2-5A-dependent RNase (RNase L), a unique endoribonuclease that requires 5'-phosphorylated 2',5'-linked oligoadenylates (2-5A), functions in the molecular mechanism of interferon action. Because this enzyme is present at very low levels in nature, characterization and analysis have been limited. The molecular cloning of human, 2-5A-dependent RNase cDNA has facilitated its expression to high levels in insect cells by infecting with recombinant baculovirus. To determine the properties of the enzyme in the absence of other proteins, the recombinant 2-5A-dependent RNase was purified to homogeneity. The purified enzyme migrated as a monomer upon gel filtration in the absence of activator and showed highly specific, 2-5A-dependent RNase activity. The precise activator requirements were determined by stimulating the purified enzyme with a variety of 2',5'-linked oligonucleotides. The activated enzyme was capable of cleaving poly(rU) and, to a lesser extent, poly(rA), to sets of discrete products ranging from between 4 and 22 nucleotides in length. Reduced rates of 2-5A-dependent RNA cleavage were observed even after removal of ATP and chelation of divalent cations. However, optimal RNA cleavage rates required the presence of either manganese or magnesium and ATP.

The requirement of 2-5A-dependent RNase (RNase L) for an activator makes it unique among the family of known ribonucleases (reviewed in Belasco and Brawerman (1993), Deutscher (1993), and Lengyel (1995)). The RNase activators consist of a series of unusual 5'-triphosphorylated 2',5'-linked oligonucleotides collectively referred to as “2-5A” (Kerr and Brown, 1978). Furthermore, 2-5A-dependent RNase is directly implicated in the molecular mechanism of interferon action, making it one of a relatively few mammalian RNases with a known biological function (Dassel et al., 1993). The discovery by Clemens and Williams (1978) of ribonuclease activity that was dependent on the addition of 2-5A, followed earlier observations involving extracts from interferon-treated cells. Studies showed that protein synthesis in these extracts was very sensitive to inhibition by double-stranded RNA (Kerr et al., 1974) and that addition of double-stranded RNA stimulated the breakdown of RNA (Brown et al., 1976; Kerr et al., 1976).

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Intrinsic Activities of 2-5A-dependent RNase

obtained from commercial sources (Calbiochem, Sigma) and subjected to additional purification as described earlier (Torrence et al., 1984). Alternatively, such core oligomers were prepared by alkaline phosphatase 5'-dephosphorylation followed by high performance liquid chromatography purification. In addition, a solution of core A2'p5'Ap2'p5'A was prepared by chemical synthesis (Imai and Torrence, 1981). Aside from the noted exceptions, the 5'-monophosphorylated 2',5'-oligoadenylates were generated by reaction of the corresponding phosphoroimidazolides (Imai and Torrence, 1985). Other oligonucleotides were prepared by described procedures (pppA'3'p5'ApA, Lesiai et al., 1983; pppA'1p'2'p'5'A, Imai et al., 1985; Imai and Torrence, 1985; A5pppp5'Ap2'Ap(A). Imai and Torrence, 1984). The bromoacylamide employed in copolymer binding studies, p5'Ap2'p5'Ap2'p5'Ap2'p5'Ap2'(bPA). Lesiai and Torrence, 1987; Torrence et al., 1992; Nolan-Sorden et al., 1990), was prepared using a solid-phase synthesis method (Lesiai et al., 1983) and was ligated to [32P]p5'C3'p as described (Nolan-Sorden et al., 1990).

Baculovirus Expression of 2-5A-dependent RNase—The cDNA encoding the entire coding sequence to the human form of 2-5A-dependent RNase was inserted in a codon-fragment of plasmid pBacPAK1 (Clontech) after filling-in the termini using Klenow fragment. Clones containing the cDNA in the correct orientation were determined by restriction enzyme analysis. The recombinant pBacPAK1/ZC5 DNA (500 ng) containing the cDNA was cotransfected into SF21 cells with 200 ng of Bsu36I-digested pBacPAK6 (Clontech) after filling-in the termini using Klenow fragment. Clones containing recombinant virus were identified by Southern blot analysis of polymerase chain reaction products obtained with Bac1 and Bac2 primers (Clontech) probed with a 300-base pair fragment of SacI-digested ZC5 DNA.

To produce recombinant 2-5A-dependent RNase, either monolayer or suspension (for large scale) cultures of SF21 cells were infected at a multiplicity of infection of 10 plaque-forming units/cell at 27°C for 3 days before harvesting. The cell pellets obtained after washing in phenyl buffered saline (pH 6.2), were frozen on dry ice and stored at −70°C. Preparative Gel Cell Extrait—Preparation of packed cell volumes of buffer A (25 mM Tris-HCl, pH 7.4, 50 mM KCl, 10% glycerol, 1 mM EDTA, 0.1 mM ATP, 5 mM MgCl2, 14 mM 2-mercaptoethanol), and 1 mg/l of leupeptin) were added to cell pellets. The cell suspensions were sonicated on ice six times for 15 at 30-s intervals. Supernatants were collected after centrifuging at 4°C for three times at 15,000 x g (once for 30 min and then twice more for 10 min each time).

Purification of 2-5A-dependent RNase—Chromatography used in the purification of the 2-5A-dependent RNase was performed with a fast protein liquid chromatography system (Pharmacia LKB Biotechnology Inc.). All purification procedures were at 4°C. Preparative gel cell extract was loaded onto a MonoQ chromatography—Crude cell extract containing about 20 mg of protein/preparation in 2 ml of buffer A was loaded onto a CL-6B sepharose column (5 x 50 mm, Pharmacia). After washing with 10 column volumes of buffer A at a flow rate of 0.3 ml/min, a linear gradient to 21% buffer B (buffer A supplemented with 1 M KCl) was performed in about 25 min. The ratio of buffer A/buffer B was then held constant while the 2-5A-dependent RNase eluted (in about 8 ml). The column fractions were monitored using SDS/PAGE and activity by densitometry.

Diagnosis of 2-5A-dependent RNase to Remove Divalent Cations—2-5A-dependent RNase (about 100 µg in 100 µl), post-Mono Q column fraction, was dialyzed at 4°C against 25 mM Tris-HCl (pH 7.4), 2.5 mM EDTA, 2.5 mM EGTA, 14 mM 2-mercaptoethanol, and 100 mM KCl; once with 400 ml for 4 h, then with 600 ml for 10 h, and finally with 200 ml of reaction buffer (EDTA and EGTA were reduced to 0.5 mM each) for another 4 h. Assays for 2-5A Binding Activities—2-5A binding activity was measured by modifications (Silverman and Krause, 1971; Nolan-Sorden et al., 1990) of the method of Knight et al. (1980). A 5'-labeled and bromine-substituted 2-5A analog, pA2'p/bpA2'p/bpA3'p[32P]Cp, about 10,000 counts/min/assay, at about 3,000 Ci/mmol, was incubated with fractions containing 2-5A-dependent RNase (or controls) on ice for 1 h. The reaction mixtures were then transferred to nitrocellulose filters which were washed twice in distilled water and dried and the amount of radioactivity bound to the membrane RNase was determined by scintillation counting (Silverman and Krause, 1987). Covariant, cross-linking of 2-5A-dependent RNase to the same 2-5A probe (about 60,000 counts/min/assay) under ultraviolet was according to Nolan-Sorden et al. (1990). Cell extracts or purified 2-5A-dependent RNase was incubated with probe for 1 h on ice and cross-linking was for an additional 1 h on ice under ultraviolet light at 336 nm. Protein separation was by SDS/10% polyacrylamide gel electrophoresis and was followed by autoradiography. Quantitation of 2-5A binding activity was by Phosphorimage analysis (Molecular Dynamics) of the dried gels. 2-5A-dependent RNase Activity Assays—RNA molecules used were poly(A)'poly(T), poly(A)'poly(U), poly(A)'poly(C)'poly(U), and poly(A)'poly(C)'poly(U) (Pharmacia) and were labeled either to degrade 50% of 12 m radiolabeled poly(U) to acid-soluble fragments in the presence of 100 µM poly(A)'poly(T), or 30°C for 30 min. For gel analysis of RNA cleavage products, 5 of reaction mixtures were boiled in the presence of gel sample buffer and then applied to 8% polyacrylamide, 8 M urea sequencing gels.

RESULTS

Baculovirus Expression of Human 2-5A-dependent RNase—To further study the structure, function, and properties of 2-5A-dependent RNase it was necessary to obtain greater levels of the enzyme that were previously possible using mammalian cells and tissues. Therefore, the cDNA to the human form of the endoribonuclease was cloned in the baculovirus vector, BacPAK6 (Clontech) under the control of the polyhedrin promoter ("Materials and Methods"). Production of the enzyme in SF21 insect cells infected by the recombinant virus was measured by both 2-5A binding and 2-5A-dependent ribonuclease assays. 2-5A binding activity was determined by covalent cross-linking of a bromine-substituted, 32P-labeled 2-5A analog to the RNase under ultraviolet light (Nolan-Sorden et al., 1990). Extract of insect cells infected with the non-recombinant virus showed no detectable 2-5A-dependent RNase by this sensitive assay (Fig. 1A, lane 1). In contrast, an intense 80-kDa 2-5A binding activity corresponding to the 2-5A-dependent RNase was detected in extract of insect cells infected with the recombinant virus (Fig. 1A, lane 2).

To measure the catalytic activity of the recombinant enzyme, ribonuclease assays were performed in the presence and absence of trimer 2-5A, i.e. pppA2'p5'ApA. The degradation of the labeled poly(U) to acid-soluble fragments was measured in these assays (Silverman, 1985). Crude extract of the insect cells infected with non-recombinant virus had no 2-5A-dependent RNase activity (Fig. 1B). These findings are consistent with a previous study in which 2-5A-dependent RNase was shown to be absent in insect cells (Cayley et al., 1982). On the other hand, the RNA was extensively degraded (93%) in extract of the recombinant virus-infected cells incubated in the presence of tetramer 2-5A at 100 nM.
enzyme is a fully functional, 2-5A-dependent RNase. It is in its "off-state" in the absence of 2-5A and in its "on-state" in the presence of 2-5A.

Purification of Recombinant, Human 2-5A-Dependent RNase—To determine the intrinsic properties of 2-5A-dependent RNase, purification of the recombinant enzyme was performed using a fast protein liquid chromatography system (Pharmacia). Three separation steps were used to obtain apparently pure enzyme (Table I and "Materials and Methods"). The expressed 2-5A-dependent RNase is clearly visible in stained gels as the major protein present in crude extract of the insect cells infected with non-recombinant virus (Fig. 2, lane 2). There was no band visible at the corresponding position in the lane containing extract from non-recombinant virus-infected SF21 cells (lane 1). The enzyme was 46 and 85% purified after the first (blue Sepharose) and second (Mono Q) separation steps, respectively (Table I and Fig. 2, lanes 3 and 4). Only a single band of protein was observed after Superose-12 chromatography (Fig. 2, lane 5). Therefore, the final Superose-12 fraction consisted of apparently homogeneous 2-5A-dependent RNase. The level of 2-5A-dependent RNase in the insect cells is determined to be 6.7% of the total soluble protein fraction on the basis of the purification data (Table I). The concentration of soluble 2-5A-dependent RNase in the insect cells is about 39 μg, in comparison mouse liver contains about 6 μg 2-5A-dependent RNase (Silverman et al., 1988).

To determine if the recombinant 2-5A-dependent RNase exists as a monomer, its migration through the Superose-12 column was compared with the elution volumes of marker proteins (Fig. 3). The elution volume of the 2-5A-dependent RNase, monitored by absorbance, 2-5A binding activity, and 2-5A-dependent RNase activity (Fig. 3), all correspond to that of a single chain of 83.5 kDa molecular mass, determined from the predicted amino acid sequence (Zhou et al., 1993). From these data we conclude that the 2-5A-dependent RNase less activator (2-5A) exists as a monomer.

Activator Requirements of 2-5A-Dependent RNase—To determine the structural requirements of 2-5A for activation of 2-5A-dependent RNase, ribonuclease activity against poly(rU) was determined as a function of oligonucleotide concentration. The purified 2-5A-dependent RNase (post-Superose-12 step) was used in these assay. Maximal activation of 2-5-dependent RNase was obtained with 1 nM pA(2'p5'A), pppA(2'p5'A)2, or pppA(2'p5'A)3 (Fig. 4A). Therefore, only a single 5'-phosphoryl group linked to the 2',5'-oligoadenylate was required for optimal activation. In contrast, the dimer species, pppA2'p5'A, failed to activate the RNase indicating a minimal requirement.

**Table I**

| Purification of 2-5A-Dependent RNase |
|-------------------------------------|
| **Fraction** | **Total protein** | **Total activity** | **Specific activity** | **Yield** | **Purification factor** |
|-------------|-----------------|-------------------|---------------------|---------|----------------------|
| Crude extract | 19.6 | 66,335 | 3.4 | 100 | 1 |
| Blue Sepharose | 2.4 | 56,507 | 23.4 | 85.2 | 6.9 |
| Mono Q | 0.95 | 40,915 | 43.3 | 61.7 | 12.8 |
| Superose-12 | 0.81 | 40,902 | 50.8 | 61.7 | 15.0 |

*Units defined under "Materials and Methods."

*In aliquots of about 200 μg per separation."
of three adenyl residues (Fig. 4A). The 2',5'-core species, lacking 5'-phosphorylated groups, were also analyzed and found to have greatly reduced activity (Fig. 4B). The trimer core species was more than 10-fold less active than the corresponding 5'-phosphorylated trimer or tetramer 2',5'-oligoadenylates (Fig. 4, A and B). The 3',5'-linked compound, pppA(3'p5'A)₃, failed to activate 2-5A-dependent RNase, even at a concentration of 1 µM (Fig. 4C). On the other hand, the 5'-blocked 2-5A analog, A₅p₅Α(2'p₅Α)₅, was equal in activity to 2-5A per se while the inosine analog of 2-5A, ppI(2'p₅Α)₆, had 10-fold reduced activity (Fig. 4C).

Degradation of Poly(rU) and Poly(rA) by 2-5A-dependent RNase into Discrete Cleavage Products—To confirm and extend previous studies on the sequence specificity of 2-5A-dependent RNase (Wreschner et al., 1981; Floyd-Smith et al., 1981), several types of nucleic acids were incubated with the purified, recombinant 2-5A-dependent RNase in the presence or absence of 2-5A. In this regard, poly(rG), poly(rC), poly(dA), and poly(dT) were not cleaved by activated 2-5A-dependent RNase (data not shown), whereas poly(rU) and, to a lesser extent poly(rA), were degraded (Fig. 5A).

To determine the lengths of the cleavage products, the RNA was analyzed on denaturing polyacrylamide gels (Fig. 5A). Interestingly, both the poly(rU) and poly(rA) were cleaved into sets of discrete products. The fragments of the poly(rU) were estimated to be 5, 7, 8, 12, 16, and 22 nucleotides in length. These were apparent after addition of 1 nM or higher concentrations of 2-5A (lanes 5-8). The poly(rA) was seen only with about 10-100-fold higher levels of 2-5A than were required to degrade poly(rU) (Fig. 5A, compare lanes 5 and 15). Specific cleavage products of poly(rA) were also seen, although to a lesser extent than with poly(rU). The poly(rA) fragments were 13, 21, and 22 nucleotides in length. Identical patterns of poly(rU) cleavage were observed with trimer, tetramer, or pentamer 2-5A, whereas the dimer species was without activity (Fig. 5B). A similar discrete pattern of poly(rU) breakdown products was observed using a crude preparation of naturally occurring mouse L cell 2-5A-dependent RNase (data not shown).

Optimal Activation of 2-5A-dependent RNase Requires Either Magnesium or Magnesium and ATP. The effects of divalent cations and ATP on 2-5A-dependent RNase activity were determined after extensive dialysis of 2-5A-dependent RNase against buffer lacking ATP and containing 2.5 mM each of EDTA and EGTA ("Materials and Methods"). Ribonuclease assays were also performed in the presence of EDTA and EGTA (at 0.5 mM each) to ensure the continued chelation of metal ions. Interestingly, 2-5A-dependent RNase cleaved poly(rU) upon addition of 100 nM 2-5A even in the absence of divalent cations and ATP (Fig. 6A, lanes 4). The basal activity is not due to contaminating divalent cation or ATP in the 2-5A because reactions were performed in the presence of a large excess of EDTA and EGTA and analysis of 2-5A by high performance liquid chromatography showed an absence of detectable levels of ATP (data not shown). Furthermore, when added individually, ATP, magnesium, or manganese had no effect on 2-5A-dependent RNase activity (Figs. 6A, lanes 6, 8, and 12). However, the combinations of either ATP plus magnesium or ATP plus manganese greatly stimulated 2-5A-dependent RNase activity (Fig. 6A, lanes 10 and 14). Calcium added alone inhibited the RNase while calcium plus ATP restored basal activity (lanes 16 and 18). Finally, zinc was inhibitory to the RNase even in the presence of ATP (lanes 20 and 22).

To determine effects of divalent cations and ATP on the affinity of the enzyme for 2-5A, 2-5A-binding assays were per-
Intrinsic Activities of 2–5A-dependent RNase produces discrete cleavage products. A, assays were with 50 ng of post-Superose-12 fraction of 2–5A-dependent RNase with either 60 nm poly(rU)-[32P]Cp or 60 nm poly(rA)-[32P]Cp as substrates for 15 min at 30 °C. B, the post-Superose-12 fraction of 2–5A-dependent RNase (100 ng/assay) was incubated in the presence or absence of 100 nM of different oligomers of 2–5A (indicated). Incubations were for 15 min at 30 °C. The RNA products were separated on an 8% polyacrylamide, 8 M urea sequencing gels.

Addition of each of the divalent cations clearly enhanced 2–5A binding activity. In this regard, magnesium or zinc enhanced 2–5A binding greater than either manganese or calcium. Therefore, although zinc strongly inhibits ribonuclease activity it nevertheless enhances 2–5A binding. Addition of ATP caused a modest (12–39%) increase in 2–5A binding activity (Fig. 6B). Effects of ATP were generally greater in the presence of divalent cations (19–39%) than in their absence (12%). These findings establish that there is a direct stimulatory effect of ATP on 2–5A-dependent RNase activity that requires either magnesium or manganese (see "Discussion").

**DISCUSSION**

To provide new insights into the functions and properties of 2–5A-dependent RNase, we have expressed the human form of the endoribonuclease in insect cells. The baculovirus system provided a high level of expression in a cell type containing no endogenous 2–5A-dependent RNase (Fig. 1). In addition, the 2–5A-dependent RNase which was produced is soluble and fully functional with respect to both 2–5A binding and catalytic activities. In the absence of its activator, 2–5A, the recombinant 2–5A-dependent RNase eluted from a gel filtration column as a monomer which had full activity (Fig. 3). Recently it was proposed that 2–5A-dependent RNase is a heterodimer of 2–5A binding and catalytic subunits (Salehzada et al., 1993); however, the present work shows that both the 2–5A binding and catalytic domains are encoded in a single cDNA expressing just one polypeptide.

**Activator Requirements of 2–5A-dependent RNase—**In accord with previous reports, optimal activation of the human form of 2–5A-dependent RNase required one 5'-phosphoryl group linked to at least three 2',5'-linked adenylyl residues (Figs. 4 and 5) (Torrence et al. (1988), Kitade et al. (1991), Kovacs et al. (1993), and references therein). For instance, absence of a 5'-phosphoryl group greatly reduced ribonuclease activity (Fig. 5).

**Fig. 5.** Degradation of poly(rU) or poly(rA) by 2–5A-dependent RNase produces discrete cleavage products. A, assays were with 50 ng of post-Superose-12 fraction of 2–5A-dependent RNase with either 60 nm poly(rU)-[32P]Cp or 60 nm poly(rA)-[32P]Cp as substrates for 15 min at 30 °C. B, the post-Superose-12 fraction of 2–5A-dependent RNase (100 ng/assay) was incubated in the presence or absence of 100 nM of different oligomers of 2–5A (indicated). Incubations were for 15 min at 30 °C. The RNA products were separated on an 8% polyacrylamide, 8 M urea sequencing gels.

**Fig. 6.** 2–5A-dependent RNase functions after chelation of divalent cations but is stimulated by ATP:magnesium and ATP: manganese. A, RNase assays were with 12 nm poly(rU)-[32P]Cp as substrate and 100 nm p, r(A2 p5'A)2 as activator for 30 min at 30 °C. Either undialyzed (lanes 1 and 2) or dialyzed (lanes 3–22) 2–5A-dependent RNase was used (“Materials and Methods”). Divalent cations were at 2.5 mM and the ATP concentration was 50 μM. B, 2–5A binding activity is stimulated by divalent cations and ATP. The covalent cross-linking of radiolabeled 2–5A probe to dialyzed 2–5A-dependent RNase was as described (“Materials and Methods”). Divalent cations were at 2.5 mM and the ATP concentration was 50 μM. Quantitation of 2–5A binding activity (lower panel) was by PhosphorImage analysis of the dried gel.
RNase—The 2-5A-dependent RNase is capable of cleaving the amino groups of the adenine bases of 2-5A has been sug-
uridylyl residues. Interestingly, degradation of poly(rU) and conclusively that the adenosine capped tetraphosphate itself result with the cloned and purified human enzyme showed there was, under these conditions, no possibility of degradation was able to activate the 2-SA-dependent endonuclease, since poly(rG), or poly(&) (Wreschner in natural RNAs and within poly(rU) but not in poly(rA), from 5 to 22 nucleotides in lengths (Figs. 5 and 337). Previous studies on the sequence specificity of 2-5A-dependent Selection of Cleavage Sites by 2-5A-dependent RNase—Previous studies on the sequence specificity of 2-5A-dependent RNase indicated that cleavage occurred after Upn sequences in natural RNAs and within poly(U) but not in poly(A), poly(rG), or poly(rC) (Wreschner et al., 1981; Floyd-Smith et al., 1981). Here we show that poly(U) and to a lesser extent, poly(A) are substrates for 2-5A-dependent RNase. Thus, the 2-5A-dependent RNase is clearly capable of cleaving after adenylyl residues in RNA even when these are not preceded by uridylyl residues. Interestingly, degradation of poly(U) and poly(A) produced cleavage products of discrete sizes, ranging from 5 to 22 nucleotides in lengths (Figs. 5 and 6A). Although the reasons for the products size distribution is unknown, the discrete cleavage products may be unable to bind to the active site of 2-5A-dependent RNase. Therefore, rejection of the discrete RNA fragments by the enzyme presumably reflects the spatial relationship between the substrate-binding site and the catalytic domain. Effects of Divalent Cations and ATP on 2-5A-dependent RNase—The 2-5A-dependent RNase is capable of cleaving RNA in response to 2-5A even after chelation of divalent cations (Fig. 6A). These findings are consistent with reports that other ribonucleases which leave 3'-phosphoryl groups also do not require divalent cations (reviewed in Deutscher (1993)). However, magnesium and manganese stimulate 2-5A-dependent RNase but only in the presence of ATP. It would appear, therefore, that ATP and 2-5A complexed with magnesium or manganese have enhanced affinities for the enzyme (Fig. 6B). Previously, it was observed that 2-5A-dependent RNase recovery after gel filtration was greatly improved by addition of ATP or ADP, and to a lesser extent AMP (Wreschner et al., 1982). Subsequent work with 2-5A-dependent RNase immobilized on 2'-5'-oligoadenylate-cellulose, also showed that ATP enhanced ribonuclease activity (Krause et al., 1986). In that report there was no difference in 2-5A-dependent RNase activ-

The abbreviations used are: Ap,A, adenosine tetraphosphate; AMP-CPP, adenosine 5'-O-(p-y-methylene)triphosphate; AMP-CP, adenosine 5'-O-(p-y-methylene)triphosphate.