Interferon Action: Binding of Viral RNA to the 40-Kilodalton 2'-5'-Oligoadenylate Synthetase in Interferon-Treated HeLa Cells Infected with Encephalomyocarditis Virus

G. GRIBAUDO,^{1,2} D. LEMBO,² G. CAVALLO,² S. LANDOLFO,² AND P. LENGYEL^{1*}

*Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511,¹ and
Department of Microbiology, Medical School, University of Turin, Turin, Italy²*

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The 40-kDa 2'-5'-oligoadenylate [(2'-5') (A)_n] synthetase isoenzyme was proven to be a mediator of the inhibition of encephalomyocarditis virus (EMCV) replication by interferon (IFN). When activated by double-stranded RNA, this enzyme converts ATP into 2'-5'-oligoadenylate [(2'-5') (A)_n], and (2'-5') (A)_n was found to accumulate in IFN-treated, EMCV-infected cells. The only known function of (2'-5') (A)_n is the activation of RNase L, a latent RNase, and this was also implicated in the inhibition of EMCV replication. Intermediates or side products in EMCV RNA replication, presumed to be partially double stranded, were shown to activate (2'-5') (A)_n synthetase in vitro. These findings served as the basis of the long-standing hypothesis that the activator of (2'-5') (A)_n synthetase in IFN-treated, EMCV-infected cells is the viral RNA. To test this hypothesis, we have generated a polyclonal rabbit antiserum to the human 40-kDa (2'-5') (A)_n synthetase. The antiserum immunoprecipitated, from IFN-treated HeLa cells that had been infected with EMCV, the 40-kDa (2'-5') (A)_n synthetase protein in complex with both strands of EMCV RNA. The immunoprecipitate was active in (2'-5') (A)_n synthesis even without addition of double-stranded RNA, whereas the immunoprecipitate from IFN-treated, uninfected cells was not. These and other results demonstrate that in IFN-treated, EMCV-infected cells, viral RNA is bound to the (2'-5') (A)_n synthetase and suggest that the agent activating the (2'-5') (A)_n synthetase is the bound viral RNA.

Double-stranded RNA (dsRNA) has multiple roles in the functioning of the interferon (IFN) system (13, 14, 22). It can induce the synthesis of IFN and of some IFN-inducible proteins in various vertebrate cells (26). Furthermore, it can activate several IFN-inducible, latent enzymes, including eIF-2 kinase and at least four different 2'-5'-oligoadenylate [(2'-5') (A)_n] synthetase isoenzymes (2). When activated by dsRNA, these isoenzymes convert ATP into (2'-5') (A)_n and pyrophosphate. The only known function of (2'-5') (A)_n is the activation of a latent RNase, RNase L.

The 40-kDa human (2'-5') (A)_n synthetase was shown to be able to mediate the inhibition of mengovirus and encephalomyocarditis virus (EMCV) replication by IFN; transfection of a cDNA specifying the enzyme under the control of a constitutive promoter converted the recipient cells into a state in which they impaired the replication of mengovirus and EMCV (3, 24). RNase L was also implicated in the impairment of replication of EMCV; it was established that a high level of (2'-5') (A)_n synthetase without a sufficiently high level of RNase L did not impair EMCV replication (11; see also reference 31). The infection of cells with EMCV was found to result in the accumulation of the products of activated (2'-5') (A)_n synthetases, i.e., (2'-5') (A)_n (8, 9, 27). These compounds, as noted above, activate the latent RNase L. In line with such an activation, rRNA cleavage (thought to be catalyzed by RNase L) was found to occur in some but not all IFN-treated, EMCV-infected cells (35). It is assumed that the (2'-5') (A)_n synthetase-RNase L enzyme system inhibits mengovirus and EMCV replication primarily by accelerating viral (and perhaps also cellular) RNA degradation (28).

So far, the only agent known to activate (2'-5') (A)_n synthetases in vitro is dsRNA. It has been generally hypothesized that the agents activating this enzyme in IFN-treated, EMCV-infected cells are intermediates or side products in viral RNA replication which are presumed to be at least partially double stranded (10). It is in accord with this hypothesis that replicating EMCV RNA can activate (2'-5') (A)_n synthetase in vitro (though much less efficiently than RNA which is fully double stranded) (16, 17).

Here, we report experiments implicating replicating EMCV RNA in the activation of (2'-5') (A)_n synthetase in IFN-treated, EMCV-infected cells. We have prepared an antiserum against a segment of the 40-kDa human (2'-5') (A)_n synthetase (7). This antiserum immunoprecipitated from IFN-treated, EMCV-infected cells the (2'-5') (A)_n synthetase in a complex with both strands of EMCV RNA. Moreover, the complex had (2'-5') (A)_n synthetase activity in vitro even without the addition of dsRNA.

MATERIALS AND METHODS

Reagents. Human recombinant IFN-α A/D (32) (specific activity, 10⁸ IU/mg of protein) was a kind gift of G. Garotta (Hoffmann-La Roche, Basel, Switzerland). Aprotinin, phenylmethylsulfonyl fluoride, hexokinase, and yeast tRNA were from Sigma Chemical Co. (St. Louis, Mo.); protein A-Sepharose CL-4B, poly(I)-poly(C), and RNases A and T₁ were from Pharmacia (Uppsala, Sweden); RNase inhibitor (Inhibit-Ace) was from 5 Prime-3 Prime, Inc. (West Chester, Pa.); and radiochemicals were from Amersham (Bucks, United Kingdom).

Growth of cells and preparation of cell extracts. HeLa cells were grown as monolayers in Eagle's medium as modified by Dulbecco (DMEM) (ICN Biomedical, Rickmansworth,

* Corresponding author.

United Kingdom) supplemented with 10% fetal bovine serum. If so indicated, subconfluent monolayers were treated with IFN (1,000 U/ml for 16 h). For the preparation of cell extracts, cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (5 mM MgCl₂, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 30 mM 2-mercaptoethanol, 10% glycerol, 0.5% Nonidet P-40, 100 μM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml); the lysate was centrifuged at 4°C and 1,500 × *g* for 10 min and at 15,000 × *g* for 30 min. The supernatant fractions were collected and stored at -80°C.

EMCV infection and UV irradiation in vivo. EMCV was a kind gift from G. Sen (The Cleveland Clinic Foundation, Cleveland, Ohio). A virus stock solution containing about 10⁹ PFU/ml (as determined by plaque assay on HeLa cell monolayers) (23) was used in all experiments. HeLa monolayers (control or treated with 1,000 U of IFN per ml for 16 h) were washed twice in DMEM (serum free) and infected at a multiplicity of infection of 2. After 1 h at 37°C, the virus inoculum was removed and the cultures were refed with 37°C DMEM containing 10% fetal bovine serum. At the indicated times after infection, the monolayers were washed three times with ice-cold Ca²⁺- and Mg²⁺-containing PBS.

Irradiation was carried out in PBS with a 15-W germicidal lamp (Sylvania G15T8) at a 5-cm distance from the cell monolayer at room temperature for 5 min. Thereafter, cell extracts were prepared as described above. Synthetic dsRNA was cross-linked to proteins in the cell extracts by UV irradiation as described above for virus-infected cells.

Immunoblot analysis. Extracts from IFN-treated or control cells were prepared as described above for growth of cells and preparation of cell extracts. Protein samples (100 μg) from the extracts were fractionated by electrophoresis in sodium dodecyl sulfate (SDS)-10% polyacrylamide gels and transferred to nitrocellulose filters by electroblotting. The filters were incubated first with an antiserum against the 40-kDa human (2'-5') (A)_n synthetase and then with ¹²⁵I-labeled protein A as previously described and visualized by autoradiography. The antiserum (7) (polyclonal) was obtained from rabbits that had been immunized with a 17-amino-acid peptide from the hydrophobic C terminus of the 40-kDa human (2'-5') (A)_n synthetase.

Labeling of cells. HeLa cells were incubated in the absence or the presence of 1,000 U of IFN per ml in methionine-free DMEM supplemented with 50 μCi of [³⁵S]methionine per ml (37 TBq/mmol) and 5% dialyzed fetal bovine serum for 16 h. Cell extracts were prepared as described above.

Immunoprecipitation. Aliquots from cell extracts were immunoprecipitated either as such or after incubation with dsRNA under the conditions indicated. The immunoprecipitation was performed in two stages. First, material binding to normal rabbit serum immunoglobulin-Sepharose CL-4B was removed from 0.5-ml aliquots of the cell extracts. Second, to the remaining material, antiserum against (2'-5') (A)_n synthetase (5 μl) was added, and the reaction mixture was incubated on ice for 1 h. Thereafter, 60 μl of a protein A-Sepharose CL-4B suspension was added. After a 1-h incubation at 4°C, the resulting immunocomplexes bound to the protein A-Sepharose CL-4B were washed three times in buffer I (20 mM Tris-Cl [pH 7.5], 50 mM KCl, 100 mM NaCl, 5 mM MgCl₂, 5 mM 2-mercaptoethanol, 0.5% Nonidet P-40, 10% glycerol) and subsequently three times in buffer II (20 mM HEPES [pH 7.5], 50 mM KCl, 5 mM magnesium acetate, 10% glycerol). Thereafter, for processing of immunoprecipitates from extracts of in vivo-radiolabeled cells, the

proteins were eluted from the protein A-Sepharose beads with Laemmli sample buffer (12) and subjected to SDS-10% polyacrylamide gel electrophoresis. The gels were subjected to fluorography to localize the labeled proteins. As size markers, the following ¹⁴C-methylated proteins were used: phosphorylase *b* (92.5 kDa), bovine serum albumin (68 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and cytochrome *c* (12.5 kDa).

Recovery of RNA from immunoprecipitates. Synthetic dsRNA or viral RNA bound to the 40-kDa (2'-5') (A)_n synthetase was recovered from the washed immunoprecipitates by resuspending the immunocomplexes bound to protein A-Sepharose (30 μl) in 200 μl of buffer III (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% SDS, 0.05% Nonidet P-40, 50 μg of yeast tRNA per ml [as a carrier]) and incubating the mixtures at 37°C for 30 min. Thereafter, an equal volume of 50 mM Tris-Cl (pH 7.5)-5 mM EDTA-2 mg of proteinase K per ml was added to each sample, and the reaction mixtures were incubated at 37°C for 30 min. Finally, after two extractions with phenol and chloroform, the RNA samples were recovered by ethanol precipitation.

Assay of (2'-5') (A)_n synthetase activity. The (2'-5') (A)_n synthetase activity associated with the washed immunocomplexes bound to protein A-Sepharose (see discussion of immunoprecipitation above) was assayed by resuspension in (2'-5') (A)_n synthetase buffer (20 mM HEPES-KOH [pH 7.4], 120 mM potassium acetate, 25 mM magnesium acetate, 10 mM 2-mercaptoethanol) supplemented with 5 mM ATP, 0.1 μCi of [α-³²P]ATP (specific activity, 450 Ci/mmol) and, if so indicated, 100 μg of poly(I)-poly(C) per ml and incubation first at 30°C for 20 h and subsequently (to terminate the reactions) at 95°C for 3 min. Further steps of the assay were as described previously (4).

Preparation of dsRNA. Synthetic dsRNA was prepared by annealing complementary sense and antisense transcripts generated in vitro. As a template, plasmid pBXELm-4-9D10 was used. This plasmid contains a 680-bp fragment from a frog non-muscle tropomyosin cDNA (34) (GenBank accession number 52243) inserted into the *Eco*RI site of the polylinker of pBluescript SK(+) (Stratagene, La Jolla, Calif.). Cleavage of this plasmid with the appropriate restriction enzymes followed by transcription with T3 and T7 polymerases (Stratagene) produced complementary single-stranded RNAs (ssRNAs). These were annealed in 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.7)-400 mM NaCl-1 mM EDTA-80% formamide at 45°C for 12 h. Thereafter, the reaction mixture was diluted with 5 volumes of 10 mM Tris-Cl (pH 7.5)-300 mM NaCl-5 mM EDTA and ethanol precipitated. To digest the residual ssRNA, the precipitate was resuspended in 10 mM Tris-Cl (pH 7.5)-300 mM NaCl-5 mM EDTA containing 2 μg of RNase T₁ per ml and 40 U of RNase A per ml and incubated at 15°C for 2 h. For deproteinization, 1% SDS and 2 mg of proteinase K per ml were added, and the reaction mixture was incubated at 37°C for 30 min, extracted with phenol and chloroform, and precipitated with ethanol. Labeled dsRNA (calculated final specific activity, about 10⁷ cpm/μg) was prepared by including [α-³²P]UTP (800 Ci/mmol) into the transcription reactions.

RNA (single or double stranded) was analyzed by gel electrophoresis on native 4% polyacrylamide gels in Tris-borate buffer as described previously (1).

Assay of binding of RNA to the immunoprecipitated (2'-5') (A)_n synthetase in vitro. After the immunoprecipitates bound to the protein A-Sepharose CL-4B (30 μl) were washed in buffers I and II (see discussion of immunoprecipitation

above), they were resuspended in 200 μ l of buffer II. Thereafter, labeled dsRNA was added without or with specific or nonspecific competitors as indicated, and the reaction mixtures were incubated with shaking at 30°C for 30 min. To remove the unbound labeled dsRNA, the immunoprecipitates were washed five times in buffer II (1.5 ml each time), and the remaining radioactive material was eluted as described above for immunoprecipitation of proteins. The radioactivity recovered was measured after precipitation with trichloroacetic acid.

Assay of EMCV-specific RNA in the immunoprecipitates. The RNA immunoprecipitated from extracts of control or IFN-treated HeLa cells infected with EMCV was recovered as described for recovery of RNA from immunoprecipitates. The RNA was denatured, serially diluted, and applied to nitrocellulose filters, using a Minifold I apparatus (Schleicher & Schuell) according to the directions of the manufacturer. The nitrocellulose filters were baked under a vacuum at 80°C for 2 h.

Hybridization with 32 P-labeled EMCV-specific dsDNA probes. Prehybridization was performed in 50% formamide (Fluka)–5 $\times$ SSC–50 mM sodium phosphate (pH 6.8)–4 $\times$ Denhardt's solution–0.1% SDS–250 μ g of sonicated salmon sperm DNA per ml at 42°C overnight. Hybridizations were carried out under the same conditions, using 3×10^6 cpm of the nick-translated DNA probe per ml for 16 h. The probe used was a 6.0-kb insert from pEM3, a cDNA clone for EMCV provided by A. Palmenberg (21). The specific activity of the probe used was about 1.3×10^8 cpm/ μ g of DNA. After hybridization, the filters were washed in 2 $\times$ SSC–0.1% SDS at room temperature for 30 min twice and in 0.1 $\times$ SSC–0.1% SDS at 50°C for 30 min twice, dried, and visualized by autoradiography, using Hyperfilm MP (Amersham) with intensifying screens at –80°C.

Hybridization with two 32 P-labeled complementary EMCV-specific ssRNA probes. Prehybridizations were performed in 50% formamide–5 $\times$ SSC–50 mM sodium phosphate (pH 6.5)–5 $\times$ Denhardt's solution–4 mM EDTA–0.2% SDS–250 μ g of sonicated salmon sperm DNA per ml–100 μ g of yeast tRNA per ml at 55°C for 4 to 6 h. Hybridizations were carried out in 50% formamide–5 $\times$ SSC–50 mM sodium phosphate (pH 6.5)–2 $\times$ Denhardt's solution–1 mM EDTA–0.1% SDS–100 μ g of sonicated salmon sperm DNA per ml–100 μ g of yeast tRNA per ml at 55°C for 16 h. Probes for single-strand-specific hybridization were generated by insertion of a 2-kb *Pst*I fragment (corresponding to the 3' part of the EMCV insert in pEM3) (21) into the polylinker of pBluescript SK(+) and transcription of the two strands of the EMCV-specific segment with T3 and T7 RNA polymerases, respectively. The specific activity of each of the two EMCV-specific ssRNA probes was about 5×10^7 cpm/ μ g of RNA. After hybridization, the filters were washed at 55°C in 2 $\times$ SSC–0.1% SDS for 15 min twice and in 0.1 $\times$ SSC–0.1% SDS for 15 min twice, dried, and visualized by autoradiography, using Hyperfilm MP with intensifying screens at –80°C.

Characterization of the EMCV-specific RNA in the immunoprecipitates by digestion with ssRNA-specific RNase T_1 . Extracts were prepared from control or IFN-treated HeLa cells 6 h after infection with EMCV. After immunoprecipitation and recovery of RNA from the immunoprecipitates, the RNA was divided into three aliquots. The first aliquot served as an undigested control; the second and third aliquots were resuspended in 20 mM Tris-Cl (pH 7.5)–20 mM MgCl₂ and digested with the ssRNA-specific RNase T_1 . However, prior to digestion, the third aliquot was denatured

by heating at 100°C for 10 min and fast cooling on ice. The second and third RNA aliquots were each digested in 25 μ l of 20 mM Tris-Cl (pH 7.5)–20 mM MgCl₂ with 80 ng of RNase T_1 at 37°C for 30 min (i.e., essentially as described previously [29]). Under the conditions of the experiments, RNase T_1 was used in large excess; it was shown to be able to digest to completion up to 1 μ g of EMCV ssRNA. Subsequently the reaction mixtures were supplemented with 1% SDS and 2 mg of proteinase K per ml and incubated at 37°C for 30 min. After the addition of 50 μ g of yeast tRNA per ml as a carrier, the reaction mixtures were extracted twice with phenol-chloroform (1:1) and ethanol precipitated. Thereafter, RNA from each aliquot was denatured (33), serially diluted, applied to nitrocellulose filters, and probed by hybridization with 32 P-labeled EMCV-specific dsDNA probes.

RESULTS

Detection of the 40-kDa (2'-5') (A_n) synthetase in cell extracts by immunoblotting and immunoprecipitation with an antiserum. The raising of a polyclonal rabbit antiserum against a 17-amino-acid-long peptide corresponding to the C terminus of the human 40-kDa (2'-5') (A_n) synthetase was described earlier (7). In an immunoblot analysis of extracts from HeLa cells, the antiserum was bound to a 40-kDa protein. In line with the fact that this enzyme is inducible by IFN, much more of this protein was detected in an extract from cells treated with IFN than in that from control cells (Fig. 1A; compare lanes 2 and 1). The same antiserum also immunoprecipitated larger amounts of this 40-kDa protein from an extract of IFN-treated cells than from that of control cells (Fig. 1B; compare lanes 4 and 3). Though several other proteins were also immunoprecipitated, the 40-kDa protein was the only one whose amount was increased after treatment with IFN. In the same experiment, we also tested whether dsRNA bound to the 40-kDa (2'-5') (A_n) synthetase interfered with the immunoprecipitation. For this purpose, extracts from [35 S]methionine-labeled control or IFN-treated cells were first incubated with poly(I)-poly(C) and then immunoprecipitated. The dsRNA bound had no appreciable effect on the specificity or extent of the immunoprecipitation (Fig. 1B; compare lanes 6 and 5 with lanes 4 and 3). This result suggests that the immunoprecipitating antibodies in the antiserum recognize in the (2'-5') (A_n) synthetase epitopes other than those to which the dsRNA is bound.

Binding of synthetic dsRNA to the 40-kDa (2'-5') (A_n) synthetase in vitro. Labeled dsRNA was generated by annealing two complementary RNA strands which were transcribed in vitro from a 0.68-kb frog tropomyosin cDNA-specific segment of a vector. The double-stranded nature of the annealed RNA was shown by its resistance to digestion with single-strand-specific RNases (Fig. 2, lanes 3 and 4) in conditions in which one of the complementary ssRNAs was completely digested (Fig. 2, lanes 1 and 2). The slight increase in mobility resulting from this treatment of the annealed RNA with RNases A and T_1 might be the consequence of the digestion of single-stranded tails (lanes 3 and 4).

Labeled synthetic dsRNA was added to extracts from IFN-treated or control HeLa cells; after incubation (to allow for the binding of the dsRNA to the enzyme), the reaction mixtures were UV irradiated (to cross-link the RNA to the enzyme protein), and immunoprecipitation was performed with the antiserum against the 40-kDa (2'-5') (A_n) syn-

FIG. 1. Immunoblotting and immunoprecipitation with the anti-serum against the 40-kDa (2'-5') (A)_n synthetase, showing that poly(I)-poly(C) does not impair the immunoprecipitation. (A) Detection of the 40-kDa (2'-5') (A)_n synthetase by immunoblotting. Extracts from control (lane 1) or IFN-treated (lane 2) HeLa cells were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. The blot was incubated first with the antiserum to the 40-kDa (2'-5') (A)_n synthetase and subsequently with ¹²⁵I-labeled protein A and was visualized by autoradiography. The position of the 40-kDa (2'-5') (A)_n synthetase is indicated by an arrow. (B) Immunoprecipitation of [³⁵S]methionine-labeled proteins from control or IFN-treated HeLa cells. Extracts from control (lanes 3 and 5) or IFN-treated (lanes 4 and 6) HeLa cells without (lanes 3 and 4) or with (lanes 5 and 6) supplementation with 100 µg of poly(I)-poly(C) per ml were incubated at 30°C for 30 min and thereafter immunoprecipitated with the antiserum to the 40-kDa (2'-5') (A)_n synthetase. Immunocomplexes were recovered by using protein A-Sepharose, washed, analyzed by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography. M, Molecular size markers (phosphorylase b, 92.5 kDa; bovine serum albumin, 68 kDa; ovalbumin, 46 kDa; and carbonic anhydrase, 30 kDa). The position of the 40-kDa (2'-5') (A)_n synthetase is indicated by an arrow. For further details, see Materials and Methods.

thetase. The RNA was recovered from the immunoprecipitate (by a procedure involving digestion of the protein by proteinase K, phenol extraction, and ethanol precipitation) and analyzed by gel electrophoresis and autoradiography. (The UV cross-linking increased the recovery of dsRNA in the immunoprecipitate fourfold [not shown].)

The results reveal that 5- to 10-fold more dsRNA was immunoprecipitated from an extract of IFN-treated cells (Fig. 2B, lane 6) than from an extract of control cells (lane 5). This was the case irrespective of whether the incubation of the dsRNA with the cell extracts was performed at 4°C (lanes 5 and 6) or 30°C (lanes 11 and 12). The RNA recovered from the immunoprecipitates gave rise to a smear upon gel electrophoresis (lanes 5, 6, 10, 11, and 12), indicating that it was partially degraded. The fact that incubation with RNases A and T₁ did not result in much further degradation (lane 13) supports the assumptions that (i) the bulk of the RNA recovered remained double stranded and (ii) the enzymes cleaving it in the cell extracts could attack dsRNA. This conclusion is in line with the result (lanes 9 and 10) indicating that an inhibitor (Inhibit-Ace) expected to inactivate a variety of ssRNA-specific RNases did not protect the RNA from degradation. Heat treatment (at 60°C for 10 min) of the extracts prior to incubation with dsRNA destroyed the

FIG. 2. Binding of synthetic dsRNA to the 40-kDa (2'-5') (A)_n synthetase in vitro, immunoprecipitation of an enzyme-dsRNA complex, and binding of dsRNA to the immunoprecipitated enzyme. (A) Characterization of the in vitro-synthesized dsRNA by treatment with RNases and electrophoresis in native 4% polyacrylamide gels. Lanes: 1, labeled ssRNA (transcribed from a tropomyosin cDNA clone); 2, ssRNA after treatment with RNases A and T₁; 3, dsRNA (produced by annealing two complementary single-stranded transcripts); 4, dsRNA after treatment with RNases A and T₁. The RNA samples were analyzed by gel electrophoresis and autoradiography. (B) Immunoprecipitation of dsRNA incubated with the HeLa cell extracts with the antiserum against the 40-kDa (2'-5') (A)_n synthetase. Labeled synthetic dsRNA corresponding to that in lane 4 of panel A was incubated with extracts from control (lanes 5, 7, 9, and 11) or IFN-treated (lanes 6, 8, 10, and 12) HeLa cells for 30 min: at 4°C (lanes 5 and 6), at 30°C in the presence of an RNase inhibitor (lanes 9 and 10), or at 30°C in the absence of the inhibitor (lanes 11 and 12). The extracts (lanes 7 and 8) were heat inactivated prior to the incubation with the dsRNA at 4°C for 30 min. After incubation, the reaction mixtures were UV irradiated (to cross-link the RNA to protein) and immunoprecipitated with the antiserum. The bound, labeled RNA was recovered from the immunoprecipitates and analyzed by gel electrophoresis and autoradiography. Half of the material eluted after an incubation with an extract of IFN-treated cells at 30°C for 30 min (as in the case of the material in lane 12) was subjected to digestion with RNases A and T₁ before analysis by gel electrophoresis and autoradiography (lane 13). (C) Binding of labeled synthetic dsRNA to the immunoprecipitated 40-kDa (2'-5') (A)_n synthetase. The synthetase was immunoprecipitated from control (lane 14) or IFN-treated (lane 15) HeLa cells. Labeled dsRNA was added to the immunoprecipitates, and the reaction mixtures were incubated at 30°C for 30 min. dsRNA bound to protein A-Sepharose (in the absence of cell extract) (lane 16). After washing the immunoprecipitates, the remaining bound radioactive material was recovered and analyzed by electrophoresis and autoradiography. The amount of labeled dsRNA added to each incubation mixture in panels B and C was 100 ng, the cell extracts were heat inactivated by incubation at 60°C for 10 min (lanes 7 and 8), and the RNase inhibitor used was Inhibit-Ace at 1 U/30 µl (lanes 9 and 10). For further details, see Materials and Methods.

dsRNA-binding ability of the enzyme (lanes 7 and 8; compare with lane 16). It was also established that preimmune serum was incapable of precipitating dsRNA in complex with the enzyme (not shown).

The ability of immunoprecipitated enzyme to bind dsRNA was also tested. The enzyme immunoprecipitated from an

FIG. 3. Binding of labeled, synthetic dsRNA to the immunoprecipitated 40-kDa (2'-5') (A_n) synthetase in vitro, showing that unlabeled dsRNA competes for binding whereas ssRNA does not. The reaction mixtures contained immunoprecipitated 40-kDa (2'-5') (A_n) synthetase from IFN-treated HeLa cells and 100 ng of labeled dsRNA per ml, together with unlabeled dsRNA at the concentrations indicated. After incubation at 30°C for 30 min, the immunoprecipitates were washed and the bound, labeled dsRNA was eluted. The RNA in the eluate was precipitated with trichloroacetic acid and counted in a liquid scintillation counter. The amount of labeled dsRNA bound to an immunoprecipitate from control cells was 2,340 cpm, whereas that bound to the immunoprecipitate from IFN-treated cells was 6,530 cpm. In the same experiment, unlabeled ssRNA generated by transcription in vitro with T7 RNA polymerase did not inhibit the binding of labeled dsRNA when tested at concentrations up to 100 $\mu\text{g/ml}$. For further details, see Materials and Methods.

extract of IFN-treated cells bound much more dsRNA than did that precipitated from an extract of control cells (Fig. 2C, lanes 14 and 15). Moreover, immunoprecipitation of the enzyme (prior to incubation with dsRNA) appeared to have removed much of the associated dsRNA-degrading activity; thus, dsRNA recovered from binding to the (2'-5') (A_n) synthetase in the crude extracts (i.e., dsRNA incubated with the enzyme in the crude extract and subsequently immunoprecipitated) gave rise to a broad smear upon gel electrophoresis (Fig. 2B, lanes 5, 6, 9, 10, 11, and 12), whereas dsRNA recovered from binding to the immunopurified (2'-5') (A_n) synthetase [i.e., (2'-5') (A_n) synthetase first immunoprecipitated from the cell extract and only subsequently incubated with dsRNA] gave rise to a narrower band in gel electrophoresis (Fig. 2C, (lane 15). The band was actually not much broader than that obtained with the dsRNA preparation used in the binding studies (Fig. 2A, lane 4). The nonspecific binding background of these experiments (i.e., binding to the antiserum protein A-Sepharose complex in the absence of cell extracts) was low (Fig. 2C, lane 16).

Characteristics of the binding of labeled dsRNA to the immunoprecipitated 40-kDa (2'-5') (A_n) synthetase. Unlabeled (tropomyosin-specific) dsRNA competed with labeled (tropomyosin-specific) dsRNA for binding to the (2'-5') (A_n) synthetase immunoprecipitated from an extract of IFN-treated HeLa cells. More than 50% inhibition of the binding of the labeled dsRNA was caused by unlabeled dsRNA at a concentration of 1 $\mu\text{g/ml}$ (Fig. 3). In the same condition, unlabeled tropomyosin-specific ssRNA did not inhibit the binding even at a concentration as high as 100 $\mu\text{g/ml}$ (not

shown). These results indicate that the RNA-binding activity of the immunoprecipitated enzyme is specific for dsRNA.

For a more quantitative assessment of the dsRNA-binding characteristics of the immunoprecipitated enzyme, the dependence of binding on the concentration of labeled dsRNA was tested (Fig. 4A). The Scatchard plot of the data (Fig. 4B) is consistent with the existence on the 40-kDa (2'-5') (A_n) synthetase of a single class of high-affinity dsRNA-binding sites with a calculated apparent K_d of about 8×10^{-10} M. This value was obtained by using a synthetic dsRNA corresponding in sequence to a 680-bp-long segment of tropomyosin cDNA.

The antiserum against the 40-kDa (2'-5') (A_n) synthetase immunoprecipitates, from an extract of IFN-treated and EMCV-infected cells, viral RNA together with the 40-kDa (2'-5') (A_n) synthetase protein. Finding that the antiserum to the 40-kDa (2'-5') (A_n) synthetase immunoprecipitated, together with the enzyme, the RNA that had been bound to the enzyme in vitro enabled us to test a long-standing hypothesis. According to this view, the agent activating the (2'-5') (A_n) synthetase in IFN-treated, EMCV-infected cells is viral RNA. After a series of experiments devoted to optimizing conditions, the test was performed in the following way. HeLa cell monolayers were exposed to 1,000 U of IFN- α per ml for 16 h, infected with EMCV at a multiplicity of infection of 2, and incubated for 2, 4, or 6 h to allow for RNA replication to take place. Thereafter, the infected cell monolayers were UV irradiated to cross-link the (2'-5') (A_n) synthetase-bound viral RNA to the (2'-5') (A_n) synthetase. The preliminary experiments revealed that such irradiation

FIG. 4. Binding of labeled synthetic dsRNA to the immunoprecipitated 40-kDa (2'-5') (A)_n synthetase in vitro, dependence on the concentration of dsRNA, and Scatchard analysis. (A) The 40-kDa (2'-5') (A)_n synthetase was immunoprecipitated from extracts of IFN-treated HeLa cells, and the immunoprecipitates were incubated with increasing concentrations of labeled tropomyosin-specific dsRNA. After 30 min at 30°C they were washed, and the residual labeled RNA was eluted, precipitated with trichloroacetic acid, and counted in a liquid scintillation counter. (B) Scatchard analysis of the data. B, Bound counts per minute; F, free counts per minute. For further details, see Materials and Methods.

greatly (up to fourfold) increased the recovery in the immunoprecipitate of dsRNA that was bound to the (2'-5') (A)_n synthetase in vitro. After preparation of cell extracts and immunoprecipitation, the RNA was recovered from the immunoprecipitates, applied to nitrocellulose filters, and hybridized with an EMCV-specific, labeled dsDNA probe. The specificity of the hybridization assay is supported by the finding that the EMCV-specific probe did not hybridize to RNA in immunoprecipitates from uninfected control or IFN-treated cells (Fig. 5, rows 1 and 2). No virus-specific RNA was detected in the immunoprecipitates from either IFN-treated or control cells 2 h after infection (rows 3 and 4). Virus-specific RNA was, however, detected in immunoprecipitates from cells infected for 4 h (rows 5 and 6). Immunoprecipitates from cells infected for 6 h (rows 7 and 8) contained more viral RNA than did immunoprecipitates from cells infected for 4 h. Furthermore, immunoprecipitates from cells that had been treated with IFN prior to infection contained much (about five times) more viral RNA than did immunoprecipitates from infected cells that had not been treated with IFN (compare row 8 with row 7 and row 6 with row 5). This is presumably a consequence of the increase in the amount of the 40-kDa (2'-5') (A)_n synthetase in IFN-treated cells.

The addition of a very large excess of dsRNA [500 μg of poly(I)-poly(C) per ml] prior to preparation of the cell extract did not decrease the amount of viral RNA in the immunoprecipitate (Fig. 5; compare rows 9 and 10 with rows 8 and 7). This finding makes it most unlikely that viral RNA binding to the (2'-5') (A)_n synthetase occurred after cell lysis.

The fact that the 40-kDa (2'-5') (A)_n synthetase binds dsRNA but not ssRNA with high affinity (Fig. 3) prompted us to test whether both plus and minus EMCV RNA strands are present in the immunoprecipitate. For this test, plus- and minus-strand viral ssRNA probes were prepared by in vitro

transcription of EMCV cDNA. Hybridization with the plus-strand probe (Fig. 6A) and the minus-strand probe (Fig. 6B) revealed the presence of both viral strands (plus and minus) in the immunoprecipitates. More of the viral strands were present in immunoprecipitates from cells 6 h postinfection than in those from cells 4 h postinfection (compare rows 4 and 3 with rows 2 and 1), and more were present in immunoprecipitates from IFN-treated cells than in those from control cells (compare rows 4 and 2 and rows 3 and 1). Finally, as expected (and revealed by densitometry), the amount of viral plus-strand RNA in the immunoprecipitates was much (3.5-fold) higher than that of viral minus-strand RNA (compare the rows in Fig. 6B with the corresponding rows in Fig. 6A).

To test whether the two complementary viral RNA strands were at least partially annealed, the immunoprecipitates were treated with the ssRNA-specific RNase T₁. After deproteinization, approximately 10% of the RNA recovered from the immunoprecipitates was resistant to digestion with RNase T₁. After denaturation, however, as expected, all of the recovered RNA could be cleaved by the enzyme.

These results are in line with the possibility that at least some of the viral RNA in complex with the (2'-5') (A)_n synthetase was double stranded in vivo. It is conceivable, however, that the proportion of EMCV RNA present in a double-stranded form in vivo was less than 10%. This is the case since the deproteinization may have extended the annealed regions of complementary strands by removing protein that blocked the annealing (see also Discussion).

The (2'-5') (A)_n synthetase-viral RNA complex immunoprecipitated from IFN-treated cells which had been infected with EMCV is enzymatically active even if not supplemented with further dsRNA. The ability of the immunoprecipitated 40-kDa (2'-5') (A)_n synthetase to bind dsRNA in vitro and the presence of both viral RNA strands (presumably partially double stranded) in the immunoprecipitate from IFN-

FIG. 5. Evidence that the antiserum against the 40-kDa (2'-5') (A_n) synthetase immunoprecipitates viral RNA together with the (2'-5') (A_n) synthetase from an extract of IFN-treated HeLa cells that had been infected with EMCV. Control or IFN-treated HeLa cells were mock infected or infected with EMCV and incubated for 2, 4, or 6 h. Thereafter, the cell monolayers were washed and UV irradiated, and cell lysates were prepared. After immunoprecipitation with the antiserum against the 40-kDa (2'-5') (A_n) synthetase, the coprecipitated RNA was eluted and purified, and serial twofold dilutions were spotted onto nitrocellulose filters. The filters were hybridized to a nick-translated EMCV-specific dsDNA probe and visualized by autoradiography. Shown are results for hybridization of labeled EMCV DNA probes to RNA from an immunoprecipitate of an extract from control cells mock infected (row 1); IFN-treated cells mock infected (row 2); control cells infected with EMCV and incubated for 2 h (row 3), 4 h (row 5), and 6 h (row 7); IFN-treated cells infected with EMCV and incubated for 2 h (row 4), 4 h (row 5), and 6 h (row 8); control cells infected with EMCV and incubated for 6 h (row 9); and IFN-treated cells infected with EMCV and incubated for 6 h (row 10). Prior to preparation of the cell extracts for the experiments shown in rows 9 and 10, the cell cultures were supplemented with a large excess of poly(I)-poly(C) (500 μ g/ml). For further details, see Materials and Methods.

treated, EMCV-infected cells prompted tests on the enzymatic activity of the immunoprecipitated enzyme. The addition of poly(I)-poly(C) to the immunoprecipitate from uninfected cells increased the (2'-5') (A_n) synthetase activity from the basal, essentially undetectable level at least 25-fold (Table 1). It is in line with inducibility of the (2'-5') (A_n) synthetase by IFN that after the addition of poly(I)-poly(C), the activity of the enzyme immunoprecipitated from IFN-treated but uninfected cells was three- to fourfold higher than that from control uninfected cells. The (2'-5') (A_n) synthetase activity of an immunoprecipitate from cells infected for 3 h was appreciable only if the extract was prepared from IFN-treated cells. However, the immunoprecipitate from cells infected with EMCV for 5 h had pronounced (2'-5') (A_n) synthetase activity. In the case of cells not treated with IFN, this level was twice as high as in uninfected cells; in the case of IFN-treated cells, it was five to six times as high as in uninfected cells. Since these (2'-5') (A_n) synthetase activities were detected in assays not supplemented with dsRNA, these observations make it likely that activation of the enzyme resulted from binding of the viral RNA. It should be noted that, at least as tested in the immunoprecipitate, the enzyme from EMCV-infected cells was not fully activated; the addition of dsRNA [poly(I)-poly(C)] to the reaction mixture resulted in an approximately 20-fold-higher activity. The specificity of the assay

system is supported by the data showing that the nonspecific background of the (2'-5') (A_n) synthetase assay (when pre-immune serum was used for precipitation) was low (only 20% of that obtained in experiments with the antiserum) (Table 1).

DISCUSSION

As outlined in the introduction, there is compelling evidence for the ability of the 40-kDa (2'-5') (A_n) synthetase to

FIG. 6. Presence of both RNA strands in the viral RNA-40-kDa (2'-5') (A_n) synthetase complex immunoprecipitated by the antiserum against the synthetase from an extract of IFN-treated HeLa cells that had been infected with EMCV; assay for possibly double-stranded regions. (A and B) Control or IFN-treated cells were infected with EMCV and incubated for 4 or 6 h. Thereafter, the monolayers were washed and UV irradiated, and extracts were prepared. After immunoprecipitation with the antiserum, the coprecipitated RNA was recovered. Serial twofold dilutions of the RNA were spotted onto a nitrocellulose filter, which was first hybridized with the labeled EMCV plus-strand ssRNA probe that anneals to the viral minus RNA strand (A). The probe was then removed, and the filter was hybridized with the EMCV minus-strand RNA probe that anneals to the viral plus RNA strand (i.e., virion RNA) (B). Shown are results for hybridization of a labeled EMCV ssRNA probe to RNA from an immunoprecipitate of an extract from control cells infected with EMCV and incubated for 4 h (row 1) or 6 h (row 3) and from IFN-treated cells infected with EMCV and incubated for 4 h (row 2) or 6 h (row 4). The filters were visualized by autoradiography. (C) Control or IFN-treated cells were infected with EMCV and incubated for 6 h. Thereafter, the monolayers were washed and UV irradiated, and extracts were prepared. After immunoprecipitation with the antiserum, the coprecipitated RNA was recovered and divided into three aliquots. The first served as an undigested control; the second and third were digested with ssRNA-specific RNase T_1 . Prior to the digestion, the third aliquot was denatured. After processing, including denaturation, serial twofold dilutions of the aliquots were applied to filters and hybridized with a 32 P-labeled EMCV-specific dsDNA probe and visualized by autoradiography. Shown are results of hybridization of a probe to RNA from an immunoprecipitate of an extract from control cells infected with EMCV and incubated for 6 h (rows 1, 3, and 5) and from IFN-treated cells infected with EMCV and incubated for 6 h (rows 2, 4 and 6). Prior to application to the filters, the RNA samples in rows 3 and 4 were digested with RNase T_1 ; those in rows 5 and 6 were first denatured and subsequently digested with RNase T_1 . For further details, see Materials and Methods.

TABLE 1. (2'-5') (A)_n synthetase activity in immunoprecipitates from control or IFN-treated, mock- or EMCV-infected HeLa cells^a

No.	Treatment of cells from which the extract was prepared			Antiserum used for immunoprecipitation	Addition of poly(I)-poly(C) (100 µg/ml) to extract	(2'-5') (A) _n accumulated (cpm) ^b	
	IFN	EMCV infection	Time (h) postinfection			Expt 1	Expt 2
1	—	—		A	+	2,452	3,365
2	+	—		A	+	10,074	11,571
3	—	+	3	A	—	120	131
4	+	+	3	A	—	272	234
5	—	+	5	A	—	185	212
6	+	+	5	A	—	506	580
7	—	+	5	P	—	117	102
8	+	+	5	P	—	102	111

^a Cells were grown, treated with IFN, and infected with EMCV (if so indicated), and cell extracts were prepared (in the case of EMCV-infected cells, 3 or 5 h after infection, as indicated). Immunoprecipitation with antiserum to (2'-5') (A)_n synthetase (A) or preimmune serum (P) was performed, and (2'-5') (A)_n synthetase was assayed. If so indicated, the reaction mixtures for the enzyme assay were supplemented with 100 µg of poly(I)-poly(C) per ml. The results of two independent experiments are presented. For further details, see Materials and Methods.

^b (2'-5') (A)_n accumulation was 108 cpm for uninfected control cells and 91 cpm for IFN-treated uninfected cells when the extracts were tested without addition of poly(I)-poly(C) and were immunoprecipitated with an antiserum to (2'-5') (A)_n synthetase.

impair the replication of EMCV (3; see also reference 24). Furthermore, there is strong support for the involvement in this impairment of both (2'-5') (A)_n, the product of the activated (2'-5') (A)_n synthetase, and RNase L, the latent RNase that is activated by (2'-5') (A)_n (11, 31). The aim of this study has been to test a long-standing hypothesis according to which the agent activating the latent (2'-5') (A)_n synthetase in EMCV-infected cells is the replicating viral RNA.

A polyclonal rabbit antiserum raised against a C-terminal peptide of the 40-kDa human (2'-5') (A)_n synthetase (7) immunoprecipitated several proteins from HeLa cells. Of these proteins, however, only one was inducible by IFN: the 40-kDa (2'-5') (A)_n synthetase. The immunoprecipitation was not impaired by dsRNA complexed to the enzyme, and the immunoprecipitated 40-kDa (2'-5') (A)_n synthetase bound dsRNA with high affinity and selectivity. The apparent K_d for dsRNA was about 8×10^{-10} M (as determined in experiments with a 680-bp-long dsRNA segment), and ssRNA, even if tested at a 1,000-fold excess, did not compete for binding.

The same antiserum was used to immunoprecipitate the (2'-5') (A)_n synthetase from an extract of EMCV-infected cells. Hybridization of the washed immunoprecipitate with an EMCV-specific probe revealed the presence of virus-specific RNA. Such RNA was detectable 4 h after infection, and its amount increased by 6 h after infection. Furthermore, treatment of the cell culture with IFN prior to infection greatly increased the amount of EMCV-specific RNA in the immunoprecipitate, presumably in consequence of increasing the level of (2'-5') (A)_n synthetase in the cells. The immunoprecipitate contained both plus and minus EMCV RNA strands. The amount of plus strands (corresponding to virion RNA) was 3.5 times higher than that of minus strands (complementary to virion RNA). This excess of plus strands over minus strands in the immunoprecipitate may reflect a similar excess of plus strands over minus strands in the course of EMCV replication (10).

A large excess of dsRNA [poly(I)-poly(C)] added to the culture before preparation of the cell extract did not diminish the amount of viral RNA in the immunoprecipitates. This makes it unlikely that viral RNA binding to the (2'-5') (A)_n synthetase was an artifact which occurred only after cell lysis. These findings demonstrate that both complementary

strands of viral RNA were bound to the 40-kDa (2'-5') (A)_n synthetase in EMCV-infected cells.

The activation of (2'-5') (A)_n synthetase in a HeLa cell extract was reported to require RNA containing an uninterrupted double-stranded region of 50 bp or longer (15), and maximal activation of a purified 100-kDa murine (2'-5') (A)_n synthetase required an approximately 80-bp-long dsRNA segment per enzyme molecule (25; see also reference 8). However, the (2'-5') (A)_n synthetase in EMCV-infected cells, at least as tested in the immunoprecipitate, was only partially activated (at 5% of the maximal level). This makes it likely that even shorter dsRNA regions occurring in intermediates or side products of EMCV RNA replication might suffice to elicit this low level of activity.

The presence of both EMCV RNA strands in the immunoprecipitate, together with the fact that in the course of replication, each strand serves as a template for the synthesis of the other strand, makes it conceivable that at least some of the strands are present in vivo in the form of partially double-stranded complexes. We have assayed for viral dsRNA in the immunoprecipitate by digesting the deproteinized RNA with the ssRNA-specific RNase T₁ and hybridizing the RNA remaining after the digestion. This corresponded to approximately 10% of the RNA hybridizable before the digestion. This value might, however, be an overestimate resulting from the annealing of regions of complementary strands upon removing proteins which blocked the annealing.

As revealed by electron microscopic examination, the extent of double-stranded regions in replicating EMCV RNA is slight (30). The occurrence of double-stranded regions in this RNA is, however, supported by the finding that a psoralen derivative (4'-aminomethyl-4,5',8-trimethylpsoralen) known to intercalate into double-stranded nucleic acids does cross-link complementary EMCV RNA strands in infected cells upon UV irradiation (10, 19).

Further studies on the structures of the EMCV RNA in complex with the (2'-5') (A)_n synthetase in vivo might reveal whether the EMCV RNA homopolymeric [poly(C)] segments (shown to affect the pathogenicity of the virus) (5) or the so-called replicative form of EMCV RNA (thought to have extended double-stranded regions and occasionally covalent linkages between complementary strands) (10) con-

tributes to the activation of (2'-5') (A)_n synthetase by EMCV RNA.

The data presented in this study indicate that the 40-kDa (2'-5') (A)_n synthetase immunoprecipitated from a culture that had been infected with EMCV was clearly active in synthesizing (2'-5') (A)_n (even when tested without the addition of dsRNA), whereas the (2'-5') (A)_n synthetase immunoprecipitated from an uninfected culture was not. This enzymatic activity of the immunoprecipitate from EMCV-infected cells, together with the results revealing the binding of viral RNA to the 40-kDa (2'-5') (A)_n synthetase in EMCV-infected cells, strongly supports the hypothesis that the agent activating the (2'-5') (A)_n synthetase *in vivo* is the viral RNA.

In conclusion, it should also be noted that there are reports on the presence of (2'-5') (A)_n in uninfected cells (6, 20). These reports suggest that (2'-5') (A)_n synthetases may have functions other than mediating the impairment of virus replication by IFNs (13, 18, 24). An examination of immunoprecipitates of the various (2'-5') (A)_n synthetase isoenzymes from uninfected cells for associated RNA may shed some light on the further functions of these enzymes.

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