(2'-5')(A)n synthetase is one of the mediators of interferon action. If activated by double-stranded RNA it converts ATP into pyrophosphate and (2'-5')(A)n. In turn, (2'-5')(A)n activates a latent endoribonuclease (RNase L) which cleaves single-stranded RNA. We report here the isolation and characterization of a homogeneous human (2'-5')(A)n synthetase. The enzyme was purified from interferon-treated HeLa S3 cells by chromatography of a ribosomal salt wash fraction on DEAE-cellulose, poly(I)-poly(C) agarose, and CM-cellulose. The purified (2'-5')(A)n synthetase can convert over 90% of ATP into (2'-5')(A)n. The enzyme is unstable but can be stabilized by certain nonionic detergents (e.g., Triton X-100). Its apparent M, = 100,000, as determined by gel electrophoresis in sodium dodecyl sulfate, and about 80,000, as determined by centrifugation through a glycerol gradient. The human (2'-5')(A)n synthetase is similar to the corresponding enzyme from mouse Ehrlich ascites tumor cells, but differs from the latter in size (100,000 versus 105,000 daltons) and in ionic conditions required for maximal activity.

Interferons are proteins produced by a large variety of vertebrate cells in response to viral infection or some other stimuli. They are secreted, bound to other cells, and alter the biochemical and immunological characteristics of these in various ways (1, 2). The exposure of cells to interferons results in the enhanced accumulation of certain messenger RNAs (3-5) and proteins (6-10), and an increase in the level of various enzyme activities. Some of these enzymes (e.g., a protein kinase and an endonuclease system) remain latent unless activated by double-stranded RNA and ATP (for reviews see Refs. 11 and 12).

Earlier, the endonuclease system (13) was divided into two complementary fractions (14). One of these fractions, when incubated with dsRNA1 and ATP gave rise to a small ther-

2'5-linked pppA(pA),, where n is between 2 and 10; EAT, Ehrlich ascites tumor; SDS, sodium dodecyl sulfate.

(2'-5')(A)n synthetase has been detected in mouse EAT and L-cells, human HeLa cells (14, 15, 18, 19), and chick embryo fibroblasts treated with interferon (20), and in tissue homogenates and sera of mice treated with interferon (21). The increase in the level of (2'-5')(A)n synthetase activity after treatment with interferon was found to be preceded by an increase in a messenger RNA that could be translated into the enzyme in Xenopus oocytes (5).

(2'-5')(A)n synthetase was also found in rabbit reticulocytes (22) and in mouse lymphocytes and other lymphoid tissues, even if not previously exposed to interferon (23). The level of the enzyme was reported to increase also in response to agents other than interferon: e.g., in chick oviducts after withdrawal of estrogens (24), and in human lymphoblastoid cells after treatment with glucocorticoids (25).

(2'-5')(A)n synthetase has been partially purified from chick embryo fibroblasts by conventional procedures (26) and from L-cells and rabbit reticulocytes by affinity chromatography on dsRNA (27, 28). We have isolated earlier homogeneous (2'-5')(A)n synthetase from interferon-treated mouse EAT cells (29). As determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, the apparent molecules weight of the mouse enzyme is 105,000, and as determined by centrifugation through a glycerol gradient it is 85,000.

The stoichiometry of the reaction catalyzed by the enzyme is (n + 1)ATP → pppA(pA)n+1 + n PP. Although PP is not cleaved the equilibrium of the reaction is strongly in favor of synthesis. At least 97% of the ATP is converted to (2'-5')(A)n (30). The synthesis of (2'-5')(A)n, by the purified enzyme depends on the presence of dsRNA (29).

The need for pure human (2'-5')(A)n synthetase for purposes of cloning, chromosomal gene mapping, and generating antibodies, prompted us to isolate the human enzyme. We report here a simple procedure for the isolation of apparently pure human (2'-5')(A)n synthetase from interferon-treated HeLa cells with a 25% yield. The human (2'-5')(A)n synthetase is similar to the corresponding mouse enzyme in several aspects. However, the two enzymes differ in molecular weight and in conditions for maximal activity.²

² Some of these findings were presented at the First Annual International Congress for Interferon Research, Washington, D.C., November, 1980.
Isolation of Human (2'-5')(A), Synthetase

Purification of (2'-5')(A), Synthetase

HeLa S3 cells were grown in a stirred suspension culture at 37 °C in minimum essential medium (Joklik modified; Grand Island Biological Co.) supplemented with 7% (v/v) calf serum. The starting cell density was 2 × 10^6 cells/ml. When the density reached 3 × 10^6 cells/ml the culture was supplemented with 300 mouse NIH reference standard units of interferon/ml; 24 h later the cells were harvested, disrupted, and a low speed supernatant fraction (S 30) was prepared following procedures described for preparing S 30 from EAT cells (53). Fifty ml of the S 30 obtained from 40 liters of cell suspension were centrifuged at 200,000 × g for 2 h. The pellet fraction (ribosomes) was resuspended in 4 volumes of minimum essential medium (Joklik modified; Grand Island Biological Co.) supplemented with 7% (v/v) calf serum. The starting cell suspension was suspended in 8 ml of buffer A (17 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.5), 30 mM β-mercaptoethanol, 4 mM Mg(OAc)₂, 1 mM EDTA, 10% (v/v) glycerol, 10 μM phenylmethylsulfonyl fluoride) containing 250 mM KOAc, stirred overnight, and resedimented by centrifugation at 200,000 × g for 2 h. The resulting supernatant fraction (12 ml; ribosomal wash fraction) was diluted by adding 9 volumes of buffer A and centrifuging at 10,000 × g for 10 min. The resulting supernatant fraction was passed through a 12-ml DEAE-cellulose column (Whatman DE-52) equilibrated with buffer B (10 mM Tris·Cl (pH 8.3), 30 mM β-mercaptoethanol, 4 mM Mg(OAc)₂, 1 mM EDTA, 10% (v/v) glycerol, 10 μM phenylmethylsulfonyl fluoride) containing 25 mM KOAc. The protein was purified by affinity chromatography, and autoradiography were as reported earlier (29,30). For the isolation procedure developed earlier that mouse interferon C (although mouse interferon A, a β type interferon) could induce (2'-5')(A), synthetase in HeLa cells (32). The level of induction using mouse interferon C (500 units/ml) was about 6-fold (32), 30% of that using a human leukocyte interferon preparation at a concentration (1000 units/ml) giving maximal induction (not shown).

To increase the yield of human enzyme we improved the isolation procedure developed earlier for the mouse enzyme (29) in several ways. The new procedure avoids dialysis entirely and requires only one gradient elution. The first step in the procedure is a differential wash of the ribosomal pellet, first with a low salt solution (including inactivation of the enzyme by heating, treatment with hexokinase to convert unreacted ATP to ADP, thin layer chromatography, and autoradiography) and then with a high salt solution (including 500 mM KOAc). Most of the enzyme is present in the high salt wash. This is passed through a DEAE-cellulose column and the resulting flow through fraction is further purified by affinity chromatography on poly(I)-poly(C) agarose (Table 1). This step, which was first used by Hovanessian and Kerr (27) for this purpose, produces an enzyme preparation consisting of about seven proteins (as revealed by acrylamide gel electrophoresis in the presence of SDS; Fig. 1, track 1). Six of these proteins are eliminated during the last step of the isolation procedure, chromatography by salt gradient elution on CM-cellulose.

RESULTS AND DISCUSSION

Isolation of (2'-5')(A), Synthetase—We have induced (2'-5')(A), synthetase in HeLa cells by treatment with a mouse interferon C (an α type interferon) preparation (31). Human interferon was not available to us in the amounts required for the project, and we have established earlier that mouse interferon C (although not mouse interferon A, a β type interferon) could induce (2'-5')(A), synthetase in HeLa cells (32). The level of induction using mouse interferon C (500 units/ml) was about 6-fold (32), 30% of that using a human leukocyte interferon preparation at a concentration (1000 units/ml) giving maximal induction (not shown).

To increase the yield of human enzyme we improved the isolation procedure developed earlier for the mouse enzyme (29) in several ways. The new procedure avoids dialysis entirely and requires only one gradient elution. The first step in the procedure is a differential wash of the ribosomal pellet, first with a low salt solution (including 250 mM KOAc), then with a high salt solution (including 500 mM KOAc). Most of the enzyme is present in the high salt wash. This is passed through a DEAE-cellulose column and the resulting flow through fraction is further purified by affinity chromatography on poly(I)-poly(C) agarose (Table 1). This step, which was first used by Hovanessian and Kerr (27) for this purpose, produces an enzyme preparation consisting of about seven proteins (as revealed by acrylamide gel electrophoresis in the presence of SDS; Fig. 1, track 1). Six of these proteins are eliminated during the last step of the isolation procedure, chromatography by salt gradient elution on CM-cellulose.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Poly(I)-poly(C) paper assay (nmol AMP polymerized/mg protein/h)</th>
<th>Yield of activity</th>
<th>Purification fold</th>
</tr>
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<tbody>
<tr>
<td>S 30*</td>
<td>1,600</td>
<td>1.2</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Riboasomal wash</td>
<td>131</td>
<td>9.7</td>
<td>100</td>
<td>68</td>
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<tr>
<td>DEAE-cellulose</td>
<td>45</td>
<td>480</td>
<td>112*</td>
<td>40</td>
</tr>
<tr>
<td>Poly(I)-poly(C) aggrose</td>
<td>0.9</td>
<td>9,500*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>0.14</td>
<td>34,000</td>
<td>25,2,800</td>
<td></td>
</tr>
</tbody>
</table>

*The poly(I)-poly(C) paper assay has been used to determine the enzyme activity. No solvent purification can be tested by the in solution assay.

**The increase in total activity after chromatography on DEAE-cellulose is thought to be due to removal of inhibitors of (2'-5') (A), synthetase and of enzymes degrading (2'-5') (A). For details see "Experimental Procedure."
Isolation of Human (2'-5')A₅, Synthetase

The purification of the enzyme is about 2800-fold with an overall yield of about 25% (Table I).

The M₀ of the protein eluted from CM-cellulose is 100,000, as revealed by gel electrophoresis in SDS in the presence of size markers, followed by staining with Coomassie brilliant blue (Fig. 1, tracks 2 and 3), or labeling with ¹²⁵I followed by gel electrophoresis in SDS and radioautography (Fig. 1, track 5). (For technical reasons, only a small amount of the purified enzyme preparation was analyzed by gel electrophoresis and staining. Thus, a small proportion of contaminating proteins, if these were resistant to labeling with ¹²⁵I, could have been missed.)

The identity of the (2'-5')A₅ synthetase with the M₀ = 100,000 protein is supported by the fact that the enzyme activity and the M₀ = 100,000 protein co-chromatograph on CM-cellulose (Fig. 2, left) and co-sediment in a glycerol density gradient (Fig. 2, right). The apparent M₀ of the enzyme under nondenaturing conditions is 80,000, as determined by comparison of the sedimentation velocity of the enzyme with those of marker proteins during the glycerol density gradient centrifugation. This result indicates that at least prior to binding to dsRNA, the enzyme is monomeric.

**Kinetics of (2'-5')A₅, Synthetase: Optimization of the Reaction Conditions**—The curves in Fig. 3 (left) reveal that under the conditions of the incubation the rate of (2'-5')A₅, synthesis decreases with time. This is apparently a consequence of the inactivation of the purified enzyme during incubation, especially when used at low concentrations. In consequence of this inactivation and the need for the formation of sufficient amounts of (2'-5')A₅ for precise assay, we optimized the composition of the reaction mixture for the following conditions. Synthesis from 1 mM ATP with 5.6 pg/ml (2'-5')A₅ synthetase were concentrated to 200 µl by ultrafiltration (using CF50 ultrafiltration membrane) and centrifuged in the Beckman SW 50.1 rotor at 48,000 rpm (210,000 × g) and 4 °C for 14 h. Fractions of 200 µl were collected. Aliquots of 10 µl from each fraction were assayed in 15-µl reaction mixtures for enzyme activity and 15-µl aliquots were used for iodination, gel electrophoresis, radioautography, and densitometry. A, B, and C indicate the positions in the gradients of the sedimentation velocity markers, catalase (M₀ = 240,000), bovine serum albumin (M₀ = 68,000), and cytochrome c (M₀ = 12,500). (2'-5')A₅, synthetase activity (percentage of ATP polymerized); C, relative absorbance of the labeled M₀ = 100,000 protein band.

**Fig. 1.** Analysis of various HeLa and Ehrlich ascites tumor (2'-5')A₅ synthetase preparations by polyacrylamide gel electrophoresis in the presence of SDS. The enzymes in tracks 1 and 4 to 7 were iodinated and detected by radioautography. Those in tracks 2 and 3 were detected by staining with Coomassie brilliant blue R250. Track 1, partially purified HeLa synthetase: poly(I)-poly(C) agarose fraction: 10 µl. Track 2, protein markers (1 µg each) (Bio-Rad) phosphorylase B (M₀ = 94,000), bovine serum albumin (M₀ = 68,000), ovalbumin (M₀ = 43,000), carbonic anhydrase (M₀ = 30,000), soybean trypsin inhibitor (M₀ = 21,000), and lysozyme (M₀ = 14,300). Track 3, purified HeLa synthetase: 0.4 µg. Tracks 4 and 7, mouse EAT synthetase: 0.12 µg. Track 5, purified HeLa synthetase: 0.12 µg. Track 6, mixture of purified HeLa synthetase (0.09 µg) and mouse EAT synthetase (0.09 µg). The data are from three separate experiments: one including track 1, the second tracks 2 and 3, and the third tracks 4 to 7. For further details see "Experimental Procedures." m, mouse enzyme; h, human enzyme.

**Fig. 2.** (2'-5')A₅ synthetase and the M₀ = 100,000 protein: co-chromatography on CM-cellulose (left) and co-sedimentation on a glycerol gradient (right). Left, (2'-5')A₅ synthetase (the poly(I)-poly(C) agarose fraction) was further fractionated on a CM-cellulose column as described under "Experimental Procedures." Aliquots (20 µl) from the (1.5 ml) fractions from the CM-cellulose column were assayed for enzyme activity and aliquots (15 µl) of the active peak fractions and some other fractions surrounding the peak were iodinated and analyzed by polyacrylamide gel electrophoresis in the presence of SDS and radioautography to locate the protein, as described under "Experimental Procedures." The amounts of the M₀ = 100,000 protein in the iodinated fractions were estimated by densitometry using a soft laser scanning densitometer (Biomed Instruments, Inc). An arbitrary value was assigned to the highest absorbance measured and the lower absorbances were related to this value. Right, 745 µl (16.4 µg) of purified (2'-5')A₅ synthetase were concentrated to 200 µl by ultrafiltration (using CF50 ultrafiltration membrane cones: Amicon). The concentrated sample was applied onto a 5-ml glycerol gradient (15-30% (v/v) in 17 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 30 mM β-mercaptoethanol, 25 mM KOAc, 4 mM Mg(OAc)₂, 1 mM EDTA, 10 µM phenylmethylsulfonyl fluoride, 0.2% Triton X-100 and centrifuged in the Beckman SW 50.1 rotor at 48,000 rpm (210,000 × g) and 4 °C for 14 h. Fractions of 200 µl were collected. Aliquots of 10 µl from each fraction were assayed in 15-µl reaction mixtures for enzyme activity and 15-µl aliquots were used for iodination, gel electrophoresis, radioautography, and densitometry. A, B, and C indicate the positions in the gradients of the sedimentation velocity markers, catalase (M₀ = 240,000), bovine serum albumin (M₀ = 68,000), and cytochrome c (M₀ = 12,500). (2'-5')A₅ synthetase activity (percentage of ATP polymerized); C, relative absorbance of the labeled M₀ = 100,000 protein band.
maximal conversion of ATP to (2'-5')(A)n was 44% under KOAc concentrations related to the activity of the homologous enzyme. The optimal KOAc concentrations for the human enzyme is 140 mM, and that of the mouse enzyme is 105,000. The purified enzyme can be stored at -60°C for several months without losing activity. However, incubation in the absence of dsRNA and ATP at 30°C for 2 h or at 0°C for 24 h, or dialysis against buffer A containing 50 mM KOAc at 0°C for 2 h, results in an almost complete loss of activity. Even an incubation as short as 2 h at 0°C results in a 49.6% decrease in activity (not shown).

Stabilization of the Enzyme by Triton X-100—The purified enzyme can be stored at -60°C for several months without losing activity. However, incubation in the absence of dsRNA and ATP at 30°C for 2 h or at 0°C for 24 h, or dialysis against buffer A containing 50 mM KOAc at 0°C for 2 h, results in an almost complete loss of activity. Even an incubation as short as 2 h at 0°C results in a 49.6% decrease in activity (not shown).

Comparison of the Human and Mouse (2'-5')(A)n Synthetase Purified from HeLa Cells and the Mouse (2'-5')(A)n Synthetase Purified from EAT Cells—The two enzymes differ from each other in several ways. The M, of the human enzyme is 100,000, and that of the mouse enzyme is 105,000. As tested at a 1 mM ATP concentration, the optimal Mg"++ for the human enzyme is 16 mM, and that for the mouse enzyme is 8 mM (not shown). The optimal KOAc is 140 mM for the human enzyme and 190 mM for the mouse enzyme.

In particular, the difference in M, and in optimal KOAc concentrations may be of use in studies on the chromosomal mapping of the (2'-5')(A)n synthetase gene involving the use of human-mouse hybrid cells. The fact that an increase in the KOAc concentration from 140 to 240 mM leaves the activity of the mouse enzyme unaffected, but diminishes the activity of the human enzyme to below 1/5 of its earlier level, should facilitate the establishment of the presence or absence of the mouse or human enzymes in extracts from interferon-treated cells.

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REFERENCES
Isolation of Human \((2'-5')A\)_n Synthetase

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