation of an RNA in the IF shut-off process is particularly attractive in light of recent findings concerning IF-induced enzymatic activities. IF treatment of cells results in the induction of at least two enzymes: an oligonucleotide polymerase which synthesizes from ATP a series of short-lived oligonucleotides [2′,5′-oligo(A)] containing 2′,5′-phosphodiester linkages (Kerr & Brown, 1978) and a protein kinase which phosphorylates the α subunit of initiation factor eIF-2 (Farrell et al., 1977). Both of these enzymes require double-stranded RNA (dsRNA) for activation. The polymerase binds strongly to dsRNA, and the 2′,5′-oligo(A) resulting from the activation of this enzyme activates, in turn, a latent endoribonuclease [see Baglioni (1979) for a review]. While this endoribonuclease is intrinsically nonspecific in the degradation of mRNA, Nilsen & Baglioni (1979) have obtained evidence which suggests that the 2′,5′-oligo(A)–endonuclease system preferentially degrades RNA-containing regions of double strandedness. The basis for the preferential degradation is presumed to be the result of binding of the 2′,5′-polymerase to the double-stranded regions, producing a regional synthesis of 2′,5′-oligo(A) and a localized activation of the endonuclease. Nilsen & Baglioni (1979) suggest that such a mechanism may be responsible for discrimination between viral and cellular mRNA in the preferential inhibition of viral mRNA accumulation.

The 2′,5′-oligo(A)–endonuclease system may be similarly involved in the shut-off of IF synthesis. Induction of IF synthesis may result in the synthesis of a translational control RNA (tcRNA) similar to the myosin tcRNA which binds to myosin mRNA, inhibiting its translation (Heywood & Kennedy, 1976). Should this be the case, then the IF-tcRNA could bind to IF-mRNA to form regions of double strandedness which would produce local activation of the endonuclease, degrading the IF-mRNA selectively as well as inhibiting translation directly. The tcRNA concept can also explain the species specificity of the translational inhibitory mechanism since sequence homology between the tcRNA and mRNA is required for binding, an expectation with homologous IF-mRNA but not with heterologous IF-mRNA.

While the initiation of IF synthesis is under transcriptional control, the results presented here together with those from other laboratories strongly suggest that the shut-off of IF synthesis is at the translational level. It should be noted that there is a significant difference in the time response of the two control systems; translational inhibition can arrest the synthesis of a protein much more quickly than termination of mRNA transcription. If a cell needs to turn off the synthesis of a protein quickly, translational control should be more efficient and responsive than transcriptional control. Mammalian cells might have good reason to quickly turn off the synthesis of IF, since IF in high concentrations has been shown to inhibit cell growth (Gresser et al., 1970; Pfeffer et al., 1979).

References

Baglioni, C. (1979) *Cell 17*, 255–264.

Barrett, J. C., Crawford, B. D., Grady, D. L., Hester, L. D., Jones, P. A., Benedict, W. F., & Ts'o, P. O. P. (1977) *Cancer Res. 37*, 3815–3823.

Bollin, E., Vastola, K., Oleszek, D., & Sulkowski, E. (1978) *Prep. Biochem. 8*, 259–274.

Cavalieri, R. L., Havell, E. A., Vilček, J., & Pestka, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4415–4419.

Colby, C., & Morgan, M. F. (1971) *Annu. Rev. Microbiol. 25*, 333–360.

De Maeyer-Guignard, J., De Maeyer, E., & Montagnier, L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1203–1207.

Dianzani, F., Ganoni, S., & Cantagalli, P. (1970) *Ann. N.Y. Acad. Sci. 173*, 727–735.

Farrell, P. J., Ballou, K., Hunt, T., & Jackson, R. (1977) *Cell 11*, 187–200.

Finter, N. B. (1969) *J. Gen. Virol. 5*, 419–427.

Graham, F. L., & Van der Eb, A. J. (1973) *Virology 52*, 456–467.

Graham, F. L., Feldheuisen, G., & Wilkie, N. (1973) *Nature (London), New Biol. 245*, 265–266.

Greene, J. J., Dieffenbach, C. W., & Ts'o, P. O. P. (1978) *Nature (London) 271*, 81–83.

Gresser, I., Brouty-Boyé, D., Thomas, M., & Macieira-Coelho, A. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1052–1058.
Grossberg, S. E., Smith, A. J., & Sedmak, J. J. (1975) in *Effects of Interferon on Cells, Viruses, and the Immune System* (Geraldes, A., Ed.) Academic Press, London.
Jankowski, W. J., Von Meunchhausen, W., Sulkowski, E., & Carter, W. A. (1976) *Biochemistry 15*, 5182–5187.
Kerr, I. M., & Brown, R. E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 256–260.
Kohase, M., & Vilček, J. (1977) *Virology 76*, 47–54.
Lab, M., & Kochran, F. (1972) *Ann. Inst. Pasteur, Paris 122*, 569–573.
Nilsen, T. W., & Baglioni, C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2600–2604.
Pfeffer, L. M., Murphy, J. S., & Tamm, I. (1979) *Exp. Cell Res. 121*, 111–120.
Pitha, P. M., & Carter, W. A. (1971) *Virology 45*, 777–781.
Sehgal, P. B., Tamm, I., & Vilček, J. (1976a) *Virology 70*, 256–259.
Sehgal, P. B., Tamm, I., & Vilček, J. (1976b) *Virology 70*, 532–541.
Sehgal, P. B., Tamm, I., & Vilček, J. (1976c) *Science 190*, 282–284.
Sehgal, P. B., Dobberstein, B., & Tamm, I. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3409–3413.
Sehgal, P. B., Lyles, D. S., & Tamm, I. (1978) *Virology 89*, 186–198.
Slate, D. L., & Ruddle, F. H. (1979) *Cell 16*, 171–180.
Stewart, W. E., II (1979) *The Interferon System*, Springer-Verlag, New York.
Tan, Y. H., Armstrong, J. A., Ke, Y. H., & Ho, M. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 464–481.
Tomkins, G. M., Gelenhiter, T. D., Granner, D., Martin, D., Samuels, H. H., & Thompson, E. B. (1969) *Science 166*, 1474–1480.
Vilček, J., & Ng, M. H. (1971) *J. Virol. 7*, 588–594.
Vilček, J., & Havell, E. A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3909–3913.

Conformational Flexibility of the 3′ Acceptor End of Transfer Ribonucleic Acid†

D. M. Cheng, S. S. Danyluk, M. M. Dhingra,‡ F. S. Ezra,§ M. MacCoss, C. K. Mitra, and Ramaswamy H. Sarma*

ABSTRACT: The intimate details of the conformational features and dynamics of the trinucleoside diphosphates CpCpA and ApCpC in aqueous solution have been arrived at by the complete analysis of their proton magnetic resonance spectra. In addition to the right-handed stacked species in which the phosphodiester torsions conform to the gauche–gauche domains, sugar puckers ^{3}E, C4′–C5′ $\simeq$ 60°, C5′–O5′ $\simeq$ 180°, C3′–O3′ $\simeq$ 205°, and $\chi_{CN} \simeq 40°$, the trimers display a variety of spatial configurations, an important one being a bulged configuration in which the central nucleotide unit is bulged out, enabling stacking interactions between the end units. It is further shown that the 3′ acceptor end of tRNA, CpCpA, displays considerable flexibility for the terminal adenine nucleotide unit. Theoretical NMR calculations demonstrate that the predominant solution conformation does not conform to the CCA terminus of tRNA as reported by four independent crystallographic studies of tRNA^{Phe}. It is shown that the preferred intramolecular order of CCA in solution is such that $\chi_1 = \chi_2 = \chi_3 = 40°$, all the three sugars are in ^{3}E, $\psi_1 = \psi_2 = \psi_3 = 60°$, ϕ_2 and ϕ_3 = 170 and 180°, respectively, $\phi_1' = \phi_2' = 205°$, and ω_1/ω_1' and ω_2/ω_2' = 240/205° and 295/265°, respectively.

The ubiquitous presence of the sequence CpCpA at the 3′ acceptor end of tRNA has been known for a long time. Less is known about geometric features in solution of this unique trinucleotide and the possible role in recognition of a variety of aminoacyl-tRNA synthetases. In an attempt to approach this problem from a structural perspective, we have investigated the conformational properties of CpCpA and ApCpC in aqueous solution, and we report our findings in this paper.

Comprehensive investigations of the conformational properties of ribodinucleoside monophosphates in aqueous solution (Lee et al., 1976; Ezra et al., 1977; Dhingra et al., 1978) have provided information about the stereochemical principles involved in the 3-D topology of these molecules. These studies revealed the presence of a coupled set of base sequence dependent conformational parameters and interdependent structural changes linked to base stacking interactions (Lee et al., 1976; Ezra et al., 1977; Dhingra et al., 1978; Evans & Sarma, 1976; Sarma & Danyluk, 1977; Cheng & Sarma, 1977). A natural extension is the investigation of the spatial configuration and conformational properties of higher oligomers. However, the higher oligomers have presented a difficult problem with respect to the complete analysis of their ^{1}H spectra. Selective deuteration studies (Kondo et al., 1975) enabled unambiguous assignment of the H1′ of each of the residues of ApApA. Computer-assisted analysis enabled Evans & Sarma (1976) to determine the signals which are coupled to each other in each of the residues in ApApA even though an unambiguous assignment of each of the protons by this method is not possible. A complete unambiguous assignment

† From the Institute of Biomolecular Stereodynamics and Department of Chemistry, State University of New York at Albany, Albany, New York 12222 (D.M.C., M.M.D., C.K.M., and R.H.S.), and the Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439 (S.S.D., F.S.E., and M.M.). *Received December 7, 1979.* This work was supported by Grant CA12462 from the National Cancer Institute, Department of Health, Education, and Welfare, Grant PCM75-16406 from the National Science Foundation, Grant 1-P07-PR00798 from the Division of Research Sources, National Institutes of Health, and the U.S. Department of Energy.

‡ Present address: Tata Institute of Fundamental Research, Bombay 400 005, India.
§ Present address: Miami Valley Laboratories, Proctor and Gamble Corp., Cincinnati, OH 45247.

0006-2960/80/0419-2491$01.00/0