Cloning, Sequencing, and Expression of Two Murine 2′-5′-Oligoadenylate Synthetases

STRUCTURE-FUNCTION RELATIONSHIPS*

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Subrata K. Ghosh, Jyotirmoy Kusari, Sudip K. Bandyopadhyay, Himadri Samanta, Rakesh Kumar, and Ganes C. Sen

From the Department of Molecular Biology, Cleveland Clinic Foundation, Cleveland, Ohio 44195

2′-5′-oligoadenylate synthetases constitute a multimeric family of interferon-inducible enzymes which need double-stranded RNA as an obligatory cofactor. We have isolated cDNA clones for two new murine synthetases. These two clones, 9-2 and 3-9, encoded proteins of 414 and 363 amino acid residues, respectively, out of which the amino terminal 346 residues were almost identical. They were also very similar to the corresponding regions of human synthetases E16 and E18. On the other hand, the carboxyl-terminal 68 residues of clone 9-2 had no homology with the carboxyl-terminal residues of E18. These murine clones had only 67% amino acid identity with the previously isolated murine synthetase clone L3.9-2 and 3-9 proteins were expressed efficiently by in vitro transcription and translation of cDNA clones containing the synthetase coding regions preceded by the 5′-untranslated region of the vesicular stomatitis virus NS gene. These in vitro synthetized proteins bound to double-stranded RNA and catalyzed the synthesis of 2′-5′ oligoadenylylates. A nested set of deletion mutants of the 9-2 clone was produced by restriction digestion and polymerase chain reaction. Functional testing of the corresponding truncated proteins revealed that a region between amino acid residues 104 and 158 was necessary for binding to double-stranded RNA and a region between residues 320 and 344 was necessary for enzyme activity. Moreover substitution of the lysine residue at position 333 by arginine did not affect the enzyme activity.

Interferons have many biological activities all of which are thought to be mediated by the products of the interferon-inducible genes (1-3). Only some of these gene products have been characterized so far. Among them are the two enzymes

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The abbreviations used are: dsRNA, double-stranded RNA; 2′-5′A, 2′-5′-oligoadenylate; IFN, interferon; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bp, base pair(s); mG, 7-methylguanosine; 7-methylguanosyltriphosphate-2′-O-methylguanosine.
kilobase mRNA whereas a 1.5-kilobase mRNA encodes 20-30 kDa synthetases which are mostly nuclear (29). The cloning of only one mouse cDNA synthetase has been reported. This clone, L3, encodes a protein of 42 kDa which has only 69% homology with human small synthetases (15, 30).

Here we report the cloning and sequencing of two additional murine synthetases. More importantly, we report the identification of specific regions of these proteins which are necessary for their enzymatic activity and for their binding to dsRNA. The newly isolated clones 9-2 and 3-9 encoded proteins of 47 and 42 kDa, respectively. The primary sequences of these cDNAs were highly homologous to the human E16/E18 clones. 9-2 and 3-9 proteins expressed in vitro dsRNA-binding and 2-5(A) synthetase enzymatic activities. Progressive deletion mutations from the carboxy-terminal of 9-2 protein established that a region between amino acid residues 104 and 158 was necessary for dsRNA-binding and a region between residues 320 and 344 was necessary for enzyme activity.

**Experimental Procedures**

**Materials**—Restriction and other enzymes and RNAs were purchased from Boehringer Mannheim, pGEM4 was from Promega. The sequencing reagents came from United States Biochemicals. Reticulo
cyte lysate and radioactive chemicals, [3]Hmethionine (specific activity 1000 Ci/mmol), [32P]ATP (specific activity 400 Ci/mmol), and [3H]UTP (specific activity 50 Ci/mmol) were from Amersham Corp. Poly(I)-poly(C)agarose, mGpppGm and Sepharose G-25 were from Pharmacia LKB Biotechnology Inc., polyethyleneimine-cellulose plates were from EM Scientific and DE81 was from Whatman. The oligonucleotides were synthesized in a solid-phase DNA synthesizer from Applied Biosystem model 380B. Polymerase chain reactions were done using a DNA thermal cycler and Taq polymerase from Perkin-Elmer Cetus.

**Isolation of cDNA Clones and their Sequence Analysis**—A Agt10 cDNA library made from IFN-treated Ehrlich ascites tumor cells (31) was screened by hybridization with a nick-translated insert of human 2-5(A) synthetase E18 clone which encodes a 46-kDa synthetase (24). The positive plaques were purified, the inserts were released from the isolated 3'-DNA by digestion with EcoRI, and subcloned in pGEM4. Sequencing was done by the dideoxy chain termination method using Sequenase. Vector primers were used first, followed by new synthetic primers whose sequences were selected from the already-sequenced regions. Nucleotide and protein sequence comparisons were done using the procedure of Krye and Doolittle (32).

**Construction of VSV NS-Synthetase Hybrid cDNA Clone**—The 5'-untranslated regions of 3-9 and 9-2 DNA clones were replaced by CCATC sequence from the 5'-untranslated region of the vesicular stomatitis virus NS gene (33). For constructing the hybrid cDNAs, a synthetic double-stranded DNA was ligated to the XhoII sites of the 3-9 and 9-2 clones which are located right after the second ATG codon. The synthetic DNA was produced by annealing two oligonucleotides: GCGCGAATTCCCATCATG and GATCCATGATGG. It was ligated to 9-2 clone which had been digested with SacI (at nucleotide 720, Fig. 1A). Its sequence was the same as that of the antisense strand of 9-2 clone except for a C residue in position 8 unless described otherwise.

**Binding of 2-5(A) Synthetase Proteins to Poly(I)-Poly(C)-Agarose**—25 ml of poly(I)poly(C)-agarose beads which had been washed with buffer A (20 mM Hepes, pH 7.5, 5 mM magnesium acetate, 10 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40 and 10% glycerol) was mixed with 25 ml of in vitro translation products diluted with buffer A (usually 5 ml translation products diluted with 20 ml of buffer A). The mixture was incubated at 30 °C for 30 min with occasional mixing. The supernatant was removed and the agarose beads were washed twice with buffer A to remove unbound proteins. The washed beads were either used for 2-5(A) synthetase enzyme assay or boiled in electrophoretic sample buffer for analysis of the bound proteins.

**2-5(A) Synthetase Assay**—Proteins bound to Poly(I)-poly(C)-agarose were incubated with 10 ml of assay buffer containing 25 mM KCl, 15 mM magnesium acetate, 2.5 mM dithiothreitol, 5 mM ATP, and 10 μCi of [α-32P]ATP. These incubations were at 30 °C for 2 h unless indicated otherwise. After the incubation, 0.25 unit of alkaline phosphatase in 10 μl of 170 mM Tris base was added along with 8 μl of 20 mM Tris-HCl, pH 8.0, and the reaction mixture (28 μl) was incubated at 57 °C for 1 h. Finally, 2 μl was spotted on a polyethyleneimine-cellulose thin layer chromatographic sheet and developed in 750 mM K2HPO4, pH 3.5 (27). The precipitated RNA was autoradiographed. For quantitating the amounts of 2-5(A) isomer synthetized, the appropriate region of the sheet was scanned, and the associated radioactivity was measured in a liquid scintillation spectrometer.

**In Vitro Transcription**—All cDNAs to be transcribed were cloned in pGEM4 in an orientation appropriate for the synthesis of sense strand RNA by SP6 RNA polymerase. Capped mRNAs were synthesized from NS-9/2/3-9 hybrid cDNAs linearized by digestion with BamHI. A typical transcription mixture of 80 μl contained 3 μg of DNA template and 30 units of SP6 RNA polymerase in a buffer containing 40 mM Tris-HCl, pH 7.9, 6 mM MgCl2, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 40 units of RNasin, 500 μM ATP, 500 μM CTP, 50 μM UTP, 500 μM mGpppGm, and 20 μCi of [3H]UTP. Transcription incubations were for 1 h at 40 °C followed by digestion with 5 units of DNase at 37 °C for 15 min. The products were deproteinized by extraction twice with phenol-chloroform and once with chloroform followed by precipitation in ethanol.

The amount of RNA synthesized was monitored by measuring 3H binding to DEAE-cellulose papers. Typically, 3-4 μg of RNA was synthesized in a standard reaction. If needed, the newly synthesized RNA was analyzed by denaturing gel electrophoresis for determining its size and purity.

**In Vitro Translation**—The in vitro synthesized RNA was translated in an mRNA-dependent protein synthesizing system prepared from nuclease-treated rabbit reticulocyte lysate. Typically, 200 ng of mRNA was translated in a 15-μl reaction mixture containing 12 μl of reticulocyte lysate, 20 units of RNasin, and 15 μCi of [3H]methionine. Incubations were at 30 °C for 90 min. Reaction products were either analyzed by electrophoresis using 10% (Fig. 3B and 6B) or 15% (Fig. 5B) polyacrylamide gels or tested for binding to poly(I)-poly(C)agarose.

**Construction of 9-2 Mutant Proteins**—The mRNAs corresponding to a nested set of deletion mutants which had progressive deletions from the 3' end were produced by in vitro transcription of 9-2 cDNA which had been cleaved at suitable restriction sites. N-344, N-278, N-232, N-222, N-158, N-104, and N-70 were synthesized from 9-2 clones which had been digested with HindIII, Ddal, ScaI, HinfII, PvuII, and HaeIII, respectively. N-320, N-300, and N-344 (Lys-333 → Arg-333) mRNAs were transcribed from specially designed clones which were engineered from 9-2 clone using specific oligonucleotide primers and polymerase chain reactions. Four 3' to 5' strand-oligonucleotides were synthesized for this purpose. SG3 was a sense-primer with a sequence corresponding to nucleotides 689 through 703 of the 9-2 clone (Fig. 1A). SG4 was an antisense primer with a sequence corresponding to nucleotides 934 through 920 of the 9-2 clone (Fig. 1A). SG5 was an antisense primer with a sequence corresponding to nucleotides 984 through 970 of the 9-2 clone (Fig. 1A). SG6 was an antisense primer corresponding to nucleotides 1060 through 1014 of the 9-2 clone (Fig. 1A). Its sequence was the same as that of the antisense strand of 9-2 clone except for a C residue in place of the T residue at nucleotide 1018. For constructing the plasmid for the production of N-304, a polymerase chain reaction was done using primers SG3 and SG4 and clone 9-2 DNA. The recessed ends, if any, of the amplified product were filled in with T4 DNA polymerase. The resultant blunt-ended DNA of 246 bp was purified by gel electrophoresis and digested with SacI to generate a 210-bp fragment. It was ligated to 9-2 clone which had been digested with SacI (at nucleotide 720, Fig. 1A) and SmaI (in the polylinker). The resultant construct was sequenced to verify expected changes in its size and purity.

**Polymerase Chain Reactions**—100 ng of supercoiled 9-2 cDNA and 40 pmol of each primer were used with 2.5 units of Thermus aquaticus
DNA polymerase. Temperature cycling consisted of an initial denaturation step for 7 min at 94 °C followed by 24 cycles of amplification. For clones N-304 and N-320, each cycle included denaturation for 1.5 min at 94 °C and annealing for 1 min at 50 °C and for the clone N-344 (Lys-333 → Arg-333) each cycle included denaturation for 2 min at 94 °C and annealing for 3 min at 50 °C. DNA synthesis in each cycle was carried out for 2 min at 72 °C.

RESULTS

Primary Structures of Two Murine Synthetases—A λgt10 library made from poly(A)+ RNA isolated from murine IFN-α/β-treated Ehrlich ascites tumor cells was used for isolation of murine 2-5(A) synthetase cDNA clones. We used a human 2-5(A) synthetase cDNA clone as a hybridization probe for this purpose. Several clones were isolated and partially characterized. Two independent clones, 9-2 and 3-9, were chosen for further analysis and the results from these experiments are reported below.

The complete nucleotide sequences of the two cDNA clones are shown in Fig. 1A. The original cDNA inserts from the λgt10 clones were released by digestion with EcoRI and subcloned in pGEM4. Sequencing was done using plasmid vector primers and synthetic primers with sequences corresponding to regions whose sequences had already been determined. As expected, the sequences of both clones were flanked by EcoRI sites and there were no internal EcoRI sites. The 9-2 clone contained 1491 nucleotides including 14 A residues at the 3' end. The 3-9 clone, on the other hand, contained 1303 nucleotides. Up to the nucleotide 1061 of the 9-2 clone, the two cDNAs had almost identical sequences but they diverged completely at the 3' side of this junction. It is notable, that even in the homologous regions there were scattered differences such as a missing triplet in 3-9 at position 385, an A in place of G at position 493, and a G in place of T at position 551 (all numbers refer to the 9-2 sequence). These differences suggest that the two mRNAs are probably the products of two highly homologous but distinct genes. Both cDNAs contained two tandem ATG codons (at position 21 of 9-2 and position 64 of 3-9) of which the second one was in a better context for serving as a translational initiation codon. Assuming the second ATG as the initiation codon, the 9-2 clone had an open reading frame of 414 amino acids and the 3-9 clone had an open reading frame 363 amino acids (Fig. 1B). The three regions of 9-2 and 3-9 clones noted for their nucleotide differences gave rise to corresponding amino acid differences. The 3-9 protein was missing an amino acid at residue 122, a Gly was replaced by an Asp at position 157 and a Gly was replaced by an Asp at position 157. There was an overall 67% identity in amino acid sequences. Since the carboxyl-terminal sequences of murine and human synthetases differed the most, we performed a hydropathy analysis of these regions (Fig. 2). As compared to E18, 9-2 contained two additional strongly hydrophobic domains at residues 346. The identical and nonidentical regions were interspersed with regions whose sequences had already been determined.

Fig. 1. Nucleotide and amino acid sequences of 9-2 and 3-9 cDNA clones. A, the nucleotide sequences of the two clones were compared. A (*) denotes the same nucleotide. The translation initiation codon and termination codons are overlined whereas two tandem polyadenylation signals in 9-2 are underlined. B, the derived amino acid sequences of the two proteins are compared. A (−) denotes the same amino acid and an (*) denotes a gap.

It is apparent that the two proteins are related but distinct. There was an overall 67% identity in amino acid sequences. The identical and nonidentical regions were interspersed with no obvious indications of conserved or nonconserved domains.

Since the carboxyl-terminal sequences of murine and human synthetases differed the most, we performed a hydropathy analysis of these regions (Fig. 2). As compared to E18, 9-2 contained two additional strongly hydrophobic domains centering at residues 390 and 402. These domains were absent from 3-9 which, however, contained a different additional hydrophobic domain around residue 355. This latter domain was also present in L3. It remains to be seen whether
A' 9-2
0)
pGEM4 clones. These mRNAs were purified and translated were compared. Positive numbers on the
comparisons. of the carboxyl-terminal regions of 9-2, 3-9,
these differences in the hydrophobic domains cause different
abscissa protein. A window size of 9 amino acid residues was used for the
sequences were aligned for the maximum fit by visual inspection. An
marks of glycosylation, myristylation, and farnesylation.
3-9 protein contained any sequence motifs which are hall-
icity and negative numbers denote hydrophobicity. Numbers on the
were, however, not high. For the purpose of improving this
weight of 42,000 (Fig. 3B, 15296
efficiently in vitro. The corresponding hybrid mRNAs were synthe-
ized using SP6 RNA polymerase and suitable linearized
zymes encoded by the 9-2 and 3-9 cDNA clones. For
this purpose, the corresponding capped mRNAs were synthe-
sized using SP6 RNA polymerase and suitable linearized
zymes encoded by the 9-2 and 3-9 cDNA clones. For
putative in vitro translation products of SP6 transcripts from the hybrid 9-2 and 3-9 cDNA clones. Lanes 1-3 are translation products analyzed di-
rectly and lanes 4-6 are analyzed after binding to poly(I).poly(C)-
agarose. Lanes 1 and 4, no added mRNA; lanes 2 and 5, 9-2 mRNA;
lanes 3 and 6, 3-9 mRNA. Numbers on the left indicate molecular
weights, in thousands, of standard proteins which migrate to the
respective positions.

Expression of the 9-2 and 3-9 Synthetases in vitro—We
wanted to establish an efficient in vitro system for expressing
the enzymes encoded by the 9-2 and 3-9 cDNA clones. For
this purpose, the corresponding capped mRNAs were synthe-
sized using SP6 RNA polymerase and suitable linearized pGEM4 clones. These mRNAs were purified and translated in vitro in a rabbit reticulocyte lysate system giving rise to the corresponding proteins. The efficiency of their translation was, however, not high. For the purpose of improving this efficiency, we designed hybrid clones in which the 5'-untranslated regions of the original 9-2 and 3-9 clones were replaced by a portion of the 5'-untranslated region of vesicular stomatitis virus NS gene (Fig. 3A) whose mRNA translates very efficiently in vitro. The corresponding hybrid mRNAs were synthesized in vitro and translated (Fig. 3B). 9-2 mRNA gave rise to a protein of an apparent molecular weight of 47,000, and 3-9 mRNA gave rise to a protein of an apparent molecular weight of 42,000 (Fig. 3B, lanes 2 and 3). These numbers matched well with the respective molecular weight of 47,627 and 41,789 calculated from the sequence information.

The in vitro synthesized proteins were tested for their ability to bind to dsRNA. Both 9-2 and 3-9 proteins bound efficiently to poly(I).poly(C)-agarose (Fig. 3B, lanes 5 and 6). The bound proteins had strong enzymatic activity as measured by thin layer chromatography of the products (Fig. 4A). The poly(I).poly(C)-agarose-bound 9-2 protein's enzyme ac-
tivity increased linearly with time for 1 h after which it tapered off (Fig. 4B).

Properties of Mutant Proteins—In the next set of experiments we generated a series of mutants of the 9-2 protein and tested their functions. All but one of these mutants belonged to a nested set with progressive deletions from the carboxyl terminus (Fig. 5A). N-344, N-278, N-232, N-222, N-158, N-104, and N-70 mRNAs were produced by transcription of the cDNAs which had been digested with the appropriate restriction enzyme shown in Fig. 5A. N-320 and N-304 mRNAs were transcribed from appropriate cDNA clones which were generated with the help of polymerase chain reaction as detailed under "Experimental Procedures." N-344 (Lys-333 → Arg-333) carried a single mutation converting the lysine residue at position 333 to arginine. This clone was also produced using polymerase chain reaction.

The mutated mRNAs were translated in vitro, and the

Fig. 2. Structural comparisons of different synthetases. A, the amino acid sequences of 9-2 and L-3 are compared. The two sequences were aligned for the maximum fit by visual inspection. An (*) in the body of the sequence denotes a gap. B, the hydropathicity of the carboxyl-terminal regions of 9-2, 3-9, E18, and L-3 proteins were compared. Positive numbers on the ordinate denote hydrophilicity and negative numbers denote hydrophobicity. Numbers on the abscissa indicate the corresponding amino acids of the respective protein. A window size of 9 amino acid residues was used for the comparisons.

Fig. 4. 2-5(A) synthetase enzyme activities of in vitro translated 9-2 and 3-9 proteins. A, autoradiography of a thin layer chromatogram. Lane 1, 9-2 mRNA; lane 2, 3-9 mRNA, and lane 3, no added RNA. B, kinetics of 2-5(A) synthesis by the 9-2 isozyme. For each time point the amounts of 2-5(A) synthesized by translation mixtures with and without 9-2 mRNA were measured. The net synthesis by the 9-2 enzyme is shown.

Fig. 3. Expression of 9-2 and 3-9 proteins in vitro. A, the sequences of the regions of VSV NS gene and the 9-2 and 3-9 cDNA clones used for designing the hybrid cDNA clones are shown. B, in vitro translation products of SP6 transcripts from the hybrid 9-2 and 3-9 cDNA clones. Lanes 1-3 are translation products analyzed directly and lanes 4-6 are analyzed after binding to poly(I).poly(C)-agarose. Lanes 1 and 4, no added mRNA; lanes 2 and 5, 9-2 mRNA; lanes 3 and 6, 3-9 mRNA. Numbers on the left indicate molecular weights, in thousands, of standard proteins which migrate to the respective positions.
resultant proteins were tested for binding to dsRNA. All deletion mutants from N-344 to N-158 bound to dsRNA. Some of these results are shown in Fig. 6B, lanes 1–5. N-104 and N-70, on the other hand, did not bind to dsRNA (Fig. 6B, lanes 6 and 7) although they were synthesized efficiently (Fig. 5B, lanes 8 and 9). These results demonstrated that a linear region between residues 104 and 158 of 9–2 protein was needed for its binding to dsRNA.

In the next experiment, the protein products of the nested deletion set produced by restriction digestions were tested for enzyme activity. Although N-344 had strong enzyme activity, N-278 was devoid of it (Fig. 6A). For narrowing this region further, the clones encoding N-320 and N-304 were produced and the encoded proteins (Fig. 6B) were tested for activity (Fig. 6C). Although both proteins bound to dsRNA (data not shown) they did not have enzyme activity (Fig. 6C), which indicated that a region between residues 320 and 344 was essential for enzyme activity. This region contained a single lysine residue at position 333. Since 2–5(A) synthetases polymerize ATP and many protein kinases contain essential lysine residues at their ATP-binding sites, we reasoned that Lys-333 may be essential for enzyme activity of the 9–2 protein. For testing this hypothesis, Lys-333 was mutated to Arg. The desired mutation was confirmed by direct sequencing of the mutated cDNA. Such mutations of essential lysine residue to arginine abolish the activity of many protein kinases. In the case of 9–2 protein however, this mutation had no effect on the enzyme activity; N-344 and N-344 (Lys→Arg) had equally strong enzymatic activities (Fig. 6D).

The results from all the experiments testing the properties of the mutant proteins are summarized in Fig. 7. The native 9–2 protein containing 414 residues bound to dsRNA and synthesized 2–5(A). A truncated protein (N-344) missing 70 residues from the carboxyl terminus had similar properties but removal of another 24 residues (N-320) abolished enzyme activity although the protein still bound to dsRNA. The dsRNA binding property was still displayed by a truncated protein (N-158) which was missing 186 residues from the carboxyl terminus of the native protein. But the removal of another 54 residues (N-104) eliminated the dsRNA binding capacity.

**DISCUSSION**

The data presented in this paper and those from the literature (15, 26, 30) indicate that even within the small size class of 2–5(A) synthetases there are many members. Combining the information from the human and the murine systems, it appears that E16 and 3–9 are the same isozyme from the two species whereas E18 and 9–2 are distinct isozymes which are different from each other and from E16/3–9. A comparison of the nucleotide sequences of the murine synthetase with those of the human synthetases provided information germane to the genesis of the corresponding mRNAs. It has been shown that the two human synthetase mRNAs E16 and E18 arise by alternative splicing of transcripts from the same gene (23). Drawing upon this information, we suggest a route for synthesis of 3–9 and 9–2 mRNAs (Fig. 8). 3–9 seems to be the murine equivalent of E16 both in its structure and in its mode of biosynthesis. 9–2 is very similar to E18 up to the crucial
suggest that the corresponding mRNAs are transcribed from the same gene. This conclusion, however, needs confirmation by direct experimentation such as isolation and transfection of the relevant gene. Moreover, the noted sequence differences in three regions of 9-2 and 3-9 and the fact that both clones were isolated from the same cDNA library may suggest that the corresponding mRNAs are transcribed from two alleles of the same gene. The murine L3 protein is related to but distinct from the others. It is encoded by a separate genetic locus which contains at least two L3-type genes (34). It remains to be seen whether both mouse and human have the same number of small family synthetase genes and whether all the isoforms are expressed in both species. Similarly, it will be informative to examine whether the L3 gene can also give rise to alternatively spliced mRNAs. Use of isoform-specific oligonucleotide probes and peptide antibodies will be necessary for these analyses.

The realization of the presence of more than one gene encoding the small synthetases suggests to us the possibility of differential transcriptional regulation of these genes. Synthetase activity has been shown to be modulated by many physiological signals, e.g. IFN-α, IFN-β, IFN-γ (11), platelet-derived growth factor (35), epidermal growth factor (36), nerve growth factor (37), dsRNA (35, 38) partial hepatectomy (39), and estrogen withdrawal from chicken oviduct (40). It is conceivable that different members of this disparate group of stimuli induce different isoforms by virtue of the corresponding genes having different transcriptional regulatory sequences responding to one signal or another.

The functional significance of the presence of so many synthetases remains obscure. Their structural differences may contribute to different subcellular distributions. We may speculate that 3-9 and 9-2 proteins will be distributed differently because of the differences in hydrophobicity at their carboxyl terminal regions. Others have noted differences in post-translations modulations of different isoforms (22). Differences have also been noted regarding the optimum concentrations of dsRNA needed for their activations as well as the lengths of 2-5(A) molecules produced by them (21, 41). The possibility remains open that some members of this large family of enzymes can use substrates other than ATP and that some members can be activated by cofactors other than dsRNA. Such activation of a synthetase by fructose 1-6 bisphosphate has been reported in the literature (42).

The most intriguing characteristics of this family of enzymes are their activation by dsRNA and their ability to catalyze 2'-5' phosphodiester bond formation. The former property can be attributed to the IFN-inducible protein kinase as well. In order to understand the mechanistic basis of these properties of the enzymes, systematic structure-function studies are needed. The studies reported here constitute the first steps toward that goal: using a simple and convenient in vitro expression system we identified a stretch of 54 amino acids between residues 104 and 158 which was needed for dsRNA-binding. However, further deletion and point mutations are needed for refining this information. It also remains to be tested whether this region by itself is sufficient for dsRNA-binding and if not, how many of the amino terminal 104 residues are needed. Our analysis has not ruled out the possibility of other putative dsRNA-binding sites situated in the region between residues 320 and 344. For the purpose of detecting these additional putative sites, we are currently testing the properties of a mutant protein from which the region between residues 104 and 158 has been deleted. Our ongoing experiments should also reveal whether this region is necessary for enzyme activity.

The studies reported here delineated another region between residues 320 and 344 to be essential for enzyme activity. Further deletions of the region between the dsRNA-binding domain, and this domain may define the minimal protein needed for dsRNA-activated enzyme activity. It is not clear whether the region between residues 320 and 344 contains the enzyme's active center and is involved in substrate binding.
Studies with other ATP-binding enzymes such as many protein kinases have identified a critical lysine residue which is needed for ATP binding (43). Mutation of this lysine residue to arginine eliminates ATP binding and kinase activity (44). 9-2 protein contains a sequence motif very similar to the one identified as responsible for ATP binding to kinases. This motif is located between residues 306 and 333. However, mutating the critical lysine at position 333 to arginine did not affect the enzyme activity of 9-2. This result suggests that either this region is not involved in ATP binding or this class of enzyme binds ATP by a mechanism different from that used by the kinases. In this context, it should be noted that exceptionally high concentrations of ATP are needed for optimal synthetase activity (28) suggesting that these enzymes have low substrate affinity. Further mutational analyses of the kind reported here coupled with direct cross-linking of the cofactor and the substrate to the enzyme, as being pursued by others (45), will be needed for conclusively identifying the different regions of the enzyme which are essential for its activity.

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