Mechanism for discrimination between viral and host mRNA in interferon-treated cells

[mechanism of interferon action/(2'-5')oligo(A) polymerase/endonuclease]

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ABSTRACT In extracts of interferon-treated HeLa cells, RNA covalently linked to double-stranded RNA (dsRNA) is preferentially degraded compared with mRNA not linked to dsRNA. This was established by following the degradation of poly(A)-containing mRNA annealed with poly(U), of poly(C)-containing encephalomyocarditis virus RNA annealed with poly(I), and of the replicative intermediate of the virus isolated from infected cells. In extracts of interferon-treated cells, dsRNA promotes the synthesis of a series of oligonucleotides, designated (2'-5')oligo(A), which in turn activate an endonuclease. Several lines of evidence suggest that the (2'-5')oligo(A) polymerase/endonuclease system is involved in the preferential degradation of mRNA linked to dsRNA. Conditions that prevent synthesis of (2'-5')oligo(A) prevent this preferential degradation, whereas addition of (2'-5')oligo(A) or conditions that favor its synthesis result in degradation of mRNA both linked and not linked to dsRNA. These results are best explained by a localized activation of the endonuclease near the dsRNA region of our model substrates. We propose that in infected cells activation of the endonuclease takes place near the replicative intermediates of RNA viruses. The replicative intermediates of encephalomyocarditis virus promote synthesis of (2'-5')oligo(A) in extracts of interferon-treated cells and are degraded to a 20S "core" resistant to digestion with RNase A. This mechanism may be responsible for discrimination between viral and cellular mRNA in interferon-treated cells.

Addition of double-stranded RNA (dsRNA) and ATP to extracts of interferon-treated cells results in the formation of a low molecular weight inhibitor of protein synthesis (1). The structure of this inhibitor is pppA(2'p5'5'A)4 (2'-5')oligo(A) or (2'-5')A, a series of oligonucleotides with an unusual 2'-5' phosphodiester linkage (2). Extracts of interferon-treated L cells adsorbed to poly(I)-poly(C)-agarose polymerize ATP into (2'-5')A (2). The synthetic dsRNA acts both as an affinity reagent and an activator for an enzymatic activity, designated (2'-5')A polymerase, which has also been shown in other cells (3–6). Treatment with interferon increases (2'-5')A polymerase activity up to several hundred-fold (3), and this increase has been correlated in HeLa cells with an inhibition of encephalomyocarditis virus (EMCV) RNA synthesis (7).

The (2'-5')A activates an endonuclease, present in extracts of both interferon-treated and control cells, which degrades in vitro cellular and viral mRNAs (4–5, 8). Degradation of cellular mRNA has not been detected in interferon-treated virus-infected cells, but accumulation of viral mRNA is specifically inhibited (9). This inhibition may be due to preferential cleavage of viral mRNA. The (2'-5')A polymerase may bind to and be activated by the dsRNA present in replicative intermediates (RI) of virus RNA viruses (4). The (2'-5')A synthesized could activate the endonuclease only near RI, resulting in preferential cleavage of viral mRNA.

In the present study we have investigated in extracts of interferon-treated and control cells the degradation of model substrates that share a characteristic structural feature with RI—i.e., single-stranded RNA covalently linked to dsRNA. These substrates were prepared by annealing poly(U) with the poly(A) sequence or mRNA or poly(I) with the poly(C) sequence of EMCV RNA (10). Similar experiments have been done with RI isolated from EMCV-infected cells. The results obtained suggest that the (2'-5')A polymerase/endonuclease system is involved in the preferential cleavage of mRNA linked to dsRNA in extracts of interferon-treated cells.

MATERIALS AND METHODS

Cells. HeLa S3 cells were grown in suspension cultures in Joklik's modified minimal Eagle's medium supplemented with 5% horse serum. The cells were treated for 20 hr with 100 NIH units of human fibroblast interferon per ml (3 × 10⁵ units/mg) provided by the Interferon Working Group of the National Cancer Institute, National Institutes of Health. Extracts from control and interferon-treated cells were prepared (11).

Poly(A)-Containing RNA. Poly(A)-containing cellular RNA was obtained from HeLa cells labeled with 50 μCi of [3H]uridine per ml for 90 min. The RNA was isolated from polysomes by oligo(dT)-cellulose chromatography (12). Labeled vesicular stomatitis virus (VSV) mRNA was synthesized as described (13). Reaction mixtures (0.5 ml) containing either 0.5 μCi of [3H]UTP or 50 μCi of [14C]UTP and 2 mM nucleotide triphosphates and 0.1 mM S-adenosylmethionine were incubated for 3 hr. Sodium dodecyl sulfate was added to 0.5%, LiCl to 0.5 M, and Tris-HCl (pH 7.5) to 10 mM. Poly(A)-containing RNA was isolated by chromatography on oligo(dT)-cellulose and sucrose gradient centrifugation (12) and further fractionated by absorption to Millipore filters (14).

EMCV RNA. Labeled EMCV RNA and RI were prepared from EMCV-infected cells as described by Spector and Baltimore (15) for poliovirus-infected cells. Briefly, infected cells were labeled from 2 to 5 hr after infection with 20 μCi of [3H]uridine per ml in the presence of 5 μg of actinomycin D per ml (7). The cells were homogenized in 10 mM NaCl, 1.5 mM Mg(OAc)₂, 10 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, and 50 μg of dextran sulfate per ml. Nuclei were removed by centrifugation and 2 M LiCl, 1% sodium dodecyl sulfate, and 10 mM EDTA were added. The precipitate obtained was dissolved and chromatographed on Sepharose 2B (15). RI in the excluded volume (about 10% of the labeled RNA) and 35S viral RNA were precipitated with ethanol and redissolved in water. The 35S RNA was further purified by sucrose gradient centrifugation (15). Identical procedures were followed for the preparation of unlabeled RI from infected HeLa cells and for the preparation of corresponding fractions from mock-infected cells.

Abbreviations: EMCV, encephalomyocarditis virus; VSV, vesicular stomatitis virus; RI, replicative intermediate; (2'-5')A, pppA(2'-5')A₄ or (2'-5')oligo(A); dsRNA, double-stranded RNA.
An annealing of Poly(U) or Poly(I) to RNA. Poly(A)-containing RNA (about 40 μg/ml, 4 × 10⁴ cpm/μg) was dissolved in 0.12 M KOAc and annealed with 1–20 μg of poly(U) per ml. The concentration of poly(U) sufficient to anneal to the poly(A) and mRNA was empirically determined by oligo(dT)-cellulose chromatography (12). Poly(A)-containing RNA, annealed with sufficient poly(U) (about 5 μg/ml) to prevent binding to oligo(dT), was used in the following experiments. Labeled EMCV RNA (5 μg/ml and about 1 × 10⁶ cpm/μg) was annealed with 20 μg of poly(U) per ml in the same way and reisolated by gradient centrifugation.

RNA Cleavage. Incubations at 30°C contained 0.3 vol of cell extract, 0.12 M KOAc, 4 mM fructose 1,6-bisphosphate, 2 mM Mg(OAc)₂, and the indicated amount of RNA. Degradation of RNA was assayed by gradient analysis or oligo(dT)-cellulose chromatography. Samples were diluted in 0.5 M LiCl/0.5% sodium dodecyl sulfate/10 mM Tris-HCl, pH 7.5, mixed with 20 μg of poly(A) per ml, and heated for 5 min at 95°C before chromatography. The poly(A) added was approximately 100-fold excess over the poly(U) annealed to mRNA. This reduced annealing of the poly(A) end of mRNA with poly(U) and essentially all the labeled mRNA bound to oligo(dT).

(2'-5')Oligo(A) Polymerase Assay. The assay for (2'-5')A synthesis has been described (16).

RESULTS

If the (2'-5')A-dependent endonuclease is activated near the site of synthesis of (2'-5')A, preferential degradation of mRNA linked to dsRNA may occur. To obtain evidence for such a mechanism, we have examined the degradation of model substrates in extracts of interferon-treated and control cells. The first model substrate was poly(A)-containing [³H]mRNA from HeLa cells. This RNA has a poly(A) sequence longer than 100 nucleotides (17). The other model substrate was VSV mRNA labeled in vitro with different isotopic precursors (see Materials and Methods). This RNA was bound to Millipore filters to select mRNA species (about 60% of the RNA synthesized) with poly(A) sequences longer than 60 nucleotides (14). These RNAs were added to extracts of control and interferon-treated cells either directly or after being annealed with poly(U). Base-pairing of the 3'-terminal poly(A) with poly(U) resulted in the formation of mRNA molecules containing a double-stranded region. We designated this RNA as mRNA-(A)ₙ(U)ₙ. Cleavage of mRNA was assayed by oligo(dT)-cellulose chromatography, which separated degraded mRNA and 3'-terminal fragments containing poly(A) from nucleolytic cleavage products. This assay was adapted to measure degradation of mRNA-(A)ₙ(U)ₙ as described in Materials and Methods.

The HeLa mRNA was not degraded to a different extent in extracts of control or interferon-treated cells unless it was annealed with poly(U) (Fig. 1). This mRNA-(A)ₙ(U)ₙ was preferentially cleaved in extracts of interferon-treated cells. Similar results were obtained with VSV mRNA. With this viral RNA it was possible to include in the same incubation mRNA labeled with one isotope together with mRNA-(A)ₙ(U)ₙ labeled with a different isotope. Preferential degradation of mRNA-(A)ₙ(U)ₙ was observed in every case in extracts of interferon-treated cells, regardless of the isotopic precursor used to label the mRNA (Fig. 1). Degradation of VSV mRNA was also investigated by sedimentation analysis (Fig. 2). In extracts of both control and interferon-treated cells, [¹⁴C]mRNA was degraded to a similar extent, whereas [³H]mRNA-(A)ₙ(U)ₙ was degraded to a greater extent in the latter extract.

Some possible explanations for the preferential cleavage of mRNA linked to dsRNA in extracts of interferon-treated cells were ruled out in the following experiments. Differential binding of mRNA to ribosomes and protection of bound mRNA was considered unlikely since both mRNAs failed to associate with ribosomes upon a 15-min incubation under the conditions of our assay (data not shown). Preferential degradation of the poly(A)-poly(U) region by a dsRNA-specific nuclease was ruled out by examining the degradation of synthetic labeled poly(A)-poly(U). No cleavage of this polynucleotide was detected in either cell extract by sedimentation analysis (data not shown). A nonspecific effect of the poly(U) associated with mRNA on its degradation was also considered. The poly(U) could prevent association of poly(A)-binding proteins with mRNA and facilitate its degradation. The results of the following experiment made this explanation unlikely.

EMCV RNA contains a poly(C) tract approximately 100 nucleotides long near its 5' end (18). Poly(I) was annealed with labeled EMCV RNA and the degradation of this RNA was analyzed upon incubation with extract of interferon-treated or control cells (Fig. 2). VSV [¹⁴C]mRNA was included in the incubations as an internal marker of RNA cleavage. In incubations with control cell extract, the poly(I)-poly(C)-containing EMCV [³H]RNA was degraded to the same extent as marker [¹⁴C]mRNA (Fig. 2E). In incubations with extract of interferon-treated cells, however, the EMCV RNA was extensively

FIG. 1. Time course of mRNA degradation in extracts of interferon-treated (A, C, and E) and control (B, D, and F) cells. Incubation mixtures contained, in 0.1 ml: (A and B) 10,000 cpm of HeLa cell [³H]mRNA not annealed with poly(U) (O--O) or annealed with poly(U) (▲—▲); (C and D) 1000 cpm of VSV [¹⁴C]mRNA (O--O) and 10,000 cpm of VSV [³H]mRNA annealed with poly(U) (■—■); (E and F) 10,000 cpm of VSV [³H]mRNA (■—■) and 1000 cpm of VSV [¹⁴C]mRNA annealed with poly(U) (Q—Q). The other components of the incubations were as indicated in Materials and Methods. Aliquots of the incubations were taken at the times indicated to measure poly(A)-containing RNA. The percentage of poly(A)-containing RNA remaining after incubation is shown on the ordinate relative to a control sample not incubated (equal 100%).
degraded (Fig. 2F). Control incubations with EMCV RNA not annealed with poly(I) showed no preferential degradation of this RNA with respect to VSV mRNA (see Fig. 4).

Several observations suggested that the (2'-5')A polymerase/endonuclease system was involved in the preferential degradation of mRNA linked to dsRNA. Preparations of HeLa and VSV mRNA-(A)ₙ(U)ₙ activated (2'-5')A polymerase in extracts of interferon-treated cells (Table 1). In this assay, synthesis of (2'-5')A was either optimized by adding 25 mM Mg(OAc)₂ and 5 mM EDTA (16) or assayed with added 2 mM Mg(OAc)₂, 1 mM ATP, and 4 mM fructose 1,6-bisphosphate to regenerate ATP (19). Under these latter conditions, which were used to assay mRNA cleavage, significant synthesis of (2'-5')A could be detected only by adding 10 μg of synthetic dsRNA per ml. The amount of (2'-5')A synthesized, however, was much less than that formed under optimal conditions (Table 1). Therefore, with mRNA-(A)ₙ(U)ₙ and low concentrations of ATP and Mg²⁺, the synthesis of (2'-5')A was at the limit of detection of our assay. Localized activation of (2'-5')A-dependent endonuclease could take place under these conditions and preferential cleavage of mRNA linked to dsRNA could be explained in this way. If preferential cleavage of mRNA linked to dsRNA results from limited and localized synthesis of (2'-5')A, addition of relatively large amounts of these oligonucleotides or of dsRNA, which promotes (2'-5')A synthesis, should eliminate this preferential cleavage. When excess (2'-5')A was added to incubations assaying degradation of mRNA, the preferential cleavage of mRNA linked to dsRNA was abolished (Fig. 3A). Inclusion of poly(I)-poly(C) in the incubation mixtures increased the degradation of [¹⁴C]mRNA to the same level as that of [³H]mRNA-(A)ₙ(U)ₙ (Fig. 3B). Increasing the concentration of this dsRNA-containing mRNA had a similar effect (Fig. 3C). As the concentration of dsRNA was increased, degradation of [¹⁴C]mRNA progressively increased.

If preferential cleavage of mRNA linked to dsRNA results from activation of endonuclease by (2'-5')A, changes in the incubation mixtures that interfere with endonuclease activity or depress synthesis of (2'-5')A should decrease this preferential cleavage. Both (2'-5')A polymerase and endonuclease require Mg²⁺ for activity (1, 20). We assayed mRNA degradation in the presence of EDTA, which chelates Mg²⁺, and found that the preferential degradation of mRNA linked to dsRNA was eliminated (Table 2). Furthermore, synthesis of (2'-5')A requires ATP as substrate. Cell extracts depleted of ATP by incubation with glucose and hexokinase (21) no longer preferentially degraded mRNA linked to dsRNA (Table 2). Conversely, when ATP was added in high concentration to the incubation mixtures, degradation of the [¹⁴C]mRNA was enhanced (Table 2). These results are consistent with the hypothesis that the (2'-5')A polymerase/endonuclease system is involved in the degradation of mRNA linked to dsRNA. Moreover, these experiments establish conditions under which RNA linked to dsRNA can be preferentially degraded in extracts of interferon-treated cells.

To test whether a “natural” substrate could be preferentially degraded, we prepared RI labeled with [³H]uridine from EMCV-infected HeLa cells (see Materials and Methods). The following criteria were applied to establish that the labeled RNA isolated was RI (22): (i) the RNA was precipitated with 2 M LiCl; (ii) it was recovered in the excluded volume by gel filtration on Sepharose 2B columns; (iii) it sedimented as a heterogeneous peak in sucrose gradients (Fig. 4A); and (iv) it was partially resistant to RNase A digestion (Fig. 4F). The labeled RI was incubated with extract of control and interferon-treated cells and its degradation was assayed by sedimentation analysis (Fig. 4). VSV [¹⁴C]mRNA was included in the incubations as an internal marker. Limited degradation of both RNAs was observed in incubations with control cell extract (Fig. 4B). In contrast, substantial degradation of RI but not of VSV mRNA was observed in incubations with extract of interferon-treated cells (Fig. 4C). A peak sedimenting at about 20 S was formed upon incubation of RI with this cell extract. A similar peak was generated by RNase A digestion of RI (Fig. 4F).
The 20S RNA formed from RI by incubation with extract of interferon-treated cells was isolated by gradient centrifugation (Fig. 4C) and further digested with RNase A. After digestion, 92% of this RNA was acid precipitable, indicating that a nucleic-resistant “core” could be produced either by incubation of RI with interferon-treated cell extract or by RNase digestion. This “core” corresponds in sedimentation and RNase resistance to the double-stranded component described for RI of picornaviruses (23).

Control experiments showed that 35S EMCV RNA was cleaved in extracts of control and interferon-treated cells to a limited and similar extent (Fig. 4D and E). This RNA was completely digested to acid-soluble nucleotides with RNase (data not shown). Similarly, the internal marker of [14C]mRNA added with RI was completely digested by RNase (Fig. 4F). Finally, RI isolated from EMCV-infected cells and 35S RNA were assayed for activation of (2'-5')A polymerase (Table 1).

The (2'-5')A synthesized in incubations with RI was identical to previously characterized (2'-5')A by chromatography on DEAE-cellulose and thin-layer plates of polyethyleneimine-cellulose before and after digestion with nucleases (2-5). The above results indicated that only RNA molecules that can activate the (2'-5')A polymerase are selectively cleaved in extracts of interferon-treated cells. The dsRNA portion of these molecules, however, is resistant to degradation.

**DISCUSSION**

Cellular and viral mRNA linked to dsRNA is markedly degraded in extracts of interferon-treated cells, but not in extracts of control cells; mRNA not linked to dsRNA is degraded to a limited extent in both cell extracts. This limited cleavage is probably due to exonuclease activity (4). The degradation of mRNA linked to dsRNA, however, is at least in part due to endonuclease activity since cleavage products sediment between undegraded RNA and the top fractions of gradients (see Fig. 4).

**Table 2. Degradation of mRNA in extracts of control and interferon-treated cells**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Additions</th>
<th>(cpm$<em>{ad}$/cpm$</em>{ox}$) $\times 10^{10}$</th>
</tr>
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<tbody>
<tr>
<td>1 Interferon</td>
<td>--</td>
<td>68 26</td>
</tr>
<tr>
<td>2 Interferon</td>
<td>5 mM EDTA</td>
<td>75 75</td>
</tr>
<tr>
<td>3 Interferon</td>
<td>2 mM ATP + 2 mM Mg(OAc)$_2$</td>
<td>43 30</td>
</tr>
<tr>
<td>4 Interferon</td>
<td>5 mM glucose + 20 units hexokinase</td>
<td>76 69</td>
</tr>
</tbody>
</table>

Incubation mixtures of 0.1 ml contained 1000 cpm of VSV [14C]mRNA and 10,000 cpm of VSV [3H]mRNA annealed with poly(U). The amount of poly(A)-containing RNA remaining after a 60-min incubation was measured by chromatography on oligo(dT)-cellulose (12) and is expressed as a percentage of that in a sample kept at 0°C.
This characteristic pattern of degradation has been shown for mRNA linked to poly(A)-poly(U) and for EMCV RNA linked to poly(C)-poly(I). The preferential degradation of these model substrates is not due to a nuclease that attacks dsRNA, because labeled poly(A)-poly(U) is not degraded in HeLa cell extracts, nor is it due to binding to ribosomes and protection of mRNA not linked to dsRNA. Degradation of mRNA linked to dsRNA takes place regardless of the location of dsRNA at the 3' or near the 5' terminus of mRNA and regardless of the base composition of the dsRNA linked to mRNA. Preferential degradation of these mRNAs requires Mg\(^{2+}\) and ATP and is abolished by the addition of (2'-5')A or of high concentrations of dsRNA or ATP. Moreover, the dsRNA linked to mRNA has to be at least 60 base pairs long because VSV mRNA not retained by Millipore filters, which contains a poly(A) tract shorter than 60 nucleotides (14), is not preferentially degraded when annealed with poly(U) (unpublished observations). A study of (2'-5')A polymerase activation by polymers of known size has shown that poly(A) shorter than 50-60 nucleotides annealed with poly(U) does not promote (2'-5')A synthesis (unpublished observations).

The (2'-5')A-dependent endonuclease can be activated under the conditions of our assays, as shown by degradation of mRNA due to the addition of (2'-5')A. These oligonucleotides can be synthesized by activating the (2'-5')A polymerase with the mRNAs that are preferentially degraded in extracts of interferon-treated cells. Therefore, it seems possible that cleavage of mRNA linked to dsRNA is due to activation of the polymerase, synthesis of limited amounts of (2'-5')A, and localized activation of the endonuclease. Conditions that favor synthesis of high levels of (2'-5')A (i.e., high concentrations of dsRNA) can presumably activate all the endonuclease present in extracts. In this case, all mRNAs are cleaved and mRNA linked to dsRNA is no longer preferentially degraded. Effective cleavage of this mRNA may require elevated levels of (2'-5')A polymerase, such as those induced by interferon (2-5), but the cleavage of mRNA linked to dsRNA in extracts of control cells (Fig. 1) may be due to the presence of a basal level of (2'-5')A polymerase in HeLa cells not treated with interferon (4).

Important factors in the postulated localization of the endonuclease are the property of this enzyme to revert to an inactive state upon removal of (2'-5')A and the rapid degradation of these oligonucleotides in cell extracts (20). High concentrations of ATP protect (2'-5')A from degradation (20). Thus, it is possible that addition of ATP in our experiments results in increased synthesis of (2'-5')A or decreased degradation of these oligonucleotides.

The results obtained with the labeled RI of EMCV agree with those obtained with model substrates. Preferential degradation of RI occurs in extracts of interferon-treated cells. Only single-stranded RNA is degraded, however, and an RNase-resistant "core" is produced upon incubation in these cell extracts. A similar "core" can be obtained by digestion of RI with RNase A under conditions where single-stranded RNA is cleaved. Again, the RI can activate (2'-5')A polymerase, and addition of large amounts of RI results in degradation of both RI and the internal marker of VSV mRNA (data not shown). These results can best be explained by a localized activation of an endonuclease at or near RI. The (2'-5')A-dependent endonuclease does not discriminate between viral and cellular mRNA (8), but the experiments presented here clearly indicate that under defined conditions a nuclease can discriminate in vitro between mRNA linked to dsRNA and mRNA not linked to dsRNA. These results make attractive the hypothesis that this mechanism is used in interferon-treated cells to preferentially cleave viral RNA linked to partially double-stranded RI. Such a mechanism would clearly be advantageous to inhibit replication of different RNA viruses. A nuclease that recognizes a specific nucleotide sequence, on the other hand, would probably not inhibit replication of all RNA viruses. Moreover, mutants resistant to such a nuclease could originate by changes in nucleotide sequence, but no mutant virus resistant to interferon has been reported.

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