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Abnormal behavior of interferon-induced enzymatic activities in an interferon-resistant cell line

(double-stranded RNA/protein kinase/oligoisoadenylate synthetase/constitutive enzymes/mechanism of interferon action)

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ABSTRACT **Interferon induces two double-stranded RNA-dependent enzymatic activities: an oligoisoadenylate synthetase that converts ATP to ppp(A2′p)$_n$5′A, and a protein phosphokinase. We have explored the level and inducibility of these two enzymes in a human cell line (HEC-1) totally insensitive to both the antiviral and the anticellular actions of interferon. The activities of both enzymes are high in untreated cells and only minor changes occur after treatment with interferon, even at high concentrations. Interferon-treated HEC-1 cells do not contain an inhibitor of the oligoisoadenylate synthetase activity. The products of this HEC-1 oligoisoadenylate synthetase consist mainly of dimers, trimers, and tetramers as found in other cell lines after interferon treatment. The synthetase level is unaffected by treating the cells with anti-interferon antiserum, indicating that the results cannot be explained by a spontaneous low production of interferon by these cells. Furthermore, virus multiplication is not inhibited, even after treatment with interferon. These observations suggest that either the two enzymatic activities do not suffice for the establishment of an antiviral state *in vivo* or that a regulatory control mechanism, lost in these cells and common for both enzymes, is required for the expression of the antiviral action of interferon. This might explain both the constitutivity of the two enzymes and the interferon resistance observed.**

Several observations by different groups have shown convincingly that treatment of tissue culture cells with interferon from the same species induces two major kinds of enzymatic activities which can be measured *in vitro* in the presence of double-stranded RNA (dsRNA) as an activator. The first one is a protein phosphokinase that has as endogenous substrate a 67,000 M_r protein (1–3) and as exogenous substrates either histones (2, 4) or the 35,000 M_r subunit of eukaryotic initiation factor 2 (5–7). The second one is a 2′,5′-oligoisoadenylate (2–5A) synthetase capable of polymerizing ATP into a series of oligonucleotides with the general structure ppp(A2′p)$_n$5′A (n = 1–12) (4, 5, 8–10). These oligonucleotides are able to activate a preexisting inactive endonuclease. Both protein kinase and 2-5A synthetase activities might contribute to the inhibition of protein synthesis that has been found in lysates from interferon-treated cells (5, 10–14).

Other biochemical events occurring after interferon treatment include the appearance of a 2′,5′-specific phosphodiesterase (15) and the synthesis of new proteins (16–19). However, the relevance of these enzymatic or biochemical modifications to the major biological effects of interferon has remained unclear (20).

Besides kinetic studies (10, 15), two types of approaches suggest that the antiviral effects of interferon could be explained by the induction of the two enzyme systems. First, introduction of 2–5A into normal cells stimulates an endonuclease activity (21, 22) and confers antiviral properties (22). Second, interferon-resistant L1210 mouse cells show no inducibility of 67,000 M_r protein phosphokinase (23).

In this paper we present evidence that is not entirely compatible with this hypothesis. We report here on the level and inducibility of some interferon-related enzymatic activities in a human endometrial cancer (HEC-1) cell line (24) which has recently been shown to be insensitive to the anticellular and antiviral actions of interferon (unpublished data).

MATERIALS AND METHODS

Materials. Human fibroblast interferon, mouse interferon, and dsRNA [poly(inosinic acid)-poly(cytidylic acid)] were kind gifts from E. De Clercq (Rega Institute, Leuven, Belgium). Anti-interferon antisera were kindly provided by A. Billiau (Rega Institute). Fetal calf serum was obtained from GIBCO and bacterial alkaline phosphatase was from Worthington.

Cell Cultures. HEC-1 cells (24), IF^r and RSa clonal transformed human cell lines established by Kuwata *et al.* (25), and mouse L929 cells were grown as monolayers in Eagle's minimal essential medium containing 10% (vol/vol) fetal calf serum. Confluent cultures were treated with human fibroblast interferon (200 units/ml unless otherwise indicated in the text) for 24 hr at 37°C in fresh medium.

Cell Extracts. Cells were detached by using 0.25% trypsin/0.17% EDTA and extensively washed with 140 mM NaCl/35 mM Tris, pH 7.5. All subsequent operations were carried out at 4°C. Cells were then homogenized in 1.5–2 vol of 20 mM Hepes, pH 7.4/10 mM KCl/1.5 mM $Mg(OAC)_2$/0.5 mM dithiothreitol (lysis buffer). The homogenate was centrifuged for 20 min at 10,000 × g and the supernatant (S10) was stored in liquid nitrogen for enzyme assays. When ribosomal extracts were used for assays, the S10 was centrifuged for 2 hr at 45,000 rpm in a Beckman 75 Ti rotor. The pellet was resuspended in lysis buffer. Protein concentrations were determined by the method of Bradford (26) with Bio-Rad reagents.

Assay of 2–5A Synthetase Activity. Cell extract (5–10 μl) was incubated for 2 hr at 30°C in 25 μl of the incubation mixture described by Minks *et al.* (27). The ^{3}H-labeled-2-5A synthesized from [^{3}H]ATP (1.4–4 Ci/mol; 1 Ci = 3.7 × 10^{10} becquerels) was isolated by DEAE-cellulose (Whatman DE-52) chromatography (27). The results are expressed as cpm recovered in 2–5A for the whole 25-μl assay mixture.

RESULTS

Interferon-Resistance of HEC-1 Cells. HEC-1 cells were tested for their sensitivity to the antiviral action of both leukocyte and fibroblast human interferons. There was no decrease of virus yield with vesicular stomatitis virus or Sindbis virus after treating the cells with interferon at up to 1000 units/ml. Virus

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Abbreviations: dsRNA, double-stranded RNA; 2–5A, a series of oligoisoadenylates [ppp(A2′p)$_n$5′A]; S10, 10,000 × g supernatant of lysed cells; TCD_{50}, median lethal tissue culture dose.

yield measured 15 hr after infection was decreased by more than 3000 times in RSa cells but was unchanged [$10^{7.5}$ median lethal tissue culture dose (TCD_{50})/0.2 ml for vesicular stomatitis virus and $10^{6.7}$ TCD_{50}/0.2 ml for Sindbis virus] in both untreated and leukocyte or fibroblast interferon (100 and 1000 units/ml for 20 hr) treated HEC-1 cells. Cell growth was also insensitive to the presence of both kinds of human interferon at up to 1000 units/ml as demonstrated by measuring either cell number for a period of 7 days or [^{3}H]thymidine incorporation after 24 hr (unpublished data).

2–5A Synthetase Activity. We found that cytoplasmic extracts from RSa cells behave as described for other cell lines (27). After treatment with fibroblast interferon at 200 units/ml these cells contain a synthetase activity that is absent from control cells, is totally dependent on the presence of dsRNA, and is linearly related to the amount of cytoplasmic protein present (Fig. 1 *A* and *B*). Furthermore, the products of this reaction had the typical characteristics of 2–5A oligomers (not shown).

In cytoplasmic extracts from HEC-1 cells we routinely found a basic level of 2–5A synthetase, usually one-third to one-fifth of the activity found in interferon-treated RSa at equivalent protein concentration. Fig. 1*D* indicates that this activity is also dependent on dsRNA and varies linearly with the amount of cytoplasmic extract used in the test. However, in HEC-1 cells, the enzyme activity was hardly affected by interferon treatment (Fig. 1*C*). The same observation was repeated in five independent pairs of cytoplasmic extracts from HEC-1 cells; activity in the treated cells was not significantly different from that found in the control cells (as compared to an at least 20-fold increase for RSa cells). The dose–response curve for 2–5A synthetase activity as a function of dsRNA concentration for extracts of interferon-treated cells was superimposable on that obtained for untreated cells, indicating a similar sensitivity to dsRNA (the concentration of dsRNA used in the assays, 20 μg/ml, is saturating for 2–5A synthetase) (not shown). This result is not compatible with a hypothetical masking of 2–5A synthetase induction in interferon-treated HEC-1 cells by a dsRNA-specific RNase.

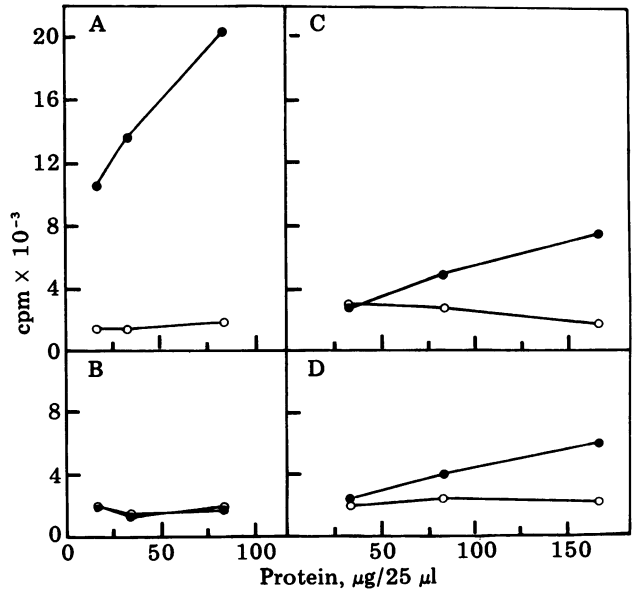

FIG. 1. Dependence of 2–5A synthetase activity on cell extract concentration in interferon-treated RSa cells (*A*), control RSa cells (*B*), interferon-treated HEC-1 cells (*C*), and control HEC-1 cells (*D*). Interferon treatment was 200 units/ml for 24 hr. The incubations were carried out for 2 hr in the absence (○) or presence (●) of dsRNA at 20 μg/ml.

Product Analysis of the 2–5A Synthetase Reaction. DEAE-cellulose chromatography in the presence of 7 M urea showed that all the radioactivity from [^{3}H]ATP incorporated in the reaction products was distributed in oligomers whose charge could be calculated (by comparison with that of markers) and corresponded with that expected for 2–5A trimers, tetramers, and pentamers (Fig. 2). After alkaline phosphatase treatment, the whole profile shifted to the left with respect to unlabeled markers (AMP, ADP, ATP) (Fig. 2*B*), as observed by others (8, 27). Alkaline phosphatase treatment of the sample improved the resolution, presumably because the heterogeneity related to a variable extent of 5′-terminal phosphorylation was abolished. Under these conditions, the profiles of radioactive oligomers obtained with extracts from control and interferon-treated HEC-1 cells were nearly identical (Fig. 2 *B* and *C*).

Thin-layer chromatography on polyethyleneimine-cellulose was used to separate [α-^{32}P]ATP-labeled 2–5A "cores" obtained by alkaline phosphatase treatment into dimers, trimers, and

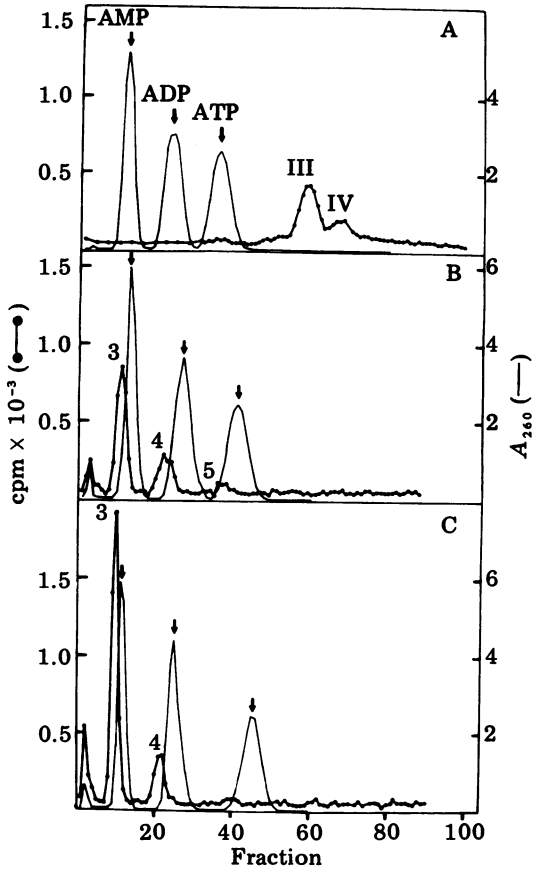

FIG. 2. DEAE-cellulose chromatography of the 2–5A oligomers synthesized in extracts from control and interferon-treated (200 units/ml, 24 hr) HEC-1 cells. (*A*) The 2–5A products synthesized in extracts from control cells and partially purified on DEAE-cellulose (27) were applied to a column (0.6 × 25 cm) of DE-52 (Whatman) equilibrated with 50 mM NaCl/20 mM Tris·HCl, pH 7.5 (28). After washing with this buffer, the column was equilibrated in the same buffer containing 7 M urea (TU buffer) and the elution (1.7-ml fractions, 18 ml/hr) was performed with a gradient of 50–300 mM NaCl (150 ml/150 ml) in TU buffer. AMP, ADP, and ATP were used as markers. (*B*) An aliquot of the products analyzed in *A* was digested with bacterial alkaline phosphatase (2 units/ml) prior to application to the same column. Elution was performed as in *A*. (*C*) The 2–5A oligomers obtained from interferon-treated cells were digested with phosphatase and subjected to chromatography as in *A*. III, Trimer [ppp(Ap)$_2$A]; IV, tetramer [ppp(Ap)$_3$A]; 3, trimer bacterial alkaline phosphatase core [(Ap)$_2$A]; 4, tetramer phosphatase core [(Ap)$_3$A]; 5, pentamer phosphatase core [(Ap)$_4$A]. These oligomers were identified according to their relative positions with respect to markers of known charge (AMP, ADP, ATP) (8, 27).

tetramers. In the presence of dsRNA, extracts from untreated and interferon-treated HEC-1 cells produced the characteristic spots corresponding to the dimers, trimers, and tetramers (Fig. 3). Their relative migration as compared to adenosine and AMP was similar to previous observations by others (9, 27). Furthermore, the spot labeled here as 2–5A dimer core comigrated exactly with commercial nonradioactive synthetic A2′p5′A marker (not shown). By contrast, with extracts from L929 cells, dimers, trimers, and tetramers were detected only when the cells were treated with interferon and dsRNA was present during the enzymatic reaction. In addition to these structural properties, HEC-1-derived 2–5A activated a 2–5A-dependent endonuclease at subnanomolar concentrations (not shown).

Search for Inhibition of the 2–5A Synthetase Activity in Interferon-Treated HEC-1 Extracts. 2–5A is known to be unstable in most cell-free systems (17, 22, 27) because it can be degraded by an interferon-induced 2′-phosphodiesterase (15, 29). If phosphodiesterase or phosphatase activities were present or induced by interferon treatment of the HEC-1 cells, it is conceivable that the resulting degradation of the substrate or of the 2–5A product could mask the induction of 2–5A synthetase activity. However, 2–5A synthetase activity in mixtures of extracts from interferon-treated HEC-1 cells and interferon-responsive IF^r cells was strictly additive (not shown); this result does not support the idea that the 2–5A synthetase activity is inhibited in interferon-treated HEC-1 cells.

Effect of Interferon Concentration on 2–5A Synthetase Activity of HEC-1 Cells. HEC-1 cells not only have an abnormally high level of "basal" synthetase activity but also are unable to respond to interferon treatment. Table 1 shows that this insensitivity is independent of interferon concentration. Maximal induction of 2–5A synthetase is normally obtained with 100–200 units of interferon per ml (27), but with HEC-1 cells even 100 times this concentration had no effect.

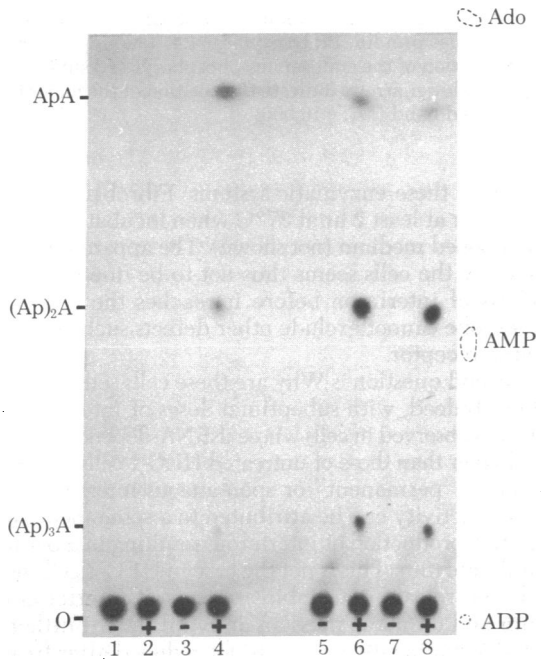

FIG. 3. Autoradiographs of ^{32}P-labeled 2–5A oligomers after thin-layer chromatography. Oligomers were synthesized in the presence (+) or absence (−) of dsRNA, in extracts from control (lanes 1 and 2) and interferon-treated (lanes 3 and 4) L929 cells and from control (lanes 5 and 6) and interferon-treated (lanes 7 and 8) HEC-1 cells. Interferon treatment was 200 units/ml for 24 hr. Aliquots (3 μl) of the 2–5A oligomers synthesized in the presence of [α-^{32}P]ATP (40 Ci/mol) were digested with bacterial alkaline phosphatase (13 units/ml) and subjected to thin-layer chromatography on polyethyleneimine-cellulose plates in 1 M acetic acid. Before the run, the plate was washed with distilled water to eliminate a large part of the labeled products resulting from the degradation of [α-^{32}P]ATP by bacterial alkaline phosphatase (17). Unlabeled adenosine (Ado), 5′-AMP, and 5′-ADP were included as markers and visualized under UV light.

Absence of Spontaneous Interferon Production by HEC-1 Cells. First, we have not detected spontaneous production of interferon in the culture medium from HEC-1 cells; the threshold of detection in the assay was 1.6 unit/ml. Second, unlike a murine mutant cell line which also has high basal 2–5A content (30), the presence of 200 units of anti-human fibroblast interferon (5 or 10 days) or anti-human leukocyte interferon (5 days) antiserum per ml did not significantly decrease the HEC-1 basal synthetase level (Table 2). Considering that HEC-1 cells have a doubling time comparable to HeLa cells, if their 2–5A synthetase has the same stability as that found in HeLa cells (27) an interruption of interferon action by effective antibody treatment should decrease the synthetase level by 80% in about 3 days (27). Finally, HEC-1 cells were sensitive to vesicular stomatitis and Sindbis virus infection (see section 1).

Presence of 2–5A-Dependent Endonuclease in HEC-1 Cell Extracts. Because in interferon-treated cell extracts (10, 31–33) and in intact cells (21, 22) the known action of 2–5A is the activation of a "latent" endonuclease, it would be of interest to know if untreated HEC-1 cells contain this endonuclease activity. Cytoplasmic extracts from several HEC-1 cell preparations contained this 2–5A-dependent endonuclease activity and 2 nM 2–5A, a concentration normally used for maximal stimulation (34), was sufficient to activate it fully (Fig. 4).

Protein Kinase Activity in HEC-1 Cells. In view of the abnormal characteristics of 2–5A synthetase in HEC-1 cells, the protein phosphokinase normally inducible by interferon also was studied. Crude ribosomes from control or interferon-treated HEC-1 cells were tested for their ability to phosphorylate endogenous proteins or exogenously added histones in the presence of [γ-^{32}P]ATP. For comparison, crude ribosomes from interferon-sensitive RSa cells were also studied.

In RSa cells, a typical pattern of phosphorylation was obtained for both the 73,000 M_r and a 16,000 M_r histone fraction (Fig. 5). Phosphorylation of these two proteins was dependent on the presence of dsRNA in the reaction mixture (500 ng/ml in these assays) and appeared only in extracts from interferon-treated cells. By contrast, in HEC-1 cells, dsRNA-dependent phosphorylation of the two proteins occurred in both control and interferon-treated cells and the extent of this dsRNA-dependent phosphorylation was not increased by interferon treatment. The dsRNA-dependent kinase activities in extracts from control and interferon-treated HEC-1 cells gave 4825 and 5230 ^{32}P cpm, respectively, for the 16,000 M_r histone fraction and 740 and 690 ^{32}P cpm, respectively, for the 73,000 M_r fraction (Fig. 5).

Table 1. Effect of interferon concentration on 2–5A synthetase activity in HEC-1 cells

Interferon, units/ml	^3H, cpm		Δ*
	No dsRNA	With dsRNA	
0	1010	19,640	18,630
50	1250	15,200	13,950
200	1440	17,930	16,490
1,000	1480	26,920	25,440
5,000	1550	23,280	21,730
25,000	1120	18,880	17,760

Cells were treated for 24 hr with the indicated dose of human fibroblast interferon ($10^{5.7}$ units/mg). The incubations were carried out for 2 hr. The results are expressed as means from duplicate assays containing 225 μg of protein.
Δ* = activity with dsRNA − activity without dsRNA.

Table 2. Effect of anti-human fibroblast or anti-human leukocyte interferon antiserum on 2–5A synthetase activity in HEC-1 cells

Treatment	dsRNA-dependent activity, cpm		
	3 days	5 days	10 days
Control	24,380	24,065	—
Antifibroblast	—	25,040	19,600
Antileukocyte	—	22,160	—

The incubations were carried out for 2 hr; antibody concentration was 200 units/ml. The results are expressed as Δ in Table 1.

DISCUSSION

The effect of interferon treatment on enzymatic activities of a human interferon-resistant cell line (HEC-1) has been investigated. Both 2–5A synthetase and a protein phosphokinase, implicated in the mechanism of inhibition of protein synthesis by interferon (10) (and perhaps in interferon action in general), have been studied. A remarkable feature of the HEC-1 cells, which are resistant to both anticellular and antiviral action of interferon, when compared to normally interferon-sensitive cells, is that the two dsRNA-dependent enzymes are already present in untreated cells at significant basic levels and that very minor changes in enzyme levels are observed after interferon treatment. Even high doses of interferon (up to 25,000 units/ml) do not affect the level of 2–5A synthetase in these cells. We found no evidence that interferon-treated HEC-1 cells contain an inhibitor of the synthetase reaction that could mask its induction by interferon.

The results presented in this paper raise two important questions. First, what is the reason for the failure of HEC-1 cells to respond to interferon? Because the two dsRNA-dependent activities appear to be normal, it seems likely that interferon resistance in HEC-1 cells results not from the disappearance or alteration of the structural genes for the two major interferon-induced enzymes but rather from an alteration in the regulation of these enzymatic systems. Fibroblast interferon was stable for at least 2 hr at 37°C when incubated in HEC-1 cell-conditioned medium (not shown). The apparent interferon resistance of the cells seems thus not to be due to enhanced proteolysis of interferon before it reaches the cell surface. However, one cannot exclude other defects such as loss of an interferon receptor.

The second question is Why are these cells sensitive to viral infection. Indeed, with suboptimal doses of interferon, virus resistance is observed in cells whose dsRNA-dependent activities are not higher than those of untreated HEC-1 cells. In some cell lines (35), a "permanent" or spontaneous interferon-related enzymatic activity can be attributed to a spontaneous (36) or constitutive production of interferon, resulting in a permanent antiviral state (30). This is not the case in HEC-1 cells because they do not release detectable amounts of interferon in the medium and do not express an antiviral state. Furthermore, their 2–5A synthetase activity is not reduced after treatment with anti-human fibroblast interferon or anti-human leukocyte interferon antiserum.

The situation thus remains paradoxical in that both interferon-induced enzymes classically associated with interferon action are present at an appreciable level in those interferon-resistant cells without the expression of an antiviral activity (inhibition of viral growth *in vivo*). A deficiency in the 2–5A-dependent nuclease might have explained the paradoxical level of 2–5A synthetase with the absence of antiviral effect in HEC-1 cells. However this endonuclease could be detected routinely in these cells.

Three explanations could account for our observations.

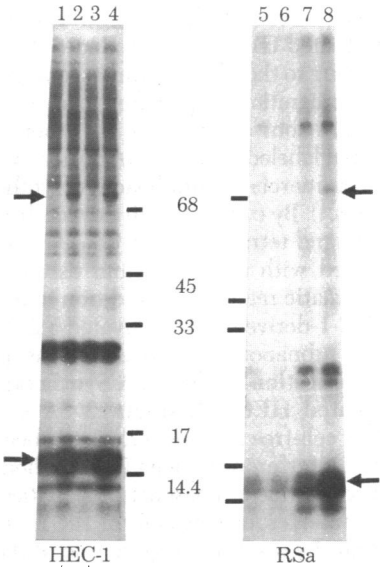

FIG. 5. Protein kinase activity of ribosomal extracts from control and interferon-treated cells. Crude ribosomes (0.35 mg of protein per ml) were incubated in 15-μl reaction mixture containing 4 μCi of [γ-^{32}P]ATP (0.5 mM), 25 mM Hepes (pH 7.5), 60 mM KCl, 8 mM $Mg(OAc)_2$, 6 mM 2-mercaptoethanol, and, when present, 500 ng of dsRNA per ml, for 10 min at 30°C and then for another period of 20 min at 30°C in the presence of histones (type II_A, Sigma) at 0.5 mg/ml. Samples were subjected to electrophoresis, as described by Zilberstein *et al.* (3), on a 10–20% polyacrylamide gradient slab gel, and autoradiographed. For the two cell lines, four lanes are presented corresponding to untreated cells (lanes 1, 2 and 5, 6) and interferon-treated cells (lanes 3, 4 and 7, 8). dsRNA (500 ng/ml) was present only in lanes 2, 4, 6, and 8. The numbers indicate the M_r of protein markers × 10^{-3}: Bovine serum albumin, 68; ovalbumin, 45; asparaginase, 33; tobacco mosaic virus coat protein, 17; lysozyme, 14.4. The upper arrows indicate the position of the endogenous phosphorylated substrate (M_r, ≈73,000). The lower arrows indicate the position of the major histone phosphorylated band (M_r, ≈16,000).

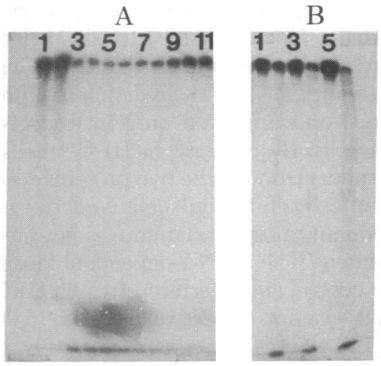

FIG. 4. Detection of 2–5A-dependent endonuclease in extracts from HEC-1 cells. Assay of endonuclease in S10 cytoplasmic extracts was carried out as described (22). ^{32}P-Labeled (15,000 cpm) 28S chicken cell rRNA (*A*, 3 × 10^4 cpm/μg; *B*, 5 × 10^5 cpm/μg) was incubated with 5 μl of lysis buffer (*A*, lanes 1 and 2), or 5 μl (*A*, lanes 3–11) or 10 μl (*B*, lanes 1–6, three independent preparations) of S10 extracts from HEC-1 cells, for 60 min at 30°C, at a final concentration of 75 mM KCl. In the assay, there was either no 2–5A (*A*, lanes 1 and 11; *B*, lanes 1, 3, and 5) or 2–5A at a final concentration of 25, 50, 25, 10, 5, 2, 1, 0.5, or 0.2 nM (*A*, lanes 2–10, respectively) or 20 nM 2–5A (*B*, lanes 2, 4, and 6). 2–5A was from L929. Its concentration was calculated from the known specific activity of [^{3}H]ATP used in the preparation and an estimation (based on thin-layer polyethyleneimine- and DEAE-cellulose chromatography) that >90% of the 2–5A oligomers are trimers. Degradation of the 28S RNA was visualized after boiling of the samples and electrophoresis in a continuous 7.5% polyacrylamide gel. An autoradiograph of the dried stained gel is presented. Under these conditions, most of the intact 28S RNA remained at the origin (*A*, lanes 1 and 2).

(*i*) The two enzymatic activities, although induced by interferon in normal cells, are not by themselves sufficient for the establishment of an antiviral state. Other cellular modifications might be required that have not been considered here. This is in agreement with the work of Wood and Hovanessian (37) who found that mouse embryonal carcinoma cells, which are not inducible by interferon for their kinase activity but are normally inducible for their 2–5A synthetase, do not express an antiviral state. In this case, 2–5A synthetase expression might not suffice for the establishment of an antiviral state. In contrast, Williams *et al.* (22) have shown that artificially introduced 2–5A into virus-infected cells can prevent viral growth. The discrepancy might be explained if 2–5A had effects other than activation of the 2–5A-dependent nuclease.

(*ii*) The absolute total cellular activity of 2–5A synthetase may not be the really important factor in interferon action. It could be that either a change in activity or in compartimentalization is required for expression of the antiviral state *in vivo*.

(*iii*) The two induced and activated enzymes or their products may interact for the establishment of an antiviral state and a coordinated tuning of both enzyme levels may be required for this kind of interaction to occur.

The concomitant loss of inducibility of both kinase and 2–5A synthetase activities in HEC-1 cells remains unexplained. The observation that *both* enzymes are spontaneously expressed in untreated cells may indicate that they share a common or a similar regulatory control. This may offer new possibilities for exploring the mechanisms of regulation of this group of enzymes. Other interferon-resistant cells—e.g., mouse L1210 cells which have lost both inducibility for the kinase (23) and synthetase activities (unpublished data), and embryonal carcinoma cells, which have lost only the inducibility for the kinase activity (37)—do not show the spontaneous enzymatic activities observed here.

The consequences, for the cell, of having a constitutive 2–5A synthetase are not yet evident. HEC-1 are rapidly growing cells, have a healthy appearance, and presumably possess normal RNA and protein metabolism. It is not known whether the enzyme can be activated intracellularly during viral infection or through other circumstances, by dsRNA or another yet unknown activator. It thus is conceivable that these cells might, under certain circumstances, produce 2–5A itself constitutively. This eventuality is amenable to investigation because an assay for intracellular 2–5A has recently been described (34).

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