Sundaralingam, M. (1975) *Ann. N.Y. Acad. Sci. 255*, 3.
Ts'o, P. O. P. (1974) *Basic Princ. Nucleic Acid Chem. 1*, 453–517.
Vetterl, V. (1976) *Bioelectrochem. Bioenerg. 3*, 338.
von der Haar, F., Schlimme, E., Erdmann, V. A., & Cramer, F. (1971) *Bioorg. Chem. 1*, 282.
Yoshikawa, M., Kato, T., & Takenishi, T. (1967) *Tetrahedron Lett. 50*, 5065.
Yount, R. G. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 1.
Zilversmit, D. B., & Davis, A. K. (1950) *J. Lab. Clin. Med. 35*, 155.

Role of 2′,5′-Oligo(adenylic acid) Polymerase in the Degradation of Ribonucleic Acid Linked to Double-Stranded Ribonucleic Acid by Extracts of Interferon-Treated Cells†

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ABSTRACT: RNA covalently linked to double-stranded RNA (dsRNA) is preferentially degraded in extracts of interferon-treated HeLa cells [Nilsen, T. W., & Baglioni, C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2600–2604]. The size of the dsRNA required for this preferential degradation has been determined by annealing poly(I) of known length to the poly(C) tract of encephalomyocarditis virus (EMCV) RNA or by annealing poly(U) to poly(A) of known length of vesicular stomatitis virus mRNA. The dsRNA must be longer than about 60 base pairs to observe the preferential degradation of RNA. Moreover, triple-stranded regions that do not activate synthesis of 2′,5′-oligo(A) and ethidium bromide, which intercalates in dsRNA and blocks 2′,5′-oligo(A) polymerase activation, prevent this degradation. Ethidium also blocks the degradation of the replicative intermediate of EMCV by extracts of interferon-treated cells. These experiments indicate that synthesis of 2′,5′-oligo(A) is required for the degradation of RNA linked to dsRNA. The 2′,5′-oligo(A)-dependent endonuclease does not cleave single- or double-stranded DNA, nor does it cleave homopolyribonucleotides. The potential role of the 2′,5′-oligo(A) polymerase/endonuclease system in the inhibition of viral RNA replication is discussed.

Interferon induces in mammalian and avian cells an oligonucleotide polymerase; this enzyme can be activated in cell extracts by double-stranded RNA (dsRNA) and converts ATP into a series of oligonucleotides designated 2′,5′-oligo(A) (Kerr & Brown, 1978). The 2′,5′-oligo(A) activates an endonuclease that degrades mRNA (Baglioni et al., 1978; Clemens & Williams, 1978). This nuclease activity may play an important role among the antiviral defense mechanisms that inhibit the replication of RNA viruses in interferon-treated cells. This is suggested by the presence of 2′,5′-oligo(A) in cells treated with interferon and infected with encephalomyocarditis virus (EMCV) (Williams et al., 1979b) and by the inhibition of EMCV and vesicular stomatitis virus (VSV) replication upon introduction of 2′,5′-oligo(A) in mammalian cells (Williams et al., 1979a; Hovanessian & Wood, 1980). Both viral and cellular RNA appear to be degraded in the presence of 2′,5′-oligo(A) under these conditions (Hovanessian et al., 1979), whereas no significant degradation of cellular RNA can in general be detected in interferon-treated cells infected by viruses [reviewed by Stewart (1979)]. If the endonuclease is activated in intact cells, it therefore discriminates between cellular and viral RNA and preferentially degrades only the latter.

A mechanism for discrimination between cellular and viral RNA by the 2′,5′-oligo(A) polymerase/endonuclease system was proposed by Nilsen & Baglioni (1979a). In cells infected by viruses that replicate by forming structures containing dsRNA, the polymerase may bind to viral dsRNA, form limited amounts of 2′,5′-oligo(A), and activate the endonuclease at the site of viral RNA synthesis. Experiments with cell extracts provided experimental support for such a mechanism. Viral replicative intermediates and RNA covalently linked to dsRNA were preferentially degraded only in extracts of interferon-treated cells (Nilsen & Baglioni, 1979a). This degradation occurred only under incubation conditions that allowed synthesis of 2′,5′-oligo(A).

In the present study we investigated the structural features of dsRNA required to observe the preferential degradation of covalently linked RNA. These structural features are essentially identical with those required for the activation of 2′,5′-oligo(A) synthesis (Minks et al., 1979b). These experiments were carried out with RNAs containing base-paired regions of known compositions and length or with ethidium bromide that intercalates with dsRNA and inhibits activation of 2′,5′-oligo(A) polymerase (Baglioni & Maroney, 1980). Finally, by assaying the degradation of different substrates, we established that the 2′,5′-oligo(A)-dependent endonuclease does not cleave homopolyribonucleotides.

Experimental Procedures

Cell Extracts. HeLa S3 cells were grown in suspension in minimal essential medium supplemented with 5% calf serum. Cytoplasmic extracts were prepared from cells treated for 17 h with 100 units/mL of human fibroblast interferon (3×10^5 units/mg of protein; obtained from the Interferon Working Group of the National Cancer Institute, NIH) or from untreated cells as previously described by Baglioni et al. (1978).

Preparation of Labeled Viral RNA. Vesicular stomatitis virus (VSV) mRNA was synthesized by in vitro transcription with replicative complexes obtained from infected cells as previously described by Toneguzzo & Ghosh (1976). The

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reaction mixtures containing 1 mCi/mL [^{3}H]UTP, 2 mM nucleoside triphosphates, and either 0.1 mM *S*-adenosylmethionine or 1 mM *S*-adenosylhomocysteine were incubated for 3 h. The poly(A)-containing mRNA was isolated by chromatography on oligo(dT)–cellulose and sucrose gradient centrifugation according to Baglioni et al. (1978). The RNA synthesized in incubations containing *S*-adenosylmethionine was also fractionated by adsorption to nitrocellulose filters as described by Gorski et al. (1974). To measure the size of poly(A), VSV mRNA was synthesized in reactions containing 1 mCi/mL [^{3}H]ATP. The mRNA was isolated as described above, digested with RNases A and T1, and analyzed by electrophoresis on 12% polyacrylamide gels as described by Rose et al. (1977). The size of poly(A) was determined according to these authors.

Labeled EMCV 35S RNA and replicative intermediate (RI) were prepared from infected cells as described by Nilsen & Baglioni (1979a). Identical procedures were followed for the preparation of unlabeled RI. The replicative form (RF) of EMCV was prepared from infected cells by treatment of cell extracts with 2 M LiCl, chromatography on Sepharose-2B, and sucrose gradient centrifugation, as described by Spector & Baltimore (1975).

Preparation of Substrates for the 2′,5′-Oligo(A)-Dependent Endonuclease. For preparation of the mRNA containing a double-stranded region, VSV mRNA (4×10^4 cpm/μg) was annealed with just enough poly(U) to completely prevent its binding to oligo(dT)–cellulose, as described by Nilsen & Baglioni (1979a). For preparation of mRNA with a triple-stranded region, the VSV mRNA was annealed with 10 times the amount of poly(U) needed to prevent binding to oligo(dT). Labeled 35S EMCV RNA (5 μg/mL; 3×10^5 cpm/μg) was annealed with 40 μg/mL of either poly(I) or poly(dI), purchased from P-L Biochemicals, and reisolated by gradient centrifugation as described by Nilsen & Baglioni (1979a). Labeled homopolyribonucleotides and poly(A)·poly(U) (purchased from Miles) were cetrifuged on 15–30% sucrose gradients in RNA gradient buffer (Weber et al., 1979) to sediment the major peak about one-third from the bottom of the tube. Two or three peak fractions were pooled, precipitated with ethanol, and fractionated again on sucrose gradients to obtain polynucleotides of uniform sedimentation. The poly(A)·poly(U) prepared in this way had an $s_{20,w} = 4 \pm 0.5$ S. The [^{3}H]poly(U) and [^{3}H]poly(C) were annealed with sufficient sized poly(A) and poly(I), respectively (purchased from Miles and P-L Biochemicals), to obtain polynucleotides 50% acid soluble upon 30-min digestion with 50 μg/mL of RNase A in 0.3 M NaCl and 0.03 M sodium citrate, pH 7.0. The sized poly(A) was previously described by Minks et al. (1979b). In the present experiments we used the polymer A_{33} that failed to activate 2′,5′-oligo(A) synthesis when annealed with poly(U) (Minks et al., 1979b). The sized poly(I) had an average $s_{20,w} = 2.5$ S; by comparing its sedimentation to that of poly(A) of known size (determined by the nucleoside/nucleotide ratio after hydrolysis), we estimated an average chain length of 45.

DNA labeled with [^{3}H]thymidine was prepared from HeLa cells as described by Nilsen & Baglioni (1979b). This DNA was sheared to an average size of about 1000 base pairs (Nilsen & Baglioni, 1979b) and fractionated by sucrose gradient sedimentation as described above. Single-stranded DNA was prepared from this DNA by alkaline denaturation and fractionated on alkaline sucrose gradients (Nilsen & Baglioni, 1979b).

Endonuclease Assay. For the degradation assay of RNA covalently linked to dsRNA, the incubations contained 0.3 volume of cell extract, 0.12 M KOAc, 4 mM fructose, 1,6-bisphosphate, 2 mM $Mg(OAc)_2$, the amount of substrate, and other additions indicated in the legends. Endonuclease activity was assayed after incubation at 30 °C either by measuring the binding of poly(A)-containing mRNA to oligo(dT)–cellulose as described by Baglioni et al. (1978) or by sucrose gradient analysis. For the measurement of the degradation of mRNA annealed to poly(U), the assay for oligo(dT) binding was modified according to Nilsen & Baglioni (1979a). For degradation assays of different substrates upon addition of 2′,5′-oligo(A), the incubations contained 0.6 volume of cell extract and the other components indicated above. Each incubation mixture was divided into three aliquots; one was kept at 0 °C and the others were incubated 60 min at 30 °C either with no addition or with 1 μM 2′5′-oligo(A) (concentration expressed in AMP equivalents). These oligonucleotides were prepared as previously described by Minks et al (1979a). The incubations were stopped by the addition of RNA gradient buffer and centrifuged on sucrose gradients. The radioactivity of gradient fractions was determined after addition of Scintiverse (Fisher Chemical Co.). The total radioactivity of peak fractions of the unincubated control and of the corresponding fractions from the incubated samples was determined to calculate percent degradation.

Assay for 2′,5′-Oligo(A) Synthesis. The assay is described by Minks et al. (1979a).

Results

Size of Double-Stranded Region Required for Preferential Degradation of Covalently Linked RNA. The 35S RNA of EMCV contains a poly(C) tract ~100 nucleotides long near its 5′ terminus (Chumakov & Angol, 1976). EMCV RNA annealed to high molecular weight poly(I) is preferentially degraded in extracts of interferon-treated cells over RNA not annealed to poly(I) (Nilsen & Baglioni, 1979a). The size of the base-paired region required for this preferential degradation was determined by annealing poly(I) of two different lengths to the poly(C) tract of EMCV RNA. Either poly(I) longer than 100 nucleotides or poly(I) about 45 nucleotides long was annealed to EMCV RNA. The 35S RNA was separated from excess poly(I) and incubated with extracts of interferon-treated or control cells as previously described (Nilsen & Baglioni, 1979a). Both RNAs were degraded to a limited extent when incubated with extract of control cells (Figure 1A,B). The RNA annealed to the long poly(I), however, was preferentially degraded upon incubation with extract of interferon-treated cells (Figure 1D), whereas the RNA annealed to the short poly(I) was not significantly degraded (Figure 1E). To these incubations was added, as an internal control, VSV mRNA labeled with a different isotope; this mRNA was not significantly degraded even in the incubation of EMCV RNA annealed to the long poly(I). The short poly(I) is stably base paired with poly(C) under the conditions of our experiment, as shown by the RNase resistance of the duplex (see Table I). These results indicate that the presence of double-stranded regions with one strand shorter than 50 bases does not result in preferential degradation of covalently linked RNA in extracts of interferon-treated cells and suggest that there is a minimum length requirement for the double-stranded region.

The preferential degradation of RNA linked to dsRNA was also shown by annealing poly(U) to the poly(A) region of mRNA (Nilsen & Baglioni, 1979a). In those experiments, VSV mRNA containing poly(A) longer than about 60 nu-

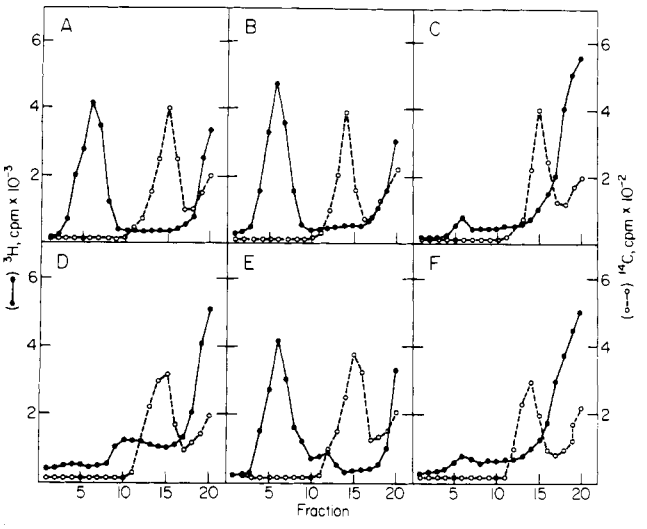

FIGURE 1: Degradation of EMCV 35S RNA annealed with different polynucleotides and incubated with extracts of control (A, B, and C) or interferon-treated cells (D, E, and F). Each 0.3-mL incubation contained the components described under Experimental Procedures, 2000 cpm of VSV [^{14}C]mRNA (○), and 20000 cpm of EMCV [^{3}H]mRNA (●) annealed with poly(I) of $s_{20,w}$ = 6–13 S (A and D), with poly(I) of $s_{20,w}$ = 2.5 S (B and E), or with poly(dI) of $s_{20,w}$ = 6–9 S (C and F). The incubation was for 60 min at 30 °C and was stopped by the addition of 0.3 mL of twice concentrated RNA gradient buffer. The samples were fractionated by 19-h centrifugation at 24000 rpm in 15–30% sucrose gradients in the same buffer.

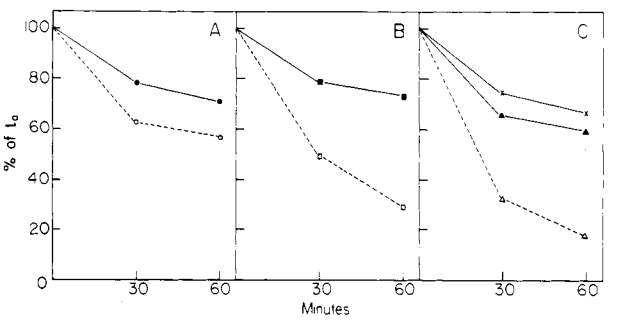

FIGURE 2: Degradation of VSV mRNA containing poly(A) of different length annealed to poly(U) and incubated in extract of control or interferon-treated cells. Each 0.1-mL incubation contained 10000 cpm of [^{3}H]mRNA annealed with poly(U) and the other components described in Experimental Procedures. (A) Degradation of mRNA synthesized in the presence of 0.1 mM *S*-adenosylmethionine incubated with extract of control (●) or interferon-treated cells (○). (B) Degradation of the same mRNA fractionated by adsorption to nitrocellulose filters in extract of inteferon-treated cells; mRNA bound to filters (□) and unbound mRNA (■). (C) Degradation of the mRNA synthesized in the presence of 1 mM *S*-adenosylhomocysteine incubated with extract of control (▲) or interferon-treated cells (△), and degradation of the same mRNA annealed with 10-fold the amount of poly(U) in extract of interferon-treated cells (×). Incubation was for 60 min at 30 °C, and aliquots were taken at the indicated times to measure poly(A)-containing RNA as described in Experimental Procedures. The percentage of poly(A)-containing RNA remaining after incubation is shown in the ordinate relative to a control sample not incubated (=100%).

Table I: Effect of Inhibitors of 2′,5′-Oligo(A) Synthesis and Substrate Specificity of the 2′,5′-Oligo(A)-Dependent Endonuclease[a]

substrate	% of unincubated: no addition	% of unincubated: 1 μM 2′,5′-oligo(A)
VSV mRNA	74	23
VSV mRNA + 0.5 mM ethidium	77	26
VSV mRNA + 1 mM 2′-dATP	75	24
VSV mRNA annealed with poly(dT)	5	4
poly(C)	2	1
poly(C)·$(I_{45})_n$	82	83
poly(U)	3	3
poly(U)·$(A_{33})_n$	76	73
poly(A)	60	58
poly(A)·poly(U)	88	89
poly(G)	93	95
double-stranded DNA	98	97
single-stranded DNA	94	95

[a] The labeled nucleic acids (10000 cpm per assay) were incubated for 60 min with extract of untreated HeLa cells as described under Experimental Procedures. In experiments with VSV mRNA, the samples were analyzed as described in the legend of Figure 2. Labeled homopolyribonucleotides of uniform sedimentation and DNA were prepared as described in Experimental Procedures and analyzed as shown in Figure 5, with the exception of poly(C) and poly(U) that were precipitated with 10% trichloroacetic acid.

cleotides was used. For the experiments described here, we prepared VSV mRNA with poly(A) of different lengths, annealed this mRNA to a standard preparation of high molecular weight poly(U), and followed its degradation in extracts of interferon-treated and control cells. This method of analysis measures the degradation of mRNA by examining its binding to oligo(dT)–cellulose (Baglioni et al., 1978). VSV mRNA transcribed with viral replicative complexes obtained from infected cells contains poly(A) varying in length from 40 to about 100 residues (see Experimental Procedures). This mRNA annealed to poly(U) is degraded slightly faster in extract of interferon-treated cells than in control cell extract (Figure 2A). The mRNA can be fractionated by adsorption to nitrocellulose filters into molecules with poly(A) longer or shorter than 60 nucleotides; only the mRNA with the longer poly(A) "tail" binds to the filters at high ionic strength (Gorski et al., 1974). This mRNA was preferentially degraded in extracts of interferon-treated cells when annealed to poly(U), whereas the mRNA with the shorter poly(A) tail was not (Figure 2B). Therefore, the enhanced degradation in extract of interferon-treated cells requires a double-stranded region longer than about 60 base pairs.

In a different experimental approach to the same problem, VSV mRNA was synthesized in the presence of the methylation inhibitor *S*-adenosylhomocysteine. The mRNA synthesized in this way with VSV virions contains very long poly(A) tails (Rose et al., 1977). We confirmed this observation for the mRNA synthesized with replicative complexes by labeling the VSV mRNA with [^{3}H]adenosine and determining by gel electrophoresis that the poly(A) obtained by RNase A digestion was longer than 100 nucleotides (data not shown). When this mRNA was annealed to poly(U) and incubated with extract of interferon-treated or control cells, it was preferentially degraded only in extract of interferon-treated cells (Figure 2C).

Structural Requirements of the Base-Paired Region. The base-paired regions of RNA molecules can be modified by forming triple-stranded polymers, by intercalating ethidium bromide, or by forming hybrid double-stranded polymers containing a deoxypolynucleotide annealed to a polyribonucleotide. Triple-stranded and hybrid polymers do not activate 2′,5′-oligo(A) synthesis (Minks et al., 1979a), and ethidium blocks this activation in a characteristic way (Baglioni & Maroney, 1980). For formation of RNA molecules containing a triple-stranded region, VSV mRNA was first annealed with enough poly(U) to prevent its binding to oligo(dT)–cellulose; additional poly(U) was then annealed to form a triple-stranded poly(A)·poly(U)·poly(U) tail (see Experimental Procedures). The mRNA with this triple-stranded tail

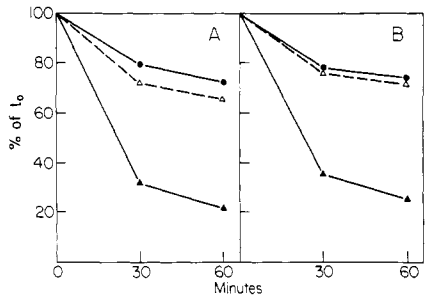

FIGURE 3: Effects of eithidium bromide and 2′-dATP on the degradation of mRNA covalently linked to dsRNA in extracts of interferon-treated cells. Incubations were as described in Figure 2 and contained VSV mRNA synthesized in the presence of *S*-adenosylhomocysteine and annealed to poly(U). (A) Degradation in extract of control cells (●) or interferon-treated cells with no addition (▲) or with 0.5 mM ethidium (△). (B) Degradation in extract of control cells (●) or interferon-treated cells with no addition (△) or with 1 mM 2′-dATP (△). The percentage of poly(A)-containing RNA remaining after incubation for the time indicated in the abscissa is shown in the ordinate relative to a control sample not incubated.

was not preferentially degraded in extract of interferon-treated cells (Figure 2C).

Addition of ethidium bromide to incubations containing VSV mRNA with a poly(A)·poly(U) tail greatly reduced the degradation of this mRNA by extract of interferon-treated cells (Figure 3A). Ethidium had no effect on mRNA degradation by control cell extract (Table I). In these experiments, ethidium was added at a concentration that inhibited the activation of 2′,5′-oligo(A) synthesis by poly(A)·poly(U) (Baglioni & Maroney, 1980). Much lower ethidium concentrations are effective in inhibiting the activation by this dsRNA than are required to inhibit the activation by poly(I)·poly(C), because of the preferential binding of the dye to poly(A)·poly(U) (Baglioni & Maroney, 1980). In subsequent experiments we studied the effect of higher ethidium concentrations on the degradation of the replicative intermediate (RI) of EMCV (Figure 4). In the extract of interferon-treated cells, the RI is preferentially degraded, presumably because of the presence of double-stranded regions that activate synthesis of 2′,5′-oligo(A) (Nilsen & Baglioni, 1979a). Addition of ethidium, however, prevented this degradation (Figure 4C,D).

When unlabeled RI isolated from EMCV-infected cells was assayed as the activator of 2′,5′-oligo(A) synthesis, ethidium was highly inhibitory (Table II). These experiments, therefore, show that preferential degradation of RI and VSV mRNA in extract of interferon-treated cells is dependent on the presence of double-stranded regions and correlates with the ability of these regions to activate synthesis of 2′,5′-oligo(A).

The source of the substrate for 2′,5′-oligo(A) synthesis is ATP present in the cell extract, and fructose 1,6-bisphosphate is added to the incubations (see Experimental Procedures) to regenerate ATP (Baglioni & Weber, 1978). The increased degradation of mRNA linked to dsRNA is dependent on the presence of ATP in the incubation, as previously shown by depleting ATP with glucose and hexokinase (Nilsen & Baglioni, 1979a). An inhibitor of 2′,5′-oligo(A) synthesis, 2′-dATP, has been described by Minks et al. (1980). Addition of this inhibitor prevented degradation of VSV mRNA with the poly(A)·poly(U) tail (Figure 3B). In this case, the 2′,5′-oligo(A) polymerase was presumably activated, but the 2′-dATP competitively inhibited synthesis of 2′,5′-oligo(A). The 2′-dATP, however, had no effect on the endonuclease activated by 2′,5′-oligo(A) (Table I).

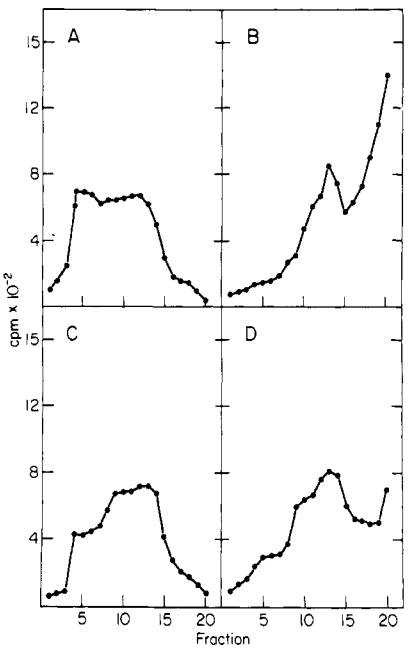

FIGURE 4: Effect of ethidium bromide on the degradation of the replicative intermediate of EMCV. Each of 0.3-mL incubation contained 10000 cpm of [^{3}H]RI and the other components described in Experimental Procedures. (A) Not incubated. (B–D) Incubated 60 min at 30 °C with extract of interferon-treated cells. (C, D) 0.5 and 0.2 mM ethidium, respectively. The incubations were stopped by addition of 0.3 mL of twice concentrated RNA gradient buffer and analyzed by 17-h centrifugation at 20000 rpm in 14 mL of 15–30% sucrose gradients in the same buffer layered over 3 mL of 60% sucrose.

Table II: Inhibition of 2′,5′-Oligo(A) Synthesis by Ethidium Bromide[a]

activator	ethidium concn (mM)			
	0	0.1	0.2	0.5
replicative intermediate of EMCV	7.1	2.4	1.1	0
replicative form of EMCV	10.6	3.9	1.6	0
poly(I)·poly(C)	21.4	19.1	10.7	5.8
poly(A)·poly(U)	21.2	0.2	0	0

[a] The assays were prepared according to Minks et al. (1979a) and contained 25 mM $Mg(OAc)_2$, 5 mM [^{3}H]ATP (125 nmol, about 200000 cpm), 0.2 volume of extract from interferon-treated cells, and the other components described for assays of endonuclease activity. The synthetic polynucleotides were tested at 10 μg/mL; viral RNAs were tested at 5 μg/mL nucleic acid. The nmol of 2′,5′-oligo(A) synthesized for 60 min in a 25-μL reaction are expressed in AMP equivalents. A control reaction without added dsRNA gave less than 0.02 nmol of nucleotides eluted with the fraction corresponding to 2′,5′-oligo(A); this background was subtracted from the data shown.

Molecules containing a base-paired region consisting of a polyribonucleotide annealed to a deoxyribonucleotide were formed by annealing poly(dI) with EMCV RNA and poly(dT) with VSV mRNA. When these RNAs were incubated in either extract of interferon-treated or control cells, they were rapidly degraded, as shown for EMC RNA in Figure 1C,F. An mRNA labeled with a different isotope and not annealed to a polydeoxynucleotide was included in the same incubations as the internal control; this mRNA was not significantly degraded. This indicates that the extensive degradation of RNAs containing hybrid base-paired regions is due to RNase H activity. The presence of such an activity was further established by showing extensive digestion of [^{3}H]poly(A)·poly(dT) upon incubation with extracts of both control and interferon-treated cells (data not shown). RNase H activity has

indeed been reported in cytoplasmic extracts prepared from HeLa cells (Ferrari et al., 1980) as well as other cell types (Hall & Crouch, 1977; Cathala et al., 1979). This nuclease does not require formation of 2′,5′-oligo(A) for activation. The extensive degradation of RNA containing a rather small hybrid double-stranded region close to one end of the molecule may seem surprising, but it can be explained by exonuclease digestion of RNA cleaved by RNase H.

The 2′,5′-Oligo(A)-Dependent Endonuclease Does Not Cleave Homopolyribonucleotides. In the experiments designed to determine the size of the double-stranded region required for preferential degradation of RNA, we have not considered the possibility that the 2′,5′-oligo(A)-dependent endonuclease cleaves dsRNA or poly(A). If the latter were the case, the binding to oligo(dT) would be impaired and the validity of the assay could be questionable. To establish whether the endonuclease cleaves dsRNA or poly(A), we investigated the degradation of these polymers in incubations with added 2′,5′-oligo(A) isolated as described by Minks et al. (1979a). These studies were further extended to other homopolyribonucleotides and to DNA to gather information on the substrate specificity of the endonuclease. Surprisingly, none of the polyribonucleotides assayed is cleaved by this enzymatic activity (Table I).

Labeled polyribonucleotides were incubated with cell extract, and the degradation to acid-soluble nucleotides was measured. Poly(C) and poly(U) were rapidly and completely degraded, whereas poly(A) was partially degraded and the other nucleic acids were not significantly degraded in a 1-h incubation. Degradation of these latter polymers by 2′,5′-oligo(A)-dependent endonuclease was then assayed by isolating a nucleic acid fraction with a relatively narrow sedimentation value by preparative gradient centrifugation (see Experimental Procedures). This fraction was incubated with HeLa cell extract with or without added 2′,5′-oligo(A) and analyzed by gradient centrifugation. No change in the sedimentation pattern was observed with either single- or double-stranded DNA, with poly(A)·poly(U), with poly(G), and with poly(A). For this latter polynucleotide, the partial degradation to acid-soluble nucleotides did not interfere with the assay since the products of degradation sedimented at the top of the gradient. Addition of 2′,5′-oligo(A) had no effect either on the sedimentation value or on the amount of acid-soluble nucleotides formed.

In the case of poly(U) and poly(C), we blocked the nuclease activity that degrades these polymers in HeLa cell extract by annealing to them short (less than 50 bases) polymers of A and I, respectively. These partially double-stranded polymers could be formed by adding enough complementary short strands to obtain about 50% resistance to RNase digestion to acid-soluble nucleotides. Digestion with RNase A resulted in a large change in sedimentation value (Figure 5), indicating that single-stranded nucleotide stretches separating double-stranded regions were digested by this endonucleolytic enzyme. In contrast, when the polymers were incubated with cell extract and 2′,5′-oligo(A), no change in sedimentation value was observed, though some nucleotides sedimenting at the top of the gradient were formed. These are presumably produced by exonucleolytic degradation of single-stranded ends of the polymers. These experiments indicated, therefore, that the 2′,5′-oligo(A)-dependent endonuclease does not cleave deoxynucleotides or homopolyribonucleotides.

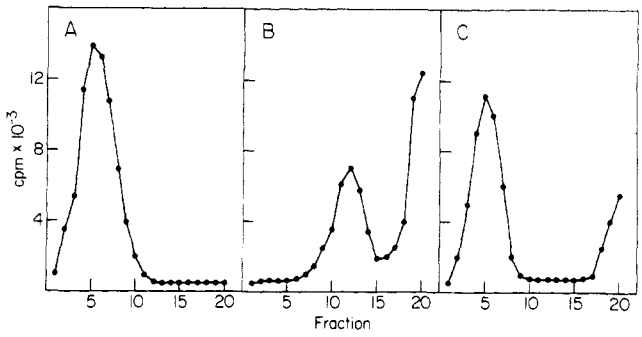

FIGURE 5: Assay for poly(U) degradation by the 2′,5′-oligo(A)-dependent endonuclease. [^{3}H]Poly(U) was annealed with poly(A) of $s_{20,w}$ = 2.1 S as described under Experimental Procedures and analyzed by sedimentation on 5–20% sucrose gradients in RNA gradient buffer for 20 h at 40 000 rpm. (A) Not incubated. (B) Incubated for 30 min at 30 °C with 50 μg/mL of RNase A in 0.3 M NaCl and 0.3 M sodium citrate, pH 7.0. (C) Incubated 60 min in extract of interferon-treated cells with 1 μM added 2′,5′-oligo(A). A parallel incubation without added 2′,5′-oligo(A) gave an identical sedimentation profile (not shown).

Discussion

We have investigated the size and structural features of dsRNA required for preferential degradation of covalently linked RNA in extracts of interferon-treated HeLa cells. Both the size and the structural features of the dsRNA are identical with those required to activate 2′,5′-oligo(A) synthesis (Minks et al., 1979b). Moreover, ethidium bromide, which inhibits activation of 2′,5′-oligo(A) polymerase by intercalating into dsRNA (Baglioni & Maroney, 1980), and the inhibitor of 2′,5′-oligo(A) synthesis, 2′-dATP (Minks et al., 1980), suppress the preferential degradation of RNA linked to dsRNA. These results provide support for a role of 2′,5′-oligo(A) polymerase in the discrimination between viral and host mRNA postulated to occur in interferon-treated cells (Nilsen & Baglioni, 1979a). In addition, the present results rule out some alternative interpretations of our findings.

The presence of extended double-stranded regions is not sufficient to render RNA molecules susceptible to nuclease digestion in extracts of interferon-treated cells. If this were the case, EMCV RNA annealed to the short poly(I) should have been degraded to the same extent as EMCV RNA annealed to the long poly(I) since the total amount of dsRNA is presumably the same in both types of molecules and only one or two discontinuities are present in the complex with the short poly(I). However, these interruptions may be sufficient to prevent activation of 2′,5′-oligo(A) synthesis (Minks et al., 1979b), and the base-pairing of the shorter poly(I) with the poly(C) tract of EMCV RNA has no effect on the degradation of this RNA.

The observation that the 2′,5′-oligo(A)-dependent endonuclease does not cleave homopolyribonucleotides provides some indication for a possible sequence recognition by this endonuclease. The results obtained with poly(C) and poly(U) are quite clear, whereas those obtained with poly(A) and poly(G) may have to take into account the secondary structure of these polymers in solution. We cannot exclude that the enzyme may cleave extended poly(A) or poly(G) while ineffective against multistranded forms of these polynucleotides. The sequence specificity of the 2′,5′-oligo(A)-dependent endonuclease remains undefined. Baglioni et al. (1980) have reported that the endonuclease cleaves all the mRNAs so far assayed but that each mRNA is cleaved at relatively few positions. However, this may be a consequence of the rapid catabolism of 2′,5′-oligo(A) and of the reversible activation of the endonuclease [see, for references, Baglioni (1979)].

The mechanism responsible for the preferential degradation of RNA covalently linked to dsRNA is not clear at this point. We have shown that dsRNA activates synthesis of 2′,5′-oligo(A) and have suggested that the concentration of these

nucleotides may be greatest near their site of synthesis; this may result in a localized activation of the endonuclease (Nilsen & Baglioni, 1979a). An alternative mechanism, also compatible with our results, is that the 2′,5′-oligo(A) polymerase may be physically associated with the endonuclease. Synthesis of 2′,5′-oligo(A) would result, in this case, in the localized activation of the enzyme complex; experiments are currently in progress to examine this possibility.

It is not yet clear whether the 2′,5′-oligo(A) polymerase/endonuclease system degrades RNA linked to dsRNA in intact cells, since cleavage of viral RNA in interferon-treated cells has not been detected. Several lines of evidence, however, suggest that degradation of viral RNA may be part of the antiviral mechanisms elicited by interferon: (i) 2′,5′-oligo(A) introduced in intact cells activates a nuclease (Williams et al., 1979a; Hovanessian et al., 1979); (ii) 2′,5′-oligo(A) is formed in interferon-treated cells infected with EMCV (Williams et al., 1979); and (iii) the replicative complexes of EMCV have been shown to contain double-stranded regions in intact cells (Nilsen et al., 1980). These viral structures are presumably responsible for the synthesis of 2′,5′-oligo(A) in intact cells and for endonuclease activation.

References

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

Baglioni, C., & Weber, L. A. (1978) *FEBS Lett. 88*, 37–40.

Baglioni, C., & Maroney, P. A. (1980) *Biochemistry* (in press).

Baglioni, C., Minks, M. A., & Maroney, P. A. (1978) *Nature (London) 273*, 684–687.

Baglioni, C., Benvin, S., Maroney, P. A., Minks, M. A., Nilsen, T. W., & West, D. K. (1980) *Ann. N.Y. Acad. Sci.* (in press).

Cathala, G., Rech, J., Huet, J., & Jeanteur, P. (1979) *J. Biol. Chem. 254*, 7353–7361.

Chumakov, K. M., & Agol, V. I. (1976) *Biochem. Biophys. Res. Commun. 71*, 551–557.

Clemens, M. J., & Williams, B. R. G. (1978) *Cell 13*, 565–572.

Ferrari, S., Yehle, C. O., Robertson, H. D., & Dickson, E. (1980) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Goski, J., Morrison, M. R., Merkel, C. G., & Lingrel, J. B. (1974) *J. Mol. Biol. 86*, 363–371.

Hall, S. H., & Crouch, R. J. (1977) *J. Biol. Chem. 252*, 4092–4097.

Hovanessian, A. G., & Wood, J. N. (1980) *Virology 101*, 81–90.

Hovanessian, A. G., Wood, J. N., Meurs, E., & Montagnier L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3261–3265.

Kerr, I. M., & Brown, R. E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 256–260.

Minks, M. A., Benvin, S., Maroney, P. A., & Baglioni, C. (1979a) *J. Biol. Chem. 254*, 5058–5064.

Minks, M. A., West, D. K., Benvin, S., & Baglioni, C. (1979b) *J. Biol. Chem. 254*, 10180–10183.

Minks, M. A., Benvin, S., & Baglioni, C. (1980) *J. Biol. Chem.* (in press).

Nilsen, T. W., & Baglioni, C. (1979a) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2600–2604.

Nilsen, T. W., & Baglioni, C. (1979b) *J. Mol. Biol. 133*, 319–338.

Nilsen, T. W., Wood, D. L., & Baglioni, C. (1980) *Virology* (in press).

Rose, J. K., Lodish, H. F., & Brock, M. L. (1977) *J. Virol. 21*, 683–693.

Spector, D. H., & Baltimore, D. (1975) *J. Virol. 15*, 1418–1431.

Stewart, W. E. (1979) in *The Interferon System*, Springer-Verlag, New York.

Toneguzzo, F., & Ghosh, H. P. (1976) *J. Virol. 17*, 477–491.

Weber, L. A., Simili, M., & Baglioni, C. (1979) *Methods Enzymol. 60*, 351–360.

Williams, B. R. G., Golgher, R. R., & Kerr, I. M. (1979a) *FEBS Lett.* 105, 47–52.

Williams, B. R. G., Golgher, R. R., Brown, R. E., Gilbert, C. S., & Kerr, I. M. (1979b) *Nature (London) 282*, 582–586.