Biochemical Pharmacology, Vol. 39, No. 5, pp. 865–871, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

IN VITRO TRANSLATIONAL ACTIVITY OF MESSENGER-RNA ISOLATED FROM MICE TREATED WITH THE INTERFERON INDUCER, POLYRIBOINOSINIC ACID·POLYRIBOCYTIDYLIC ACID*

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(*Received* 18 *August* 1989; *accepted* 10 *October* 1989)

Abstract—Treatment of mice with interferon and interferon inducers causes down regulation of a number of hepatic proteins. In a previous publication it was demonstrated that these treatments depress hepatic protein synthesis and increase protein degradation, particularly of the endoplasmic reticulum Gooderham NJ and Mannering GJ, *Arch Biochem Biophys* **250**: 418–425, 1986. In the present study the effects of polyriboinosinic acid·polyribocytidylic acid (poly IC) treatment on mouse hepatic RNA levels and the translation of this RNA in a cell-free system were examined. Poly IC treatment of mice increased hepatic poly (A^+)RNA levels. The translation of isolated poly(A^+)RNA was evaluated at various intervals after the administration of poly IC. Translation was marginally increased at 3–6 hr after treatment and depressed after 12–18 hr. Antibodies were employed to examine the effects of poly IC treatment on specific polypeptides in order to evaluate the *in vitro* translation of mRNAs for tyrosine aminotransferase and albumin; translation of these proteins was biphasic with pronounced depression. These studies indicate that *in vivo* interferon may regulate gene expression by altering levels of hepatic proteins via increased transcription and decreased translation.

Extensive research has shown that in addition to their potent antiviral activity, interferons can influence numerous biochemical reactions [1]. One such effect of interferon therapy relates to the depression of oxidative drug metabolism [2–4]. Drug oxidation is predominately catalysed by the cytochrome P-450 family of isozymes located in the endoplasmic reticulum. These enzymes metabolize both xenobiotic and endogenous compounds; changes in the levels of these enzymes can alter biochemical, pharmacological and toxicological processes. We and others have shown that interferon inducers and interferons depress drug oxidation by lowering levels of hepatic cytochrome P-450 and NADPH cytochrome P-450 reductase [1, 5–9] but the mechanism involved has not been determined. In cell culture studies, interferon has been shown to cause alterations in protein synthesis and degradation [10]. We investigated the possibility that a similar mechanism might account for the *in vivo* depression of the hepatic cytochrome P-450 system by interferon induction. In a previous publication we described effects of polyriboinosinic acid·polyribocytidylic acid (poly IC) treatment on mouse hepatic protein biochemistry [1]. These studies indicated that in the 24 hr after poly IC administration hepatic microsomal protein degradation was temporarily enhanced and protein synthesis was decreased. We have now attempted to gain a better understanding of the mechanism by which interferon alters protein synthesis by examining the functionality of isolated hepatic messenger RNA. It was hoped that these studies would provide information regarding interferon mediated control of gene expression and help explain why treatment of animals with interferon or interferon inducing agents alters hepatic enzyme homeostasis.

* This work was supported by National Institutes of Health Grant GM 28171.

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MATERIALS AND METHODS

Materials. Polyriboinosinic acid·polyribocytidylic acid (poly IC) was purchased from the Sigma Chemical Co. (St Louis, MO). Guanidine HCl and rabbit reticulocyte lysate translation kits were obtained from Bethesda Research Laboratories (Gaithersburg, MD). L-2[^{3}H]Leucine, Protosol tissue solubilizer, Enhance scintillant and Aquasol II scintillant were obtained from New England Nuclear Corp. (Boston, MA). Oligo(dT)cellulose was purchased from Collaborative Research (Waltham, MA). Protein A-sepharose was purchased from Pharmacia (Piscataway, NJ). Mouse serum albumin, rabbit anti-mouse serum albumin and rabbit preimmune IgG were obtained from Cappel Worthington (Cochranville, PA). Kodak X-Omat AR film was purchased from Northern X-ray (Minneapolis, MN).

Animals. Male Swiss–Webster mice (20–25 g) were purchased from Taconic Farms, Inc. (Germantown, NY). They received autoclaved mouse chow supplied by Taconic Farms Inc., given *ad lib.* and were maintained in a controlled diurnal lighting cycle (12 hr on, 12 hr off). Poly IC (10 mg/kg) was injected i.p. in 0.9% saline.

Isolation of RNA. Groups of at least five mice per time point were killed by cutting the throat and exsanguinating. All animals were killed as close to

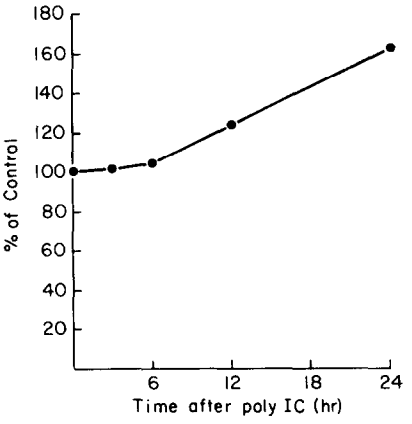

Fig. 1. Effect of administered poly IC on levels of mouse hepatic poly (A^+)RNA. Mice were treated with poly IC (10 mg/kg) and killed at indicated times (at least five mice per time point). Their livers were perfused *in situ* with 1.15% KCl, pooled and their total hepatic RNA was isolated. From each pool of total hepatic RNA at least two separate isolations of poly(A^+)RNA were performed. Quantification showed recoveries from each pool to be within 5%. The entire experiment was repeated twice. The points are the means of at least four poly(A^+)RNA determinations from two separate experiments. Control value = 99.27 ± 0.50 μg poly(A^+)RNA/g liver.

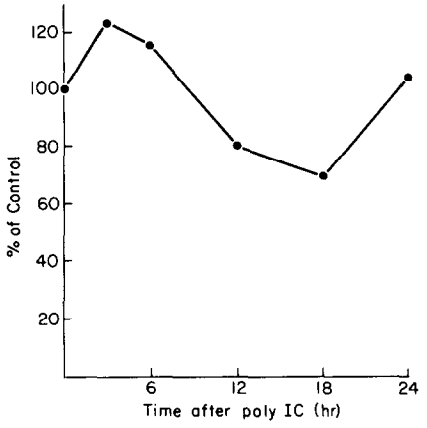

Fig. 2. Effect of administered poly IC on *in vitro* translation of mouse hepatic poly(A^+)RNA. Mice treated with poly IC (10 mg/kg) were killed at the indicated times (at least five mice per time point) and their hepatic poly(A^+)RNA was isolated. Poly(A^+)RNA (2 μg per time point) was used for cell-free translation in the presence of L-[^{3}H]leucine. Total translation was determined by the extent to which ^{3}H was incorporated into trichloroacetic acid precipitable polypeptides. Points are the means of at least four assays from each of two separate experiments. Control value = 204,841 ± 19,661 dpm/μg poly(A^+)RNA.

9.00 am as possible. Livers were rapidly perfused *in situ* with 1.15% KCl. Gall bladders were excised, the livers removed and pooled and immediately homogenized in about 10 vol. of cold guanidine HCl/dithiothreitol/sodium acetate buffer, pH 5.0 (8 M/1 mM/10 mM) by using 3 × 5 sec pulses of a Waring blender. Total hepatic RNA was isolated by using the procedure described by Cox [11]. RNA purity was assessed by comparing the A_{260}/A_{280nm} ratio and using the A_{260nm} extinction coefficient of 0.025 μg RNA mL^{-1} cm^{-1}.

Isolation of poly(A^+)RNA. Total hepatic RNA isolated by guanidine HCl extraction as described above was used for the isolation of poly(A^+)RNA. Total RNA (100 A_{260} units) was dissolved in Tris/EDTA (20 mM/1 mM, pH 7.5), heated at 70° for 5 min, cooled on ice and adjusted to 0.5 M NaCl. Poly(A^+)RNA was isolated by using an oligo (dT) column as described by Aviv and Leder [12]. The recovery of poly(A^+)RNA was quantitatively consistent (SE of 0.5%). The poly (A^+)RNA was dissolved in sterile water and stored at −80°.

In vitro translation. Poly(A^+)RNA isolated from mice treated with poly IC was used to program cell-free protein synthesis in a rabbit reticulocyte lysate system. The poly(A^+)RNA was heated at 70° for 1 min and then rapidly chilled on ice immediately before adding to the assay mixture. Incubations were usually carried out for 60 min at 37° with 2 μg of poly(A^+)RNA. Blank control incubations in which sterile water was substituted for poly(A^+)RNA were routinely employed. In addition, positive control incubations using purified rabbit globin mRNA were always performed. In certain experiments the incubation time and concentration of poly(A^+)RNA were varied. The reaction was terminated by rapid freezing to −80°.

Products of *in vitro* translation were quantified by measuring acid-precipitable radioactivity by a modification of the method of Pelham and Jackson [13] as described in the reticulocyte batch analysis sheet provided with the reticulocyte lysate preparation.

Immunoprecipitation. Immunoprecipitation experiments were performed as described by Negishi and Nebert [14], using protein A-sepharose. Non specific binding to the antibodies was minimized by preincubation with preimmune IgG/ protein A-sepharose. Immunoprecipitate aggregates were stored at −20° until used for electrophoretic analysis or ^{3}H-scintillation counting.

Electrophoresis, fluorography and densitometry. *In vitro* translation mixture (10 μL) or immunoprecipitation aggregates suspended in 40 μL water were dissolved in Tris–HCl buffer, pH 6.8 (250 mM) containing sodium dodecyl sulphate (0.4%), glycerol (30%) and β-mercaptoethanol (340 mM). The solutions were heated at 100° for 10 min and the supernatant (15 μL of translation mixture or 50 μL of immunoprecipitate) were applied to sodium dodecyl sulphate polyacrylamide gels (8.5%) for electrophoresis by the method of Laemmli [15]. After electrophoresis, gels were impregnated with scintillant (Enhance) and dried. Electrophoretic patterns were visualized by fluorography by using pre-flashed Kodak X-Omat film at −80° as described by Bonner and Laskey [16].

In some experiments the fluorographic pattern was evaluated by densitometry by using a Gilford 2400 Spectrophotometer fitted with a linear transporter device.

Other assays. Microsomal cytochromes P-450 and

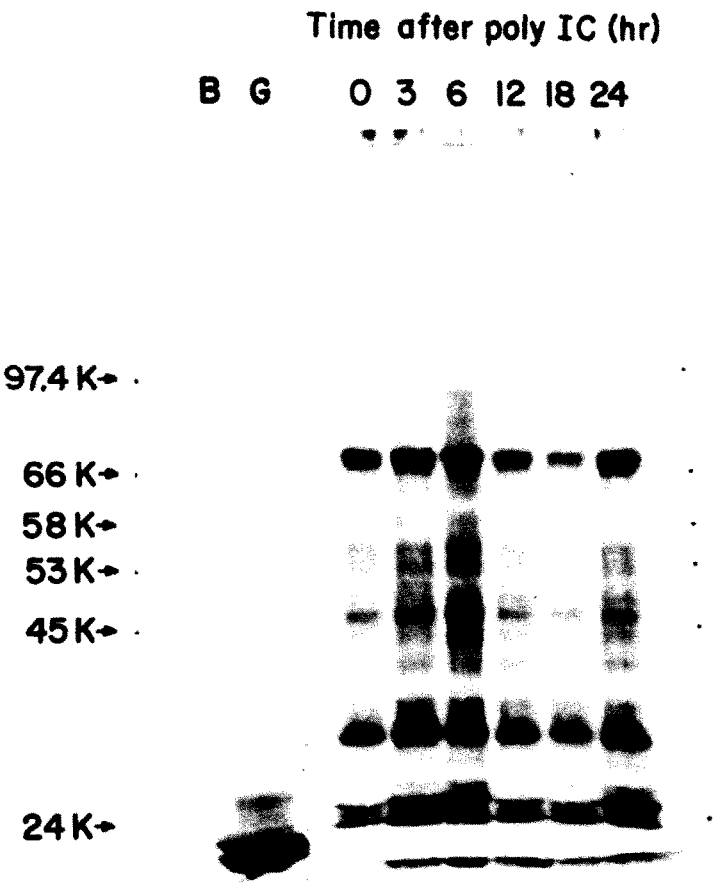

Fig. 3. SDS/PAGE fluorograph of *in vitro* translation products of mouse hepatic poly(A$^+$)RNA. Mice were treated with poly IC and hepatic poly(A$^+$)RNA was isolated as described in Fig. 1. Poly(A$^+$)RNA was used to program translation using reticulocyte lysate as described in Materials and Methods. B, blank assay without exogenous poly(A$^+$)RNA; G, assay programmed with purified rabbit globin mRNA as control; 0–24 are total translation products obtained with reticulocyte lysate programmed with poly(A$^+$)RNA (2 μg) isolated from mice at indicated times after poly IC treatment. Numbers on the vertical axis represent molecular weight standards.

b_5 were measured by the methods of Matsubara *et al.* [17] and Omura and Sato [18], respectively. Protein was determined by the method of Lowry *et al.* [19] using bovine serum albumin as the standard.

RESULTS

Microsomal cytochrome P-450 levels were used to monitor the effects of poly IC on liver protein homeostasis. Poly IC (10 mg/kg) produces its maximum effect on the depression of the cytochrome P-450 system 24 hr after its administration [3]. In the present study, at the 24 hr time point the cytochrome P-450 level was found to be 75.2 ± 2.1% of the control value (0.70 ± 0.03 nmol P-450/mg microsomal protein, $N = 6$, $P < 0.001$).

Since depression represents perturbation of the steady state of the enzyme level, the effect of treatment of mice with poly IC on RNA levels was examined. An increase in the level of poly (A$^+$)RNA (messenger RNA) was observed during the 24 hr study (Fig. 1). At the 24 hr time point the level of poly(A$^+$)RNA was 60% greater than at the 0 hr time point.

The poly (A$^+$)RNA isolated at various times after poly IC treatment was used to program cell-free translation. Using exactly the same amount of poly(A$^+$)RNA (2 μg), total translation of these preparations varied with time as measured by the incorporation of L-[^{3}H]leucine into trichloroacetic acid precipitable polypeptides (Fig. 2). Translation of poly(A$^+$)RNA isolated 3–6 hr after poly IC administration generated more product that poly(A$^+$)RNA isolated at the 0 hr time point (control). Poly(A$^+$)RNA isolated from animals exposed to poly IC for longer periods (12–18 hr) yielded less translation products than the control.

In order to determine to what extent the translation of a wide variety of hepatic proteins was affected by poly IC, the electrophoretic pattern (SDS/PAGE) of total translation mixtures were examined. Figure 3 is a fluorograph of the resulting gel. Lane B is an assay blank in which water was used in place of poly(A$^+$)RNA; no translation products are

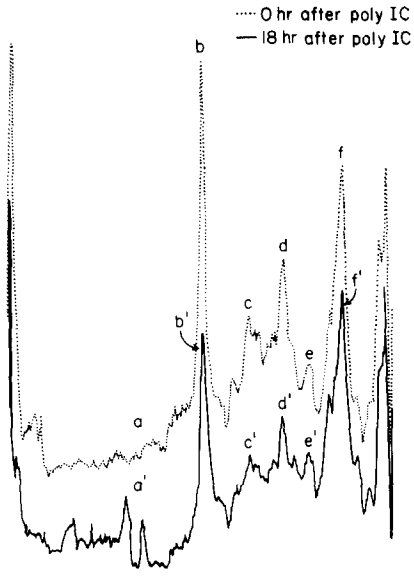

Fig. 4. Densitometry scan of SDS/PAGE fluorograph of *in vitro* translation of mouse hepatic poly(A^+)RNA. (....) 0 hr after poly IC; (———) 18 hr after poly IC. See text for explanation of areas.

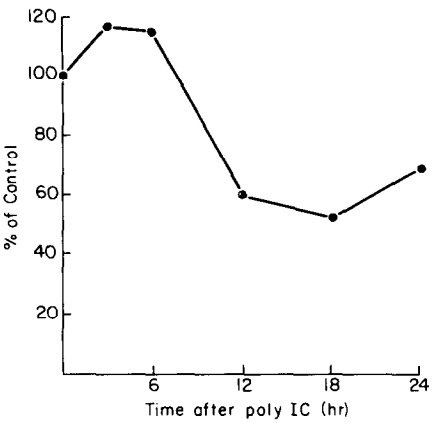

Fig. 5. Effect of administered poly IC on immunoprecipitable pre-proalbumin translated *in vitro* from mouse hepatic poly(A^+)RNA. Hepatic poly(A^+)RNA from mice treated with poly IC was translated *in vitro* as described in Fig. 2. Pre-proalbumin polypeptide precipitated from translation mixtures was quantified by ^{3}H-scintillation counting as described in Materials and Methods. Points are the means of at least four determinations from each of two separate experiments. Control value = 41,703 ± 2306 dpm/μg poly(A^+)RNA.

evident. Lane G is a positive control in which purified rabbit globin mRNA was used to program translation. Lanes 0–24 are total translation products obtained by using poly(A^+)RNA (2 μg) obtained at various times (0–24 hr) after poly IC treatment of mice. Signal intensities relative to the zero hour control were increased at short poly IC exposure times (3–6 hr), whereas at the later time points (12–18 hr), signal intensities were decreased. The protein band around 70,000 Da is particularly increased 3–6 hr after poly IC, but is decreased after 18 hr exposure. Similarly, the signals in the 45,000–55,000 Da region, where cytochrome P-450 polypeptides are known to migrate, are decreased 18 hr after poly IC treatment. Densitometry scans of lanes 0 and 18 hr allow comparison of the signals generated at the various molecular weights (Fig. 4). Relative to bands from preparations obtained at time zero, bands derived from samples collected 18 hr after poly IC administration show increased protein levels around 100,000 Da (region a, a^1), decreased signal intensities at 70,000 Da and 45,000–55,000 Da (b,b^1; c,c^1; d,d^1) and areas of little or no change (e.g. 35,000 Da, f,f^1).

Having examined the global effects of poly IC treatment on translation of the isolated poly(A^+)RNA, we turned our attention to the effect on individual polypeptides. Albumin, the major secretory protein of mammalian liver, comprises about 10% of the protein synthesized by the liver [20]; its level is depressed after poly IC treatment [7]. Tyrosine aminotransferase is an intracellular enzyme with a short half-life whose activity is initially induced and then depressed after poly IC treatment [1]. Specific antibodies were used to examine the effects of poly IC treatment on translation of these proteins. Pre-proalbumin levels were decreased to 60% of the control value 12–18 hr after poly IC (Fig. 5). The decreased level of pre-proalbumin was also evident at the 24 hr time point (75% of 0 hr control). Examination of the pre-proalbumin immunoprecipitates by SDS/PAGE demonstrated that the mouse albumin antibody was specific for a 71,000 Da polypeptide (Fig. 6).

Similar studies which used an antibody to mouse tyrosine aminotransferase also showed decreased levels of immunoprecipitable tyrosine aminotransferase polypeptide 12 hr after poly IC administration (Fig. 7). The duration of impairment of translation did not appear to be as long as that for albumin.

DISCUSSION

In previous publications we demonstrated that treatment of mice with the potent interferon inducer, poly IC, depressed cytochrome P-450 mediated enzyme activities [3], and that this was associated with alterations in hepatic endoplasmic recticulum protein synthesis [1]. In the current study we attempted to reveal the molecular events that accompany these alterations in hepatic protein biochemistry by examining the effect of poly IC on mouse hepatic messenger RNA. A single treatment of poly IC (10 mg/kg) caused an increase in the level of isolated mRNA during the 24 hr period of the study. One contribution to this increase in mRNA is likely to be a requirement for *de novo* synthesis of antiviral proteins induced by interferon. Additionally, or alternatively, the inhibitory effect of interferon on protein synthesis [21, 22] may lead to an accumulation of mRNA (i.e., decreased degradation of mRNA) or cause the cell to over-supply mRNA by a feedback mechanism.

Cell-free translation of mRNA isolated from mice

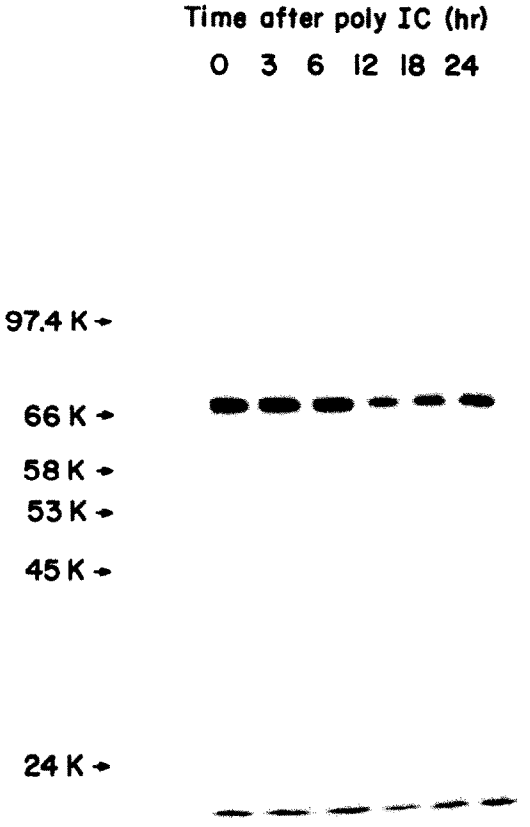

Fig. 6. SDS/PAGE fluorograph of pre-proalbumin immunoprecipitates derived from *in vitro* translation of mouse hepatic poly(A^+)RNA. Pre-proalbumin immunoprecipitates obtained as described in Fig. 5 were electrophoresed as described in Materials and Methods. Values on the vertical axis represent molecular weight standards.

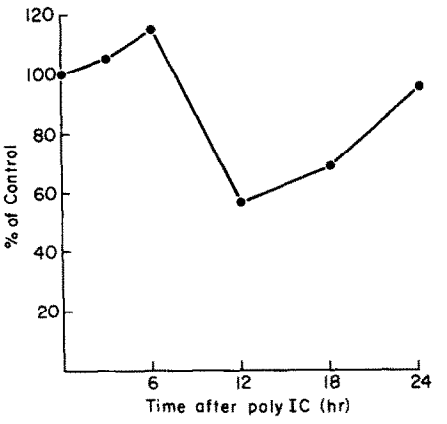

Fig. 7. Effect of administered poly IC on immunoprecipitable tyrosine aminotransferase translated *in vitro* from mouse hepatic poly(A^+)RNA. Hepatic poly(A^+)RNA from mice treated with poly IC was translated *in vitro* as described in Fig. 2. Tyrosine aminotransferase polypeptide was immunoprecipitated from translation mixtures and quantified by ^{3}H-scintillation counting as described in Materials and Methods. Points are the means of at least four determinations from each of two separate experiments. Control value = 21,125 ± 3800 dpm/μg poly(A^+)RNA.

treated with poly IC demonstrated that such treatment could alter the total translation of a known amount (2 μg) of poly(A^+)RNA. These findings compare very favourably with the results of our previous report [1] with poly IC treated mice, in which hepatic tyrosine amino transferase activity was initially found to be increased (up to 6 hr post-poly IC) then decreased. These observations have been extended by a recent report by Moochhala *et al.* [23] in which purified interferon treatment caused a biphasic effect on hepatic cytochrome P-450 which was very similar to that observed in our previous publication [1].

Moochhala *et al.* [23] reported that the initial increase in enzyme activity that occurred 3 hr after animal treatment with interferon could be totally prevented by treating the animals with puromycin, thus confirming the effect was translation mediated. The results of the present experiments support the case for the biphasic effect of interferon treatment on hepatic protein synthesis. Whether the interferon mediated effects on translation are a consequence of altered structure, mis-coding or impaired binding of the mRNA to ribosomes cannot be determined from these experiments. Interferon is known to exert translational control by means of at least two induced enzyme systems [21, 22]. In one of these systems, interferon, in the presence of double stranded RNA, induces 2′-5′-oligoadenylate synthetase, which synthesizes adenosine oligomers with 2′-5′-phosphate linkages. The oligomers activate a latent endonuclease which can cleave mRNA and ribosomal RNA. The second interferon induced enzyme activity is a protein kinase, which in the presence of double stranded RNA, phosphorylates ribosomal initiation factor eIF-2 and thereby inhibits protein synthesis. This latter enzyme cannot account for the results of the current study because a reconstituted translation system using exogenously supplied ribosomes was employed. Collectively, the two enzyme systems are believed to provide the mechanism whereby interferons regulate *in vivo* gene expression at the level of translation [21, 22].

In the present study the decrease in translation of poly(A^+)RNA obtained from mice pretreated with poly IC might be explained on the ability of interferon to prevent the formation of cap structures of mRNA [24]. Most cellular and viral mRNAs have, at the 5′ terminus, a 7-methyl guanosine (m^7G) linked to the penultimate nucleotide in the 2′-OH position of the ribose moiety. Efficient translation of cellular and viral mRNA depends on the m^7G being present at the 5′ terminus [25, 26]. mRNA of vesicular stomatitis virus grown in the HeLa cells treated with interferon was found to be under-methylated in the 5′ terminal guanosine [24]. This hypomethylated mRNA does not associate with polysomes and is therefore not translated. Other examples exist in which uncapped mRNA can be correctly translated in cell free systems, but with lowered efficiency [25].

The results of our studies are in agreement with

the findings of Nilsen *et al.* [10, 27] and of Benavente *et al.* [28]. Nilsen *et al.* employed cultured HeLa cells pretreated with interferon and then infected with reovirus. They observed that this treatment caused a large fraction of the cellular mRNA to be degraded [27]. However, total cellular protein synthesis did not appear to be affected, which the authors suggest indicates an increased production of mRNA [10]. In our experiments we employed mice treated with the double stranded RNA, poly IC; this induces interferon *in vivo* and provides double stranded RNA to activate the 2′-5′ oligoadenylate synthetase and the endonuclease. The impaired translation of our mRNA preparations is perhaps the result of activity of these enzymes and is consistent with the findings of Benavente *et al.* [28] who showed that mouse L-cells pretreated with interferon and infected with vaccinia virus caused indiscriminant degradation of both cellular and viral mRNA. Our experiments also indicate that after poly IC administration, levels of hepatic poly(A^+)RNA are increased; this is compatible with the suggestion of Nilsen *et al.* [10] that increased production of mRNA is required to maintain overall cell protein synthesis.

Under conditions of cellular stress, e.g. viral attack or poly IC exposure, the body would be expected to inhibit viral (double stranded RNA) infestation by producing interferon and thereby invoking interferon control of gene expression. The resulting inhibition of translation would retard viral replication. For a cell to survive inhibition of translation by interferon, it would be required that the translation of essential cellular proteins remain largely unimpaired. Increased transcription of mRNAs for essential cellular proteins would have the effect of diluting the viral mRNA pool. This would favour translation of cellular proteins by competitive inhibition, under conditions of partially inhibited translation [29]. An increased translation of essential cellular mRNAs in preference to "luxury" cellular mRNAs would be a further refinement. Under such circumstances, the rate of loss of non-essential proteins would depend upon their rate of degradation. Loss of specific proteins or even specific isoenzymes, may be explained in these terms. However, it should be pointed out that loss of specific protein could also be the result of decreased synthesis of specific mRNAs, increased breakdown of specific mRNAs, decreased efficiency of translation or breakdown of the protein.

We therefore suggest that treatment of mice with the interferon inducer poly IC may selectively affect the translation of some proteins more than others, e.g. depression of the 71,000 Da protein and those of molecular weight 45,000–55,000 Da.

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