P69 is an isozyme of the medium size class of human 2'-5' oligoadenylate synthetases. In this study, recombinant P69 was expressed and used for enzymological and structural investigations. Bacterially expressed P69 was inactive whereas the same protein expressed in insect cells was highly active. Whether this difference could be due to differential post-translational modifications of the protein was investigated. Mutations of appropriate residues showed that myristoylation of the protein was not necessary for enzyme activity. In contrast, inhibition of glycosylation of P69, by tunicamycin treatment of the insect cells, produced an enzymatically inactive protein. Recombinant P69 produced in insect cells was purified by affinity chromatography. It was a dimeric glycoprotein, very stable and completely dependent on double stranded (ds) RNA for activity. The enzyme catalyzed the non-processed synthesis of 2'-5'-linked oligoadenylate products containing up to 30 residues. 2'-O-Methylated dsRNA was incapable of activating P69 and a 25-base pair dsRNA was as effective as larger dsRNA. This expression system will be useful for large scale production of P69 and its mutants for structural studies.

2'-5' Oligoadenylate (2-5(A)) synthetases are a family of enzymes that polymerize ATP into a series of 2'-5'-linked oligoadenylates (1, 2). Trimers and higher oligomers of 2-5(A) can activate a latent ribonuclease, RNase L, by causing its dimerization (3). Cellular synthesis of the 2-5(A) synthetases is induced by interferons and viral infection of interferon-treated cells causes activation of the enzymes by viral dsRNA, production of 2-5(A), activation of RNase L, and degradation of RNA. This chain of events can prevent the replication of certain classes of viruses, such as picornaviruses, but other viruses are not affected by the 2-5(A) synthetase/RNase L pathway (4).

According to their sizes, the 2-5(A) synthetases can be divided into three classes: large, medium, and small, and within each class there are many isozymes (2). Multiple genes, alternative splicing of the mRNAs, and differential post-translational modification of the proteins add to the diversity of these enzymes. For example, in mouse, there are at least three genes encoding small synthetases and 9-2 and 3-9 isozymes are encoded by two alternately spliced mRNAs of the same gene (5–7). Similarly, the human medium synthetase gene gives rise to two alternatively spliced mRNAs encoding the isozymes P69 and P71 (8). The three classes of the enzymes are structurally related and highly conserved across the species. The small synthetases are homotetramers, the medium synthetases are dimers, and the large isozyme is a monomer (9). These enzymes are interesting to study for many reasons: their interferon inducibility, their activation by dsRNA, their ability to synthesize 2'-5'-linked oligonucleotides, and their cellular actions. Structure-function and enzymological studies of 2-5(A) synthetases have not progressed well in the past because of the lack of an efficient expression system for recombinant proteins. Recently, we have used a bacterial system and a insect cell/baculovirus system effectively for producing a small isozyme of 2-5(A) synthetase and its mutants (10, 11). The same systems were used in the current study for producing the recombinant medium synthetase P69. The recombinant P69 protein was purified and used for enzymological studies.

Bacterially produced P69 was enzymatically inactive but recombinant P69 produced in insect cells was highly active, completely dependent of dsRNA, non-processive, and stable. The recombinant protein was a dimer and had sugar modifications that were required for its activity. A 25-bp dsRNA, but not a 15-bp dsRNA, could activate P69 maximally and free 2'-OH groups on the dsRNA were required for its activity.

MATERIALS AND METHODS

Cloning of Human P69—Total RNA was prepared using RNAzol (Tel-Test Inc.) from human Daudi cells treated with 200 units/ml interferon a for 12 h. This RNA were reverse transcribed by priming with random hexamers using reverse transcriptase kit (Life Technologies, Inc.) according to the manufacturer’s instructions, followed by PCR using primers (primer 1: GGCCTATCTAGTGAGGAAATGGG-GAGGTC; primer 2: CCGGGTCGACTTAAAGCTTGATGACTTTTACCCGGAG; primer 3: TAAAGCTTGATGACTTTTACC-)

Site-directed Mutagenesis—To be able to use convenient unique restriction enzyme sites on full-length P69 cDNA, it was cloned in pET28a vector with NdeI and SalI, which were incorporated in the PCR primers for the convenience of subsequent cloning, and cloned into the same sites of PET28a vector (Novagen), which puts a hexahistidine tag at the NH2 terminus of P69. Expression of P69 in Bacteria—Bacterial expression and partial purification of NH2-terminal hexahistidine-tagged P69 was done in a similar way as described earlier for 9-2 (11). The yield of pure P69 protein from bacterial expression system was 5 μg/50 ml of culture.
(GGAGATATACCATGGGGAATGCGGAG) containing a NcoI site over-lapping with protein start codon and a reverse primer downstream of BamHI site (nucleotides 873–892) were used to PCR a ~900 bp fragment. The PCR product was Klenow filled and cloned into the SmaI site of pBluescript KS+ . The insert was then excised out from pBluescript KS+ using SmaI and HindIII and reinserted into the SmaI site of pET28a-P69 construct. The full-length cDNA insert from the above construct was excised out with XhoI and SalI and subcloned into the EcoRI-SalI site of pGEM4. The resulting construct, called pGM13, contained full-length P69 cDNA without any NH2-terminal tag, and could be transcribed from the SP6 promoter of pGEM4. The G2A and G2D mutants were made using the pGM13 construct as template for PCR with similar forward primers as before having a NcoI site and the mutation (G to A or D) and the same reverse primer downstream of BamHI site (nucleotides 873–892). The 900 bp PCR products were Klenow filled and cloned into the SmaI site of pBluescript KS+. The mutated inserts were then excised out from pBluescript KS+ with NcoI and BamHI, swapped with the corresponding fragment on wild type cDNA in pGM13.

Expression and Purification of P69 from Insect Cells—The insect cell expression of P69 was done using the Bac-to-Bac Baculovirus expression system (Life Technologies) in a similar way as described earlier (10, 13). The NH2-terminal hexahistidine-tagged P69 insert from pET28a was cloned into the XhoI and XhoI site of pFastBac. The resulting construct was transformed into DH10Bac cells. Cells were harvested after 30 h post-transfection, and the baculovirus expression system instruction manual. High Five (H5) cells (Invitrogen) were infected with the recombinant virus containing hexa-histidine-tagged P69 at a multiplicity of infection of 10 and were harvested 32 h post-infection. Cells were washed twice with phosphate-buffered saline, resuspended in lysis buffer (1 ml of buffer for cells from one 150-mm plate) containing 300 mM NaCl, 20 mM Tris-Cl, pH 7.4, 10% glycerol, 5 mM β-mercaptoethanol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml apritinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin, and lysed by sonication of six 15-s pulses. Clarified cell lysate was prepared by centrifuging at 30,000 × g for 15 min and applied to Ni-NTA beads (Qiagen). For 1 ml of cell extract, a 0.2-ml bead suspension was used. Prior to loading with the cell extract, beads were washed twice in the binding buffer (300 mM NaCl, 20 mM Tris-Cl, pH 7.4, 10% glycerol, 10 mM imidazole 5 mM β-mercaptoethanol, 0.3% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml apritinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Cell extract was diluted 1:1 (v/v) with the binding buffer, the final imidazole concentration was adjusted to 10 mM and applied to the beads for binding at 4 °C with shaking on a rotary shaker. After 2 h, beads were centrifuged and washed for 15 min each: once with binding buffer, three times with binding buffer containing 1M galactose. Bound proteins were eluted by boiling beads in 1 ml of reaction mixture to assay the activation of P69 activity. The 2-μl aliquot of each alternate fractions were concentrated by Ni-affinity chromatography (Fig. 1B), and subcloned for further analysis.

dsRNA Activation of P69—Synthetic dsRNA of various lengths were made by transcribing the multicloning site of pBluescript KS+ as described earlier (17). The purified dsRNAs were used in a standard reaction mixture to assay the activation of P69 activity. The 2′-O-methylated dsRNA (36 bp) was obtained as two complementary pure single stranded RNA using commercial nucleic acid synthesis service from Operon Technologies. The single stranded 2′-O-methylated RNAs were hybridized to form the dsRNA (11).

RESULTS

Bacterially Expressed P69 Is Inactive—For the purpose of preparing recombinant P69 protein, we cloned its full-length coding sequence by reverse transcription-polymerase chain reaction using primers derived from the published sequence (8). The newly cloned cDNA was expressed in the rabbit reticulocyte lysate system and the in vitro synthesized protein was enzymatically active (Fig. 1A). Since we have previously expressed active 9-O-isozyme in Escherichia coli, (11), bacterial expression of P69 was first attempted. A hexahistidine-tagged protein was expressed in bacteria and it was purified by Ni-agarose affinity chromatography (Fig. 1B). Importantly, the bacterially expressed P69 protein was enzymatically inactive although similarly expressed and purified 9-2 isozyme was active (Fig. 1C). Unlike 9-2, native P69 from interferon-treated human cells is a myristoylated protein (9). Since myristoylation occurs in rabbit reticulocyte lysate, but not in E. coli (18, 19), the lack of activity of bacterially expressed P69 could be due to the lack of myristoylation of the protein. To explore whether myristoylation is indeed needed for enzymatic activity, appropriate mutants of P69 were generated. Myristoylation of a protein is directed by the sequence present at its NH2 terminus (20) and mutation of the Glys residue at position 2 is known to destroy the proteins ability to be myristoylated (18, 21). Using site-directed mutagenesis, such mutants of P69, G2A, and G2D
Recombinant Medium 2-5(A) Synthetase

were generated and the corresponding proteins were synthesized in vitro. The mutant proteins were as active as the wild type protein (Fig. 2A) thus demonstrating that myristoylation is not required for enzyme activity. Another possible reason for a bacterially expressed protein to be inactive could be the presence of the extraneous hexahistidine tag at its NH₂ terminus. This possibility was also ruled out by the demonstration that (His)₆P69 made in vitro was enzymatically active (Fig. 2B). Although the reason for the observed inactivity of the bacterially expressed P69 was not revealed, the above series of experiments established that the presence of the polyhistidine tag at the NH₂ terminus or the absence of myristoylation does not affect the protein’s enzyme activity.

Recombinant P69 Expressed in Insect Cell Is Active—Since bacterial expression of active recombinant P69 was ineffective, we decided to express it in insect cells using the baculovirus system. A recombinant baculovirus, encoding (His)₆P69, was constructed for this purpose. Recombinant P69 protein was produced in the infected cells in quantities large enough to be easily detected by Coomassie Blue staining of proteins in total cell extracts (data not shown). The P69 protein was purified from the soluble supernatant of the cell extract using Ni-agarose affinity chromatography. This single step purification process was very efficient yielding an apparently homogeneous P69 preparation, as shown in Fig. 3A. A 290-fold purification of the activity was obtained by this method with about 30% yield (Table I).

Unlike the bacterially expressed P69, the recombinant P69 produced in insect cells was highly active (Fig. 3B). A series of 2-5(A) oligomers is synthesized by 2-5(A) synthetases. Using denaturing gel electrophoresis for analyzing the products by size, we have previously observed that small 9-2 isoform is capable of synthesizing dimers to hexamers of 2-5(A) (10). In contrast, the recombinant P69 protein was very efficient in synthesizing higher oligomers (Fig. 3B). With increasing lengths of incubation time, more of the higher oligomers were synthesized. Up to 30-mers of 2-5(A) could be detected after 9 h of incubation.

Recombinant P69 Is a Dimeric Glycoprotein—Native P69 is a dimer (9). The same was true for the recombinant P69 purified from insect cells. Gel filtration analysis revealed that the purified recombinant P69 had an apparent molecular mass of 160 kDa (Fig. 4A). Since the calculated molecular mass of the polyhistidine-tagged P69 is 80.8 kDa, it showed that the protein exists primarily as a dimer.

The protein sequence of P69 indicates the presence of four potential N-glycosylation sites in the protein. That information, coupled with our failure to produce active protein in bacteria that lack protein glycosylation activity, prompted us to examine the glycosylation status of P69 expressed in insect cells. Because glycoproteins are known to bind to lectins, purified P69 was challenged to bind to two different lectins (Fig. 5B). P69 bound to both wheat germ agglutinin and ConA-coupled Sepharose but not to the Sepharose matrix itself. These data strongly suggest that P69 has sugar modifications. To determine if glycosylation of the protein is needed for its enzyme activity, unglycosylated P69 was synthesized in tunicamycin-treated insect cells because tunicamycin is known to inhibit all sugar modifications of glycoproteins. P69 was purified from tunicamycin-treated and untreated cells (Fig. 5A) and, as expected, the protein purified from the drug-treated cells did not bind to ConA-Sepharose (Fig. 5) thus demonstrating its lack of glycosylation. The unglycosylated protein was enzymatically inactive (Fig. 5C) indicating that sugar modification of the P69 protein is required for producing an active enzyme.

P69 Is a dsRNA-activated Non-processive Enzyme—The enzymatic characteristics of the recombinant protein were determined in the next series of experiments. In these experiments, thin layer chromatography of phosphatase-treated products was used for measuring the total amounts of 2-5(A) production. Under the experimental conditions used, enzyme activity increased linearly with increasing protein concentration and length of incubation (Fig. 6, A and B). The protein was enzymatically active even after 30 h of incubation (data not shown).
A substrate concentration curve revealed that the $K_m$ for ATP of this enzyme was 2.1 mM (Fig. 6C). A similar experiment with increasing concentrations of the cofactor, dsRNA, demonstrated that the purified protein was totally inactive without dsRNA and for the maximum enzyme activation, 5–10 mg/ml of poly(I):poly(C) was required (Fig. 6D). The various characteristics of the recombinant P69 protein are listed in Table II.

For other isozymes it has been suggested that 2-5(A) oligomerization is non-processive (22). The kinetics of synthesis of different oligomers by P69 (Fig. 3B) indicated that the same was also true for this isozyme. To directly examine the processivity of P69, the experiment shown in Fig. 6 was carried out. No 2-5(A) oligomers remained bound to the protein (lane 2, Fig. 7) and the profiles of the products made by fresh enzyme (lane 1, Fig. 7) and preincubated enzyme (lane 4, Fig. 7) were identical.

A 25-bp dsRNA Can Activate P69 Maximally—The nature of the dsRNA required for P69 activation was investigated in the next series of experiments. Perfect dsRNA of defined lengths

**TABLE I**

| Fraction             | Protein | Specific Activity $^a$ | Purification $^{\text{-}\text{fold}}$ | Yield $\%$ |
|----------------------|---------|-----------------------|---------------------------------|-----------|
| Clarified cell lysate| 50 mg   | 0.66                  | 1                               | 100       |
| Affinity purified protein | 50 μg  | 190                   | 290                             | 28.7      |

$^a$ Expressed as nanomoles of ATP polymerized/μg of protein/h.

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A 25-bp dsRNA Can Activate P69 Maximally—The nature of the dsRNA required for P69 activation was investigated in the next series of experiments. Perfect dsRNA of defined lengths

**FIG. 3.** Purification of active P69 in insect cells. A, NH$_2$-terminal hexahistidine-tagged P69 protein was expressed in insect cells and purified as discussed under “Materials and Methods,” electrophoresed on a 8% SDS-polyacrylamide gel, and visualized by Coomassie Blue staining. B, activity assay of purified recombinant P69. For each lane, 30 ng of protein was incubated in a 10-μl reaction with 50 μg/ml poly(I):poly(C) and 1 mM ATP for indicated time. 2-5(A) products were analyzed by gel electrophoresis.

**FIG. 4.** Physical characterization of purified P69. A, the recombinant P69 exists as a dimer. Purified proteins were analyzed by chromatography on a Sephacryl S300 column. Proteins present in each alternate fraction was analyzed as described under “Materials and Methods” and presented as percent of total. The positions of the different molecular weight markers are noted at the top. B, P69 binding to lectins. Purified protein (200 ng) was used for binding to wheat germ agglutinin-Sepharose (lane 1), ConA-Sepharose (lane 2), or Sepharose MB (lane 3). After washing, samples were eluted with SDS-PAGE loading buffer and analyzed by Western blotting. In lane 4, purified protein (20 ng) was loaded as positive control.

**FIG. 5.** Effect of glycosylation on P69 activity. A, affinity purified P69 from tunicamycin treated (lane 1) and untreated (lane 2) cells. H5 cells were treated with 10 μg/ml tunicamycin, P69 proteins from treated and untreated cells were purified as described under “Materials and Methods.” A Coomassie Blue-stained electrophorogram is shown. B, lectin binding of unglycosylated (lane 1) and glycosylated (lane 2) P69. 200 ng of purified protein for each sample was used for binding to ConA-Sepharose as described above. After binding, samples were analyzed by Western blotting with anti-His antibody. C, activity assay of unglycosylated and glycosylated P69. 1 μg/ml purified protein for each sample was assayed; products were analyzed and quantified.
were synthesized by in vitro transcription and their ability to activate P69 was tested. When tested at a high concentration (20 μg/ml), a 25-bp dsRNA was as active as longer dsRNAs (Fig. 8A), whereas a 15-bp dsRNA was less efficient in activating the enzyme. Moreover, higher oligomers of 2-5(A) were more efficiently synthesized in the presence of the 25-bp or longer dsRNA as compared with the 15-bp dsRNA (Fig. 8, A and B). Quantitation of total 2-5(A) synthesis in the presence of different concentrations of poly(I):poly(C), and the products were analyzed by thin layer chromatography.

### DISCUSSION

The 2-5(A) synthetases are interesting enzymes primarily because of their two unique properties: they are activated by dsRNA and they, unlike any other DNA or RNA polymerases, catalyze the formation of 2’-5’ phosphodiester bonds. Pursuit of mechanistic and structural investigation of these enzymes has been hampered by the lack of an ample source of pure proteins. Although synthesis of 2-5(A) synthetases can be induced in mammalian cells by interferon treatment, many isozymes are induced together and the cells do not tolerate their sustained expression at high levels. For these reasons, we sought to synthesize by in vitro transcription and their ability to activate P69 was tested. When tested at a high concentration (20 μg/ml), a 25-bp dsRNA was as active as longer dsRNAs (Fig. 8A), whereas a 15-bp dsRNA was less efficient in activating the enzyme. Moreover, higher oligomers of 2-5(A) were more efficiently synthesized in the presence of the 25-bp or longer dsRNA as compared with the 15-bp dsRNA (Fig. 8, A and B). Quantitation of total 2-5(A) synthesis in the presence of different concentrations of various dsRNAs revealed that 25-bp dsRNA was as good as the longer RNAs at every concentration tested (Fig. 8C). Their product profiles at each concentration were also very similar (data not shown). But for 15-bp dsRNA, the maximum level of synthesis was about 25% of that achieved by others (Fig. 8C) and synthesis of the higher oligomers was very inefficient (Fig. 8B). Curiously, for all RNAs, the product profile changed with the activator concentration, i.e. the formation of the higher oligomers was favored at higher concentrations of the dsRNA (Fig. 8B). Moreover, a low concentration of the 25-bp RNA (0.1 μg/ml) produced the same product profile as a high concentration (10 μg/ml) of the 15-bp RNA (Fig. 8B).

A different property of the dsRNA was tested in Fig. 8D. Another dsRNA-activated enzyme, PKR, cannot be activated by 2’-O-methylated dsRNA (23). To examine if this property is shared by P69, a 36-bp 2’-O-methylated dsRNA was synthesized. Even at 50 μg/ml, this RNA could not activate the P69 protein (Fig. 8D). Thus, it appears that free 2’-OH groups on the ribose moieties of the dsRNA are required for proper interactions with the P69 isozyme of 2-5(A) synthetases.

**Table II**

| Property          | Values          |
|-------------------|-----------------|
| ATP $K_{cat}$     | 2.1 mm          |
| dsRNA optimum     | 10 μg/ml        |
| Specific activity | 190 nmol/h/μg   |
| $k_{cat}$         | 7 s⁻¹           |
| Product profile   | pppA(2’p5’A)₁⁻₃₀ |

**Fig. 6.** Kinetic properties of purified P69. A, dependence of 2-5(A) synthesis on enzyme concentrations. Different amounts of proteins were incubated for 4 h under standard assay conditions, followed by product analysis by thin layer chromatography. B, time course of 2-5(A) synthesis. For each point, 1 μg/ml protein was assayed for the indicated length of time. C, synthesis of 2-5(A) as a function of substrate concentration. 0.8 μg/ml protein was assayed for 4 h. The $K_m$ for ATP (2.1 mM) was determined by fitting the data points with Michaelis-Menten equation. The inset shows the reciprocal plot of the same data. D, double-stranded RNA activation profile of P69. Purified protein (1 μg/ml each) was incubated for 4 h in presence of different concentrations of poly(I):poly(C), and the products were analyzed by thin layer chromatography.
As follows. 10 μl of reaction mixture was diluted with 40 μl of binding buffer (20 mM Tris-Cl, pH 7.5, 20 mM magnesium acetate, 2.5 mM dithiothreitol), applied to Ni-NTA beads, and incubated for 2 h at 4 °C. Following the incubation, beads were washed twice with the binding buffer, and resuspended in 10 μl of binding buffer of which 5 μl, which is 5 times compared with lane 1, were analyzed by gel electrophoresis (lanes 2 and 3). To show that the enzyme remained bound to Ni-NTA agarose, the bound enzyme was reincubated for 3 h with 50 μg/ml poly(I)poly(C) and 5 mM ATP, and the products were analyzed in lane 4.

Fig. 7. Non-processive synthesis of 2-5(A) by P69. Four samples containing 1 μg/ml protein were incubated in standard reaction mixture for 3 h. At the end of incubation, one reaction mixture was directly analyzed by gel electrophoresis (lane 1), and the other three were subjected to Ni-NTA agarose (lanes 2 and 4) or agarose (lane 3) binding as follows. 10 μl of reaction mixture was diluted with 40 μl of binding buffer (20 mM Tris-Cl, pH 7.5, 20 mM magnesium acetate, 2.5 mM dithiothreitol), applied to Ni-NTA beads, and incubated for 2 h at 4 °C. Following the incubation, beads were washed twice with the binding buffer, and resuspended in 10 μl of binding buffer of which 5 μl, which is 5 times compared with lane 1, were analyzed by gel electrophoresis (lanes 2 and 3). To show that the enzyme remained bound to Ni-NTA agarose, the bound enzyme was reincubated for 3 h with 50 μg/ml poly(I)poly(C) and 5 mM ATP, and the products were analyzed in lane 4.

We have previously expressed a small isozyme, 9-2, in both bacteria and insect cells (11, 13). Both systems produced enzymatically active 9-2 proteins although the bacterially expressed 9-2 protein had a lower specific activity (10). As reported here, P69, a medium isozyme was also expressed efficiently in both of these expression systems. But surprisingly, the bacterially expressed 9-2, 25 μg/ml, was however, lower than the optimum for 9-2, 25 μg/ml. Both proteins were also extremely stable: their enzymatic activity was maintained even after 20 h of incubation at 30 °C. There was, however, a major difference in one property of the two isozymes: the P69 isozyme synthesized much longer 2-5(A) chains than the 9-2 isozyme, which synthesized mostly dimers and trimers. Although, for 9-2, long incubations produced some higher oligomers, but nothing longer than hexamers could be detected as its product activity (10). In contrast, up to 30-mer of 2-5(A) was synthesized by the P69 isozyme. The mechanistic basis of this observed difference in the properties of the two isozymes is unclear at this time.

The possible basis of the ability of P69 to synthesize higher 2-5(A) oligomers could be that the enzyme was highly processive. Our experimental results, however, point to the contrary. No oligomer remained bound to the protein after their synthesis, suggesting that chain lengthening proceeds through multiple initiation events. The presence of all intermediates, from dimers to the highest oligomer, in the products produced during a given length of incubation, and the observed uniform gradient in the amounts of longer to shorter oligomers, also indicate that the reaction is non-processive. Our observation is in contrast to the recent report of Marie et al. (27) suggesting that the natural P69 may be a processive enzyme. Their conclusion was based on the negative observation that isolated dimers were poor acceptors for chain elongation. This observation is explainable by our finding that ATP is a much better acceptor than dimers or higher oligomers of 2-5(A). As a result, high concentrations of ATP favors dimer formation and inhibit the formation of higher oligomers (data not shown). In contrast, our conclusion that the enzyme reaction is non-processive is in tune with the earlier observation by Justesen et al. (22) that the enzyme purified from the reticuloocyte lysate could efficiently use dimers and trimers of 2-5(A) as acceptors.

We used the recombinant purified P69 protein for studying the nature of its activator. The protein was inactive as such and single-stranded RNA, DNA-RNA hybrid, or dsDNA could not activate it (data not shown). Double-stranded RNAs were the only activators and there were no specific sequence requirements for this activity. We have previously shown that the small isozyme can be activated by dsRNAs as short as 40 bp and by HIV-1 TAR RNA (28) and adenoviral VA1 RNA (11) which are not perfect dsRNAs. Results presented here show
that the P69 isozyme can be activated maximally even by a 25-bp dsRNA. Moreover, unlike 9-2, P69 was activated equally well by shorter or longer dsRNAs even at subsaturating concentrations. However, the shortest dsRNA tested, 15-bp dsRNA, behaved differently. It was a poor activator even at high concentrations. It suggests that more than one complete helical turn of the dsRNA may be necessary for optimum P69 recognition and its activation. This observation is reminiscent of the length requirement of dsRNA for activation of PKR, another class of dsRNA-activated enzyme (29). Similarly, like PKR (23), the lack of activation of P69 by 2'-O-methylated dsRNA indicates that free 2'-OH groups on the RNA are necessary for its interaction, a conclusion supported by its lack of activation by dsDNA or DNA-RNA hybrids. These observations revealed remarkable mechanistic similarities between the two classes of dsRNA-activated enzymes, 2-5(A) synthetase and PKR. Such similarities were somewhat unexpected in view of the known differences of their relevant properties: the dsRNA binding motifs of PKR are not present in P69; PKR, but not P69, is inhibited at high concentrations of dsRNA and dsRNA-binding of PKR is much stronger as revealed by the lack of dissociation of the PKR-dsRNA complex even at 0.5 M NaCl (30). As discussed above, it is not apparent why P69, but not 9-2, can synthesize longer 2-5(A) oligomers. Since our data indicates that 2-5(A) chain elongation is a non-processive reaction, binding of shorter 2-5(A) molecules, as acceptors of the next AMP, may be more efficient for P69 than 9-2. In this respect, it is interesting to note that higher 2-5(A) oligomer formation by P69 was also impaired at low dsRNA concentrations or when the activator was the short 15-bp dsRNA (Fig. 7, A and B). Thus, it appears that optimal interaction of dsRNA with the P69 protein not only dictates its ability to catalyze the 2'-5'-phosphodiester bond formation but also influences its choice of acceptor molecules such as ATP or 2-5(A) oligomers.

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FIG. 8. dsRNA activation characteristics of P69. A, product profile of 2-5(A) synthesis by different sized dsRNA. The assay was done under standard conditions in the presence of 20 μg/ml dsRNA for 3 h. B, product profiles of 2-5(A) synthesized in response to 15- and 25-bp dsRNA at various concentrations (0.1, 0.5, 2.5, 5, 10, and 20 μg/ml) for 3 h. C, activation of P69 by different sized dsRNA. Double-stranded RNAs of various lengths (15 bp (a), 25 bp (b), 55 bp (c), and 112 bp (d)) were synthesized as described earlier (17) and used to test their activation properties in standard assay conditions (5 mM ATP, 3 h). D, effects of 2'-O-methylation. Product profiles of 2-5(A) synthesized in presence of 50 μg/ml 34-bp dsRNA (lane 1) or 50 μg/ml 36-bp dsRNA with 2'-O-methyl modifications.
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