Proc. Natl. Acad. Sci. USA
Vol. 77, No. 6, pp. 3489–3493, June 1980
Cell Biology

Regulation of the stability of poly(I)·poly(C)-induced human fibroblast interferon mRNA: Selective inactivation of interferon mRNA and lack of involvement of 2′,5′-oligo(A) synthetase activation during the shutoff of interferon production

(FS-4 cells/superinduction/priming)

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Communicated by Igor Tamm, March 21, 1980

ABSTRACT **The inactivation of interferon mRNA during the shutoff phase of interferon production in poly(I)·poly(C)-induced human fibroblast cultures is selective. We have determined that the shutoff of interferon production, which takes place from 3 to 8 hr after the beginning of induction, is not associated with an appreciable decline in the rate of bulk cellular protein synthesis or of cellular protein secretion. While the amount of translatable interferon mRNA declined markedly during the shutoff phase, the level of translatable bulk cellular mRNA and the stability of [^{3}H]uridine-labeled mRNA were unaffected. Superinduction with actinomycin D selectively stabilized interferon mRNA with no apparent effect on the stability of bulk cellular mRNA. Furthermore, an activation of the 2′,5′-oligo(A) synthetase/endonuclease system does not appear to be involved in the shutoff phenomenon. Uninduced FS-4 cells contained a low basal level of 2′,5′-oligo(A) synthetase activity, which was unchanged in poly(I)·poly(C)-induced cells during the shutoff phase. Treatment of FS-4 cells with interferon for 16–18 hr prior to induction increased the enzyme activity by approximately 200-fold. However, this did not inhibit interferon production after induction with poly(I)·poly(C) alone or after superinduction with cycloheximide or actinomycin D or both. Furthermore, the rates of decay of interferon production were comparable in cells with either a basal or an increased level of 2′,5′-oligo(A) synthetase. Thus a 200-fold increase in 2′,5′-oligo(A) synthetase level did not affect either the stability of interferon mRNA or the efficacy of interferon superinduction by metabolic inhibitors.**

Exposure of confluent cultures of diploid human fibroblasts (FS-4) to poly(I)·poly(C) leads to prompt activation of interferon mRNA transcription and a burst of interferon synthesis (1–3). The rate of interferon secretion peaks by approximately 3 hr but then declines rapidly, and the production is shut off by 6–8 hr. The half-life of interferon mRNA during the shutoff phase is approximately 0.5 hr (4, 5). Appropriate treatment of induced FS-4 cultures with inhibitors of RNA and protein synthesis markedly enhances total interferon yields (superinduction) primarily but not solely by prolonging interferon production. The half-life of interferon mRNA in superinduced cultures is in the range of 6–8 hr (4, 5).

In both poly(I)·poly(C)-induced and superinduced cultures, transcription of interferon mRNA is largely completed within 3 hr of induction (6). Yet the rapid shutoff of interferon production in induced cultures is not due to the cessation of transcription but rather to the existence of a posttranscriptional mechanism that degrades or inactivates interferon mRNA (refs. 2, 4, 5, and 7; also see Fig. 3). This conclusion is based on an extensive investigation of the phenomenon of interferon superinduction (2, 4, 5, 7). The interferon mRNA molecule appears itself to be quite stable, as indicated by its long half-life in superinduced cultures. There is considerable support for the hypothesis that inhibitors of RNA and protein synthesis used in superinduction schemes interfere with a posttranscriptional regulatory mechanism that degrades or inactivates interferon mRNA (1–9). The shutoff and superinduction of human fibroblast interferon production represent striking examples of the regulation of mammalian gene expression by alterations in mRNA stability in accordance with the McAuslan–Tomkins hypothesis (10–12).

We have investigated the specificity with which the shutoff mechanism inactivates interferon mRNA. We present evidence that this posttranscriptional mechanism does not affect bulk cellular mRNA. Because poly(I)·poly(C)-induced FS-4 cultures are in contact with large amounts of interferon that they have themselves produced, it is conceivable that an induction of 2′,5′-oligo(A) synthetase (13–16) in response to the interferon produced may be involved in the inactivation of interferon mRNA. In cell-free systems 2′,5′-oligo(A) synthetase is greatly stimulated by double-stranded RNA to synthesize 2′,5′-oligo(A), which in turn activates an endonuclease (13–16). It has been reported that the levels of 2′,5′-oligo(A) synthetase in chicken oviduct tissue increase during hormone withdrawal at a time when mRNA degradation is at a peak (17), which raises the possibility that 2′,5′-oligo(A)-dependent endonuclease may be involved in regulating the stability of specific cellular mRNA species. To evaluate the possible involvement of 2′,5′-oligo(A)-mediated endonuclease activation in the regulation of interferon production, we have investigated (*i*) the levels of 2′,5′-oligo(A) synthetase during poly(I)·poly(C) induction, and (*ii*) the functional stability of interferon mRNA in FS-4 cells with a low basal as well as 200-fold increased level of 2′,5′-oligo(A) synthetase. The data obtained suggest that activation of the 2′,5′-oligo(A) synthetase/nuclease system is not involved in regulating the stability of interferon mRNA.

MATERIALS AND METHODS

The procedures for growing FS-4 cells in 35- or 150-mm Falcon petri dishes, for induction of interferon with poly(I)·poly(C) (P-L Biochemicals, 20 μg/ml), for harvesting cells by trypsinization, for extraction of RNA, for poly(U)-Sepharose chromatography, and for the assay of interferon mRNA in *Xenopus laevis* oocytes (10–15 oocytes per assay) have been reported (2–7, 18). The procedures for monitoring the rates of cellular protein synthesis have also been described (5–7, 18). Cell-free translation of polyadenylylated cellular mRNA was carried out by using a rabbit reticulocyte lysate translation kit ([^{3}H]leucine, NEK 002, New England Nuclear). Interferon was assayed on

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GM-258 cells by a modified semimicro method using vesicular stomatitis virus as the challenge virus (19–21). Interferon titers are expressed in terms of the 69/19 reference standard for human interferon. Human fibroblast interferon [specific activity 10^7 reference units (U)/mg of protein] was kindly supplied by J. Horosewicz and W. Carter, Roswell Park Memorial Institute, Buffalo, NY. [^{3}H]Uridine (25 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) and L-[^{3}H]leucine (110 Ci/mmol) were obtained from New England Nuclear, cycloheximide from Calbiochem, and actinomycin D from Merck, Sharp and Dohme.

Assay of 2′,5′-Oligo(A) Synthetase Activity. FS-4 cultures in 150-mm petri dishes were washed twice with 10 ml of chilled buffer A (35 mM Tris·HCl, pH 7.5/146 mM NaCl) and then once with 5 ml of buffer B [25 mM Tris·HCl, pH 7.5/120 mM KCl/5 mM magnesium acetate/7 mM 2-mercaptoethanol/10% (vol/vol) glycerol]. The cells were scraped with a rubber policeman and then lysed by adding 0.4 ml of buffer B containing 0.75% Nonidet P-40 detergent. The cell lysates were allowed to stand on ice for approximately 15 min in centrifuge tubes with occasional mixing. The lysates were centrifuged at 1000 × *g* for 10 min and the supernatant fractions obtained were further centrifuged at 27,000 × *g* for 10 min. The final supernatant fractions (S27) were divided into aliquots and stored in liquid N_2. The poly(I)·poly(C) column procedure (22) was used to test for 2′,5′-oligo(A) synthetase activity. Briefly, cell extracts (300 μl) were applied to poly(I)·poly(C)-agarose columns (50–60 μl, P-L Biochemicals), mixed with the column bed, and incubated for 30 min at 4°C and then for another 5 min at room temperature with occasional mixing. The flow-through fraction was collected and recycled twice through the column. The columns were then washed with approximately 25 ml of buffer containing 10 mM Hepes–KOH at pH 7.5, 90 mM KCl, 1.5 mM magnesium acetate, 7 mM 2-mercaptoethanol, and 20% (vol/vol) glycerol in the cold and then with 2–3 ml of the same buffer containing 10 mM magnesium acetate. Reaction mixtures (70 μl) containing 10 mM Hepes–KOH at pH 7.5, 90 mM KCl, 10 mM magnesium acetate, 10 mM 2-mercaptoethanol, 20% glycerol, 5 mM [^{3}H]ATP (approximately 7 × 10^5 cpm, New England Nuclear), creatine phosphate at 2 mg/ml, and creatine kinase at 0.3 mg/ml were added to the columns, followed by incubation at 30°C for approximately 16 hr. The ^{3}H-labeled oligoadenylates formed were fractionated on DEAE-cellulose columns as described (22).

RESULTS

Specificity of the shutoff mechanism

Exposure of confluent FS-4 cultures to poly(I)·poly(C) leads to a rapid induction of interferon (Fig. 1). The rate of interferon production peaks by approximately 3 hr and interferon production is then rapidly shut off by 6–8 hr. Fig. 1 shows that as the rate of interferon secretion after induction of FS-4 cultures with poly(I)·poly(C) reaches a peak and then declines, the rates of bulk cellular protein synthesis and of cellular protein secretion remain unchanged.

Fig. 2 shows that while the levels of cellular polyadenylylated interferon mRNA, as assayed in oocytes in several independent experiments, decrease during the shutoff phase (also see refs. 4, 5, and 23), the levels of bulk cellular mRNA assayed in reticulocyte lysates remain constant. On the basis of the data shown in Fig. 3 it is unlikely that bulk cellular mRNA levels remain constant because of continuous synthesis of mRNA while interferon mRNA levels decrease solely because synthesis of interferon mRNA is terminated by 3 hr after induction. FS-4 cultures were induced with poly(I)·poly(C) and labeled with

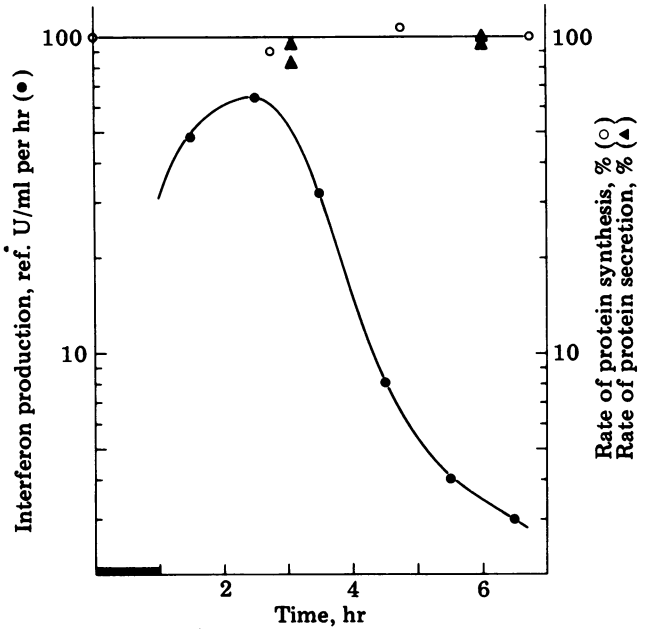

FIG. 1. The rates of interferon production (●), of cellular protein synthesis (○), and of cellular protein secretion (▲) in confluent FS-4 cell cultures in 150-mm petri dishes induced with poly(I)·poly(C) (20 μg/ml) for 1 hr. The rate of interferon production was determined by hourly changes of the culture medium (10 ml per dish). The rates of protein synthesis and protein secretion were monitored in duplicate at different times during the experiment and are expressed relative to corresponding cultures that did not receive poly(I)·poly(C). The rate of protein synthesis was assayed by pulse-labeling cultures with [^{3}H]leucine (5 μCi/ml) for 10 min and monitoring the trichloroacetic acid-precipitable cellular radioactivity, while the rate of cellular protein secretion was estimated by labeling cultures in duplicate with [^{3}H]leucine (10 μCi/ml) for 30 min and monitoring the appearance of trichloroacetic acid-precipitable radioactivity in the culture medium during the next 30 min. One hundred percent protein synthesis corresponds to 343 cpm/culture, whereas 100% protein secretion corresponds to 1743 cpm/culture. The solid bar from 0 to 1 hr in this and subsequent figures represents the period of exposure to poly(I)·poly(C).

[^{3}H]uridine for 2.5 hr. The survival of ^{3}H-labeled polyadenylylated mRNA was monitored during the shutoff phase. The results indicate that the shutoff mechanism does not affect bulk cellular mRNA but it inactivates or degrades interferon mRNA in a selective manner. Furthermore, the selectivity does not appear to relate to the fact that interferon mRNA codes for a secretory protein. The rate of cellular protein secretion is unaffected during the shutoff phase (Fig. 1).

Earlier studies have revealed that superinduction of interferon production by inhibitors of RNA synthesis (actinomycin D and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) leads to a stabilization of interferon mRNA (4, 5, 23). The results presented in Fig. 3 support this conclusion and suggest that this stabilization may be selective. Whereas the shutoff of interferon production was blocked by treatment of induced cells with actinomycin D between 2 and 3 hr after induction, the survival of bulk [^{3}H]uridine-labeled polyadenylylated cellular mRNA was unaffected. The levels of phenol-extractable cellular polyadenylylated mRNA in untreated and actinomycin D-treated induced FS-4 cultures, as measured by translation in rabbit reticulocyte lysates, were comparable. Furthermore, the level of translatable cellular mRNA in actinomycin D-treated cultures decreased in a manner consistent with the loss of [^{3}H]uridine-labeled polyadenylylated RNA described in Fig. 3.

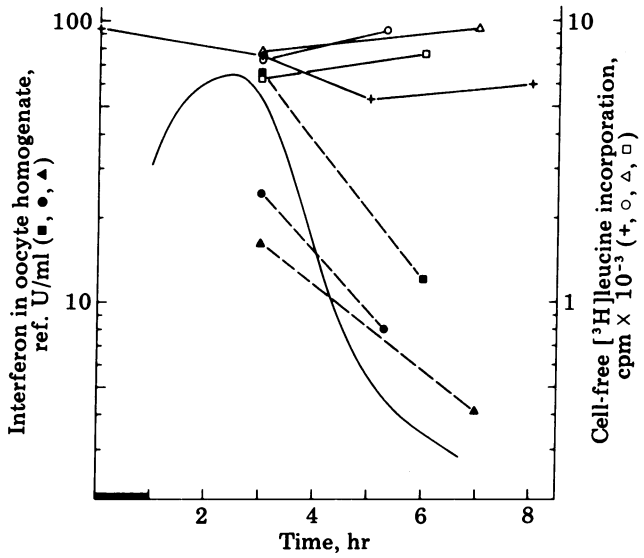

FIG. 2. Comparison between the cellular content of interferon mRNA (closed symbols) and of total translatable mRNA (+ and open symbols) during the shutoff phase. Groups of four to five FS-4 cultures (150-mm dishes) induced with poly(I)·poly(C) were harvested at the indicated times and cellular polyadenylylated RNA was isolated by poly(U)-Sepharose chromatography. Each poly(A)-containing RNA sample was dissolved in 5 μl of sterile distilled water. A 2-μl aliquot was assayed for interferon mRNA by microinjection into *X. laevis* oocytes (closed symbols), whereas total cellular mRNA was assayed by translation of a second 2-μl aliquot in a rabbit reticulocyte lysate (25-μl reaction volume) and monitoring the stimulation of [³H]leucine incorporation (data expressed as cpm in 10 μl of the translation reaction) (open symbols). The solid curve corresponds to the rate of interferon production illustrated in Fig. 1. The circles, squares, and triangles each illustrate three separate experiments.

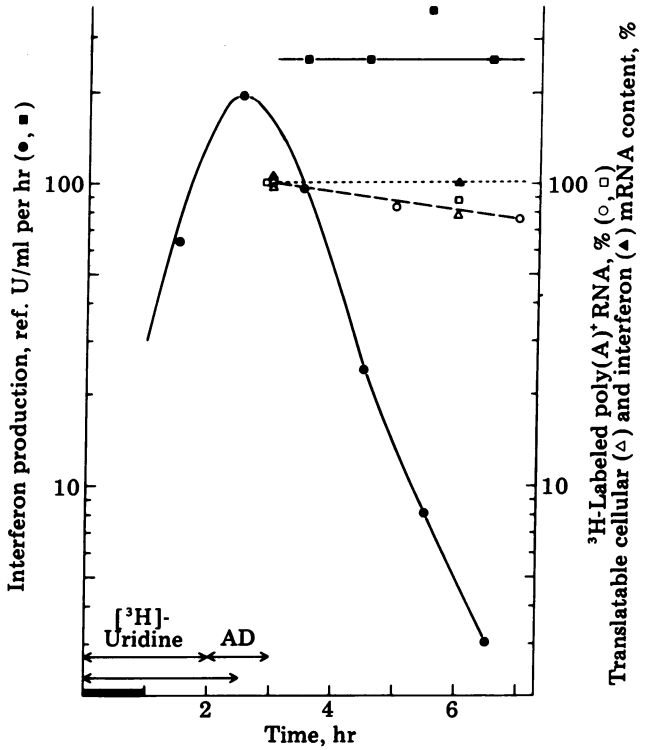

FIG. 3. Stability of [³H]uridine-labeled polyadenylylated cellular RNA during shutoff (○) and superinduction (□) of interferon production. FS-4 cultures in 150-mm dishes were labeled with [³H]uridine (20 μCi/ml) for the first 2.5 hr of poly(I)·poly(C) induction (lowermost double headed arrow, ↔), and the cultures were washed thoroughly and incubated in fresh unlabeled medium. At each indicated time, a culture was harvested and the amount of ³H in poly(A)-containing cellular RNA was estimated by poly(U)-Sepharose chromatography. Each determination was carried out in duplicate. Additional cultures (five per group) that had been labeled with [³H]uridine (10 μCi/ml) for 2 hr were treated with actinomycin D (2 μg/ml) between 2 and 3 hr after the beginning of induction and the survival of ³H in poly(A)-containing RNA was monitored thereafter in superinduced cultures. The cellular content of ³H-labeled polyadenylylated RNA is expressed as a fraction of the level in cells harvested 3 hr after the beginning of induction (363,225 cpm/culture for ○; 106,074 cpm/culture for □). Separate unlabeled cultures were used to monitor the rate of interferon production after poly(I)·poly(C) induction (●) or after superinduction with actinomycin D (AD) (■). The poly(A)-containing RNA samples from actinomycin D-treated cultures were dissolved in 5 μl of distilled water and aliquots (2 μl each) were assayed for cellular mRNA content by translation in a rabbit reticulocyte lysate (25-μl reaction volume; 100% corresponds to 784 cpm/10-μl reaction volume; △) and for interferon mRNA content by microinjection into oocytes (100% corresponds to 96 reference units of interferon per ml in oocyte homogenate; ▲). A corresponding sample of mRNA from induced actinomycin D-free cultures stimulated protein synthesis in reticulocyte lysates by 923 cpm/10-μl reaction volume.

Is 2′,5′-oligo(A)-mediated nuclease activation involved in the shutoff of interferon production?

To investigate the possibility that induction of 2′,5′-oligo(A) synthetase in response to the interferon produced may cause inactivation of interferon mRNA through nuclease activation, we determined whether the levels of 2′,5′-oligo(A) synthetase were increased during the shutoff phase, and we determined the effect of elevated levels of 2′,5′-oligo(A) synthetase on the functional half-life of interferon mRNA.

Levels of 2′,5′-Oligo(A) Synthetase in Poly(I)·Poly(C)-Induced Cultures. The levels of 2′,5′-oligo(A) synthetase were measured in extracts of uninduced and in poly(I)·poly(C)-induced FS-4 cultures. The enzyme levels were estimated 5 hr after the beginning of induction because the shutoff of interferon production takes place between 3 and 8 hr after induction. In parallel, 2′,5′-oligo(A) synthetase activities were measured in interferon-treated uninduced cultures, in interferon-treated poly(I)·poly(C)-induced cultures, and in interferon-treated superinduced cultures. Fig. 4 shows that there is a low basal level of 2′,5′-oligo(A) synthetase activity in confluent FS-4 cultures. Five hours after the start of poly(I)·poly(C) induction this low level is virtually unchanged. Exposure of FS-4 cultures to human fibroblast interferon at a concentration of 250 reference units/ml for 17 hr caused a 200-fold increase in 2′,5′-oligo(A) synthetase levels. The data in Fig. 4 leave open the possibility that the low basal level of 2′,5′-oligo(A) synthetase after exposure to poly(I)·poly(C) may be sufficient for the synthesis of enough 2′,5′-oligo(A) to activate the endonuclease, which in turn could degrade interferon mRNA if the shutoff mechanism were highly sensitive to 2′,5′-oligo(A) levels. We therefore investigated the stability of interferon mRNA in FS-4 cultures that had been pretreated with interferon in order to increase the level of 2′,5′-oligo(A) synthetase.

Interferon Production in FS-4 Cultures with Basal and Elevated 2′,5′-Oligo(A) Synthetase Levels. Cultures of FS-4 cells with high 2′,5′-oligo(A) synthetase levels were obtained by exposure of cells to human fibroblast interferon. Table 1 summarizes our initial results. In experiment 1 we treated cultures with interferon (250 reference units/ml) for 2.5 hr in an attempt to mimic conditions just prior to the shutoff phase of ordinary induction. Interferon-treated and untreated control cultures were then induced with poly(I)·poly(C) alone or in combination with cycloheximide or actinomycin D or both. Interferon production between 3.5 and 24 hr in control cultures

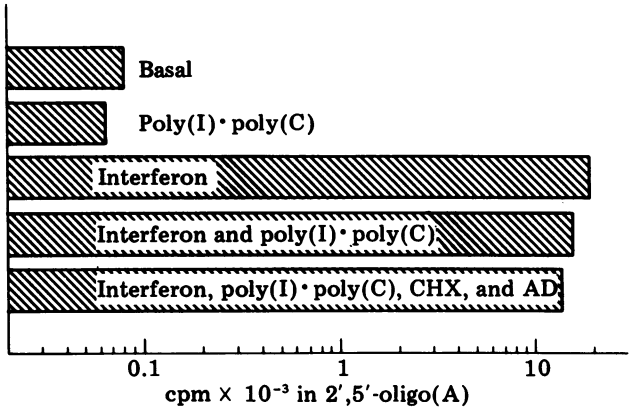

FIG. 4. Levels of 2′,5′-oligo(A) synthetase activity in FS-4 cells. The enzyme was assayed in: (*i*) uninduced cultures; (*ii*) cultures induced with poly(I)·poly(C) 5 hr earlier; (*iii*) interferon (250 reference units/ml, 17 hr)-treated cultures; (*iv*) cultures treated with both interferon and poly(I)·poly(C) as in *ii* and *iii* above; (*v*) cultures treated with interferon, poly(I)·poly(C), and superinduced with cycloheximide (CHX) (50 μg/ml, 0–3.5 hr) and actinomycin D (AD) (2 μg/ml, 3.0–3.5 hr).

was indistinguishable from that in cultures that had been exposed to interferon for 2.5 hr prior to induction. In experiments 2 and 3 we treated cultures with interferon overnight (conditions similar to those in Fig. 4) and then induced with poly(I)·poly(C) with or without superinducing inhibitors. Interferon yields from cultures pretreated with interferon were generally higher than those from control cultures. Experimental conditions that gave a 200-fold increase in 2′,5′-oligo(A) synthetase levels did not reduce interferon yields.

In order to compare the stability of interferon mRNA in FS-4 cultures with low and high levels of the synthetase, we treated cultures with interferon overnight, induced them with poly(I)·poly(C) (with or without superinduction with cycloheximide and actinomycin D), and monitored the rate of interferon secretion by hourly medium change. We have previously documented the fact that the rate of interferon secretion in FS-4 cultures correlates well with the content of extractable, polyadenylylated, translatable interferon mRNA (refs. 4 and 5; also see Fig. 5). Fig. 5 summarizes the results obtained. Experiments described in Figs. 4 and 5 were carried out simultaneously.

Table 1. Interferon production by interferon-treated FS-4 cell cultures*

Exp.	Induction† in combination with	Interferon yield,‡ ref. U/ml			
			Interferon-treated		
		Control	25 ref. U/ml, 18 hr	250 ref. U/ml 2.5 hr	250 ref. U/ml 18 hr
1	—	200		300	
	CHX	1,000		1,000	
	AD	2,000		3,000	
	CHX and AD	10,000		10,000	
2	—	30	200		200
	CHX	300	1,000		1,000
	AD	300	1,000		2,000
	CHX and AD	3,000	10,000		10,000
3	—	100			600
	CHX	600			2,000
	AD	1,000			3,000
	CHX and AD	6,000			10,000

* Confluent FS-4 cultures in 35-mm petri dishes were used.

† Poly(I)·poly(C), 20 μg/ml in 1 ml of Eagle's minimal essential medium for 1 hr at 37°C. Where indicated, cycloheximide (CHX) was present at 50 μg/ml from 0 to 3.5 hr, or actinomycin D (AD) at 2 μg/ml from 3.0 to 3.5 hr, or both.

‡ Interferon yield (3.5–24 hr) in 1 ml of Eagle's minimal essential medium supplemented with 2% (vol/vol) fetal bovine serum.

It is clear from Fig. 5 that despite a documented 200-fold increase in 2′,5′-oligo(A) synthetase levels there is little difference in the apparent half-life of interferon mRNA in cultures with low or high synthetase levels. The slopes of the lines describing the decay of the rate of interferon secretion in both ordinarily induced and superinduced cultures are similar in the control and interferon-treated cultures. As expected, the half-life of interferon mRNA in superinduced cultures is longer than that in ordinarily induced cultures. There is an 8-fold higher level of interferon synthesis and a similar increase in extractable interferon mRNA levels in interferon-treated cultures (priming, ref. 24). However, the levels of bulk cellular mRNA assayed by translation in reticulocyte lysates are similar in interferon-treated and untreated cultures (not shown). The results suggest that it is unlikely that 2′,5′-oligo(A)-mediated nuclease activation is involved in regulating the half-life of interferon mRNA.

DISCUSSION

We have presented evidence indicating that the mechanism that degrades or inactivates human fibroblast interferon mRNA in poly(I)·poly(C)-induced cultures does not affect bulk cellular mRNA. Furthermore, poly(I)·poly(C) induction does not alter the levels of cellular 2′,5′-oligo(A) synthetase up to 5 hr after induction. Finally, the stability of interferon mRNA is unaffected by a 200-fold increase in the level of the synthetase. Induced cells that contain a 200-fold higher level of 2′,5′-oligo(A) synthetase respond equally well to superinduction by inhibitors of RNA and protein synthesis compared to those with low basal levels of the synthetase. These data suggest that 2′,5′-oligo(A)-mediated nuclease activation may not be involved in the inactivation of interferon mRNA during the shutoff phase.

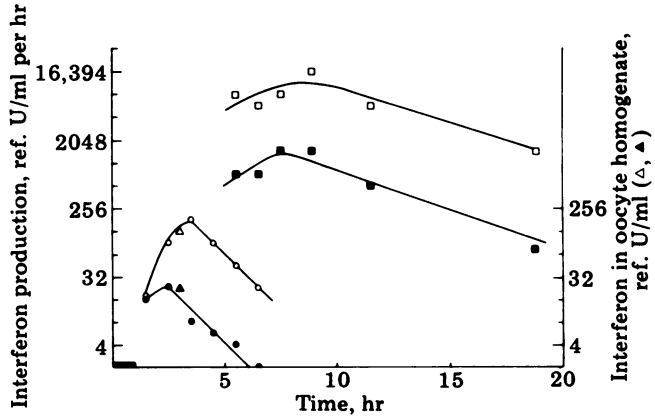

FIG. 5. Rate of interferon production in FS-4 cell cultures in 150-mm dishes treated with interferon (250 reference units/ml for 17 hr). FS-4 cell cultures treated with interferon (open symbols) or untreated (closed symbols) were induced with poly(I)·poly(C) alone (○, ●) or in combination with cycloheximide (50 μg/ml, 0–3.5 hr) and actinomycin D (2 μg/ml, 3.0–3.5 hr) (□, ■). The rate of interferon production was monitored by changing the culture medium at appropriate intervals. Interferon-treated and untreated cultures (four per group) were also induced with poly(I)·poly(C) and harvested 3 hr later, and the cellular content of interferon mRNA was assayed by microinjection of the polyadenylylated RNA fraction into oocytes (△, ▲).

The observation that interferon mRNA is inactivated selectively during the shutoff phase suggests that the underlying mechanism must also be selective. This contrasts with the 2′,5′-oligo(A)-activated endonuclease, which degrades a variety of RNAs, including bulk cellular mRNA in cell-free systems (14–16). Furthermore, the direct exposure of mammalian cell cultures to 2′,5′-oligo(A) leads to a marked though transient reduction in the rate of synthesis of bulk cellular protein (25, 26). This effect is apparently due to a degradation of bulk cellular mRNA by the 2′,5′-oligo(A)-activated endonuclease. Though a possible mechanism for the selective action of 2′,5′-oligo(A)-activated endonuclease on specific mRNA species under special cell-free conditions has been proposed (27), the data in Figs. 4 and 5 taken together argue against the involvement of this endonuclease in the shutoff mechanism.

The regulation of the expression of specific mammalian genes by alterations in the stability of specific mRNA species appears widespread (2, 5, 10–12, 23, 28–33). For example, Guyette *et al.* (32) have shown that treatment of rat mammary gland organ cultures with prolactin increases the rate of casein mRNA transcription 2- to 4-fold but increases the half-life of casein mRNA by 17- to 25-fold. The enhancement of the stability of casein mRNA is a specific effect, because the stability of ^{3}H-labeled bulk cellular mRNA is not altered after prolactin treatment. The increase in casein mRNA transcription together with a dramatic enhancement of mRNA stability quantitatively accounts for the accumulation of casein mRNA in mammary gland cells exposed to prolactin. In a striking parallel, we have shown that superinduction of poly(I)·poly(C)-induced interferon production involves a 3- to 4-fold increase in the apparent rate of interferon mRNA synthesis and a 14-fold increase in the stability of the molecule (4, 5, 33).

Our data (Fig. 5) show that the enhanced production of interferon after priming of FS-4 cells with interferon is associated with a corresponding increase in the level of interferon mRNA.

We thank Dr. Igor Tamm for helpful discussions. We also thank Mr. Ira Augenzucker and Ms. Leota Mehra for technical assistance. This investigation was supported by grant AI-16262 from the National Institute of Allergy and Infectious Diseases and by Institutional Grant CA-08748-15 from the National Cancer Institute. P.B.S. is the recipient of a Junior Faculty Research Award from the American Cancer Society.

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