Activation of 2',5'-Oligo(A) Polymerase and Protein Kinase of Interferon-treated HeLa Cells by 2'-O-Methylated Poly (Inosinic Acid)•Poly(Cytidylic Acid)

CORRELATIONS WITH INTERFERON-INDUCING ACTIVITY*

(Received for publication, January 28, 1980)

Michael A. Minks, Deborah K. West, Susan Benvin, James J. Greene, Paul O. P. Ts'o, and Corrado Baglioni

From the Department of Biological Sciences, State University of New York at Albany, Albany, New York 12222, and the Division of Biophysics, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland 21205

An oligonucleotide polymerase and a protein kinase which require double-stranded RNA (dsRNA) for activation are induced in HeLa cells by human fibroblast interferon. The polymerase synthesizes a series of oligonucleotides from ATP, whereas the kinase phosphorylates a polypeptide of $M_r = 72,000$ and the $\alpha$ subunit of initiation factor eIF-2. Partially or fully 2'-O-methylated derivatives of poly(inosinic acid)•poly(cytidylic acid) (rI•rC) were used to determine the structural requirements of dsRNA in the activation of these two enzymes. While fully methylated polymers failed to activate either enzyme, partially methylated polymers activated the enzymes in specific manners. The activation of the kinase by the rI•rC analogues was affected more severely by the level of methylation than was the activation of the polymerase. Moreover, fully methylated analogues blocked the activation of the kinase by rI•rC, but not the activation of the polymerase. These observations are consistent with a biphasic model for enzyme activation similar to that proposed for interferon induction, which required the recognition of a relatively small region of rI•rC as the last step. Differences in the activation of the polymerase and kinase are explicable on the basis of the polymerase requirement for a smaller recognition region of the rI•rC duplex than the kinase. Dependence of polymerase activation on the level of methylation shows striking similarities with the interferon inducing activities of these analogues, suggesting a possible relationship between polymerase activation and interferon induction.

Interferons are glycoproteins secreted by virus-infected animal cells (1). These glycoproteins interact with other cells to render them resistant to a variety of viral infections (2). Although the precise mechanisms by which interferon is induced and an antiviral state established remain unclear, it seems likely that double-stranded RNA (dsRNA) plays an important role in both processes. Double-stranded RNA of natural or synthetic origin is a very effective interferon inducer (3) and is also a potent inhibitor of protein synthesis in cell-free systems prepared from interferon-treated cells (4–7). This effect of dsRNA is due to the activation of a protein kinase and an oligonucleotide polymerase which are present in elevated levels in interferon-treated cells (see Ref. 8 for references). The protein kinase inhibits protein synthesis by phosphorylating the $\alpha$ subunit of initiation factor eIF-2, whereas the other enzymatic activity polymerizes ATP into oligonucleotides containing adenosine residues linked by 2',5'-phosphodiester bonds (8). These oligonucleotides, designated 2',5'-oligo(A), activate a latent endonuclease present in both control and interferon-treated cells which degrades mRNA (9, 10).

The structural requirements of dsRNA necessary for interferon induction have been studied in great detail (3, 11). The molecular characteristics of active interferon inducers are: (i) a minimum molecular weight for both strands of dsRNA; (ii) resistance to degradations by nucleases; (iii) absence or presence of a minimum number of mismatched base pairs; (iv) presence of the 2'-OH group in both strands of a duplex (3). This latter structural requirement has been studied in detail by Greene et al. (12), who have prepared a series of dsRNAs with increasing degree of 2'-O-methyl substitution in either strand. The interferon-inducing activity of these dsRNAs correlated significantly with the presence of clusters of at least 6 or more ribosyl residues in the methylated strand (12).

We have recently investigated some of the structural requirements of dsRNA for the activation of the protein kinase and 2',5'-oligo(A) polymerase induced by interferon (13). Activating dsRNA is characterized by a minimum molecular weight for both strands and by the absence or presence of a minimum number of mismatched base pairs. These structural characteristics are similar to those required for interferon induction. This observation prompted us to investigate 2'-O-methylated dsRNA for activation of the protein kinase and 2',5'-oligo(A) polymerase. Activation of these enzymes declines with increasing methylation of either strand of dsRNA, with the fully 2'-O-methylated derivatives unable to activate either enzyme. Some interesting differences between the activation of the protein kinase and that of 2',5'-oligo(A) polymerase have been observed, with the protein kinase requiring larger clusters of ribosyl residues than the 2',5'-oligo(A) polymerase.

MATERIALS AND METHODS

Details of the experimental procedures which are similar to those

* This work was supported by Grant AI-11887 from National Institutes of Health to C. B. and by Grant GM 16066 from National Institute of General Medical Science to P. O. P. T. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviation used is: dsRNA, double-stranded RNA.
previously published (14–18) are presented in the miniprint supplement.

RESULTS

In the presence of ATP and rI-rC, a polypeptide of M, = 72,000 co-sedimenting with the ribosomes of interferon-treated HeLa cells is phosphorylated with high specificity (19). The level of dsRNA-dependent protein kinase activity has previously been quantitated by measuring the phosphorylation of this polypeptide (16). In this investigation we compared the ability of rI-rC, to activate the kinase with that of its partially or completely 2′-O-methylated analogues. A standard ribosome preparation, obtained from cells treated for 17 h with 100 units/ml of interferon, was incubated with different polymers and fractionated by gel electrophoresis (Fig. 1). In order to quantitate kinase activity, autoradiographs of the gels were scanned at 560 nm and the relative areas under the peaks corresponding to the M, = 72,000 polypeptide determined (16). Highly methylated polymers were essentially inactive, whereas polymers with less than 20% methylation in either strand were fully active; rI-rC, with 20% methylation in the C strand was partially active (Fig. 2A).

The same polymers were assayed for their ability to activate the 2′,5′-oligo(A) polymerase (Fig. 2B). Fully methylated polymers were inactive, whereas polymers with less than 20% methylation were fully active. Interestingly, polymers with intermediate contents of methylated nucleotides were partially active and there were differences in the effect of methylation of the C strand compared to the I strand. The activity of dsRNA 40% methylated in the C strand was lower, for example, than that of dsRNA 60% methylated in the I strand (Fig. 2B). Both these polymers were inactive, however, in the kinase activation assay. Activation of this latter enzyme is therefore much more sensitive to the effect of partial methylation than is the activation of the 2′,5′-oligo(A) polymerase. A similar effect of I or C strand methylation on the interferon-inducing activity of these polymers has previously been observed with human fibroblasts (12, 20).

Some possible reasons for the lower activity of the partially methylated polymers were investigated. Differential degradation of the methylated dsRNAs was ruled out in experiments measuring the time course of 2′,5′-oligo(A) synthesis (Fig. 3A). The rate of synthesis remained constant for at least 2 h with all the polymers assayed, indicating that the reduced amount of 2′,5′-oligo(A) synthesized with methylated dsRNA was not due to a decrease in activator concentration.

The different pattern of activation observed in the kinase versus the polymerase assay can also be due to the different conditions employed to test these enzymatic activities. Maximal synthesis of 2′,5′-oligo(A) is obtained in HeLa cell extracts with 25 mM Mg(OAc)2 and high dsRNA concentrations, whereas the kinase is assayed at 2 mM Mg(OAc)2 and low concentrations of dsRNA (13–16). To establish whether the Mg2+ concentration has some effect on the relative activation of the 2′,5′-oligo(A) polymerase by partially methylated rI-rC, analogues, we measured 2′,5′-oligo(A) synthesis at different Mg(OAc)2 concentrations (Fig. 3B). At low Mg2+ concentrations the methylated analogues were less active than rI-rC, and maximal activity with these polymers was observed with 15 mM Mg(OAc)2.

Another possible explanation for the lower activity of partially methylated rI-rC, is that it contains fewer sequences of unmethylated nucleotides of the length required for the activation of the 2′,5′-oligo(A) polymerase. If this were the case, addition of increasing amounts of partially methylated rI-rC, should result in increased activity. In an experiment measuring the increase in 2′,5′-oligo(A) synthesis with dsRNA concentration, maximal synthesis at different plateaus was obtained with the same concentration of methylated and unmethylated rI-rC, (Fig. 4). The maximal activity of partially methylated dsRNA was lower, however, than that of unmethylated dsRNA. Similar results were obtained in the kinase assay (data not shown).

In order to establish whether the 2′-O-methylated or 2′-deoxy derivatives of rI-rC, compete with dsRNA for activation of the 2′,5′-oligo(A) polymerase, these polymers were...
Protein Kinase and 2',5'-Oligo(A) Polymerase

FIG. 3. Time course (A) and dependence on Mg(OAc)₂ concentration (B) of 2',5'-oligo(A) synthesis promoted by 2'-O-methylated poly(I)-poly(C) derivatives. All the incubations contained 20 ng/ml of dsRNA. A, 150 μl incubations contained 5 mM [³²P]ATP (600,000 cpm) and 25 mM Mg(OAc)₂. 25 μl were withdrawn at the indicated times and analyzed for 2',5'-oligo(A) content as described under "Materials and Methods." B, 25 μl reactions containing 5 mM [³²P]ATP and the Mg(OAc)₂ concentration indicated in the abscissa were incubated for 60 min and analyzed for 2',5'-oligo(A) formation. • • • poly(I)-poly(C); Δ • • Δ, poly(I)-poly(C) 43% methylated in the C strand; ■ ■ ■, poly(I)-poly(C) 67% methylated in the I strand. The counts per min of ATP converted into 2',5'-oligo(A) are indicated in A or the relative conversion in B (100% activity corresponds to the conversion of 45 nmol of ATP into 2',5'-oligo(A) from an input of 125 nmol).

Fig. 4. Effect of increasing concentration of 2'-O-methylated poly(I)-poly(C) derivatives on the synthesis of 2',5'-oligo(A). Incubations prepared as described under "Materials and Methods" contained 150,000 cpm of [³²P]ATP. The counts per min of ATP converted into 2',5'-oligo(A) are indicated. A, • • • unmethylated poly(I)-poly(C); △ • △ 22% methylated C strand; ■ ■ ■ 43% methylated C strand. B, • • • unmethylated poly(I)-poly(C); △ • △ 58% methylated I strand; ■ ■ ■ 68% methylated I strand.

assayed in competition experiments (Table I) (see Miniprint). A constant input of rI-rC₉ was challenged with increasing amounts of polymers containing a partially or fully methylated strand or a deoxy strand. No decrease in 2',5'-oligo(A) polymerase activity was observed, even at an input ratio of inactive to active dsRNA of 45:1. In these experiments, the active dsRNA was assayed at a nonsaturating concentration (2.5 μg/ml; see Fig. 4) and at 25 mM Mg(OAc)₂. The same results were obtained when the competition experiments were repeated at 2 mM Mg(OAc)₂ (data not shown). These observations suggest that the 2',5'-oligo(A) polymerase has a much lower affinity for inactive rI-rC₉ analogues than for active rI₉-rC₀.

The protein kinase was assayed in similar competition experiments with opposite results (Table I). When increasing amounts of inactive rI₉-rC₀ analogues were added to incubations containing a constant input of rI₉-rC₀, a progressive decrease in kinase activity was observed. Some competition was also observed with the RNA/DNA hybrids which do not activate the kinase (21) and have negligible activity in the 2',5'-oligo(A) polymerase assay (14). Competition was observed in this case only at a polynucleotide concentration inhibitory for kinase activation (15, 16). These results show a further difference between the activation of the kinase and that of the 2',5'-oligo(A) polymerase.

The simplest interpretation of experiments assaying the activation of dsRNA-dependent enzymatic activities is that a minimum length of unmodified base pairs is required for the binding, or activation of these enzymes, or both. The activity of different polymers should in this case be related to the probability of occurrence of such sequences in each polymer. The distribution of methylated residues in the polymers assayed in the experiments described above is not random, since clustering of methylated residues has been observed by sequence analysis of the methylated polynucleotides (12). However, when these polymers were synthesized in reactions containing dimethyl sulfoxide as solvent, a more uniform distribution of methylated residues was observed (12). Runs of unmethylated residues were less frequent in these polynucleotides, therefore, than in polynucleotides synthesized in completely aqueous medium. Accordingly, when tested in the 2',5'-oligo(A) polymerase assay these latter polymers had higher activity than the polymers synthesized in dimethyl sulfoxide (Table I). The difference in the activation of the protein kinase, however, was negligible.

The relative frequency of runs of unmethylated nucleotides in the polymers synthesized by the two different procedures is reported in Table II together with the relative activity of the corresponding polymers in the kinase and 2',5'-oligo(A) polymerase assay. The activity of this latter enzyme measured at high Mg⁺⁺ concentration is approximately correlated with the frequency of runs of fewer unmethylated nucleotides than that measured at lower Mg⁺⁺ concentration, whereas the activity of the kinase seems correlated with the frequency of longer runs of unmethylated nucleotides. These trends indicate still further differences in the structural requirements of rI₉-rC₀ for the activation of the kinase versus the activation of the 2',5'-oligo(A) polymerase.

**DISCUSSION**

Double-stranded RNA may play a major role in both the induction of interferon (3, 11) and in the activation of two interferon-induced enzymes (8). To establish whether these two processes involve a similar recognition mechanism, we have tested in enzymatic assays rI₉-rC₀ analogues that are fully or partially methylated in one strand and which have previously been shown to induce interferon synthesis in a characteristic pattern (12, 20). Polymers in which one strand is fully methylated do not induce interferon synthesis (12) and do not activate either protein kinase or 2',5'-oligo(A) polymerase (Fig. 2). These enzymes differ, however, in their pattern of activation with partially methylated dsRNA. The 2',5'-oligo(A) polymerase is activated by rI₉-rC₀ with a relatively high content of methylation, whereas the kinase is not (Fig. 2). Methylation of the C strand of rI₉-rC₀ reduces the activation of 2',5'-oligo(A) polymerase more severely than methylation of the I strand. This effect of ribose methylation has previously been observed for interferon-inducing activity and has been explained by a greater clustering of similar residues in the methylated I strand than in the methylated C strand (12). Since the methylated sequence is inactive in induction or activation, the biological activity must depend on the uninterrupted, unmethylated rI₉-rC₀ sequences. Thus, the differences in the activation pattern of the two enzymatic
activities investigated may be explained by a requirement of the protein kinase for a longer stretch of unmethylated base pairs than that required for the activation of 2',5'-oligo(A) polymerase. Such a correlation between frequency of clusters of unmethylated nucleotides and enzyme activity has been observed by De Clercq et al. (11).

We have previously reported that dsRNA at least 40 to 60 base pairs long is required for the activation of 2',5'-oligo(A) polymerase and protein kinase using mismatched analogs of rL_3 rC_3 (13). The present observation that smaller clusters of unmethylated nucleotides may activate these enzymes can be reconciled with our previous report by assuming that a minimum length of base pairs is required for enzyme binding to dsRNA and that 6 to 12 base pairs (0.5 to 1 helical turn) can activate these enzymes. A similar explanation has been proposed by Greene et al. (12) for the interferon-inducing activity of methylated rL_3 rC_3. The induction process may occur as a biphasic event (22), involving first the binding of a large segment of dsRNA of the proper topology to a cellular receptor, followed by the triggering of interferon induction by a smaller region of dsRNA (12). Binding to the larger segment, although an obligatory step, may not lead to activation. Methylated polymers, for example, compete for activation of the protein kinase, but it cannot be activated when more than 20% of the residues are 2'-O-methylated.

Under the present conditions of the enzyme assays (nonlimiting for ATP substrate, linearly dependent on enzyme with a constant reaction rate), addition of saturating amounts of partially methylated dsRNA produces a reaction rate lower than that produced by saturating amounts of unmethylated dsRNA. In fact, the data in Fig. 4 suggest that the difference between the analogues and rL_3 rC_3 is not due to their "affinities" toward the enzyme but due to the efficiencies of the enzyme-dsRNA complex in the reaction. It should be pointed out that "affinity" is used here to interpret the experimental data, but that the extent of binding of dsRNA to the enzyme that synthesizes 2',5'-oligo(A) cannot be estimated by the rate of formation of these oligonucleotides.

The striking similarities in the requirement of free 2'-OH groups for interferon induction and 2',5'-oligo(A) polymerase activation raise the possibility that this enzyme may be involved in the cellular recognition of dsRNA. An exception to the requirement for free 2'-OH groups has, however, been reported by De Clercq et al. (23). An analogue of rL_3 rC_3, in which the 2'-OH of the rL_3 strand is replaced by an azido group was an active interferon inducer in human fibroblast cultures but was less active than rL_3 rC_3 in other cell types (23). It seems possible that the 2'-azido analogue is structurally related to rL_3 rC_3, and it will be of interest to establish whether it activates the synthesis of 2',5'-oligo(A) in cell extracts.

REFERENCES
1. Lindenmann, J., Burke, D. C., and Isaacs, A. (1957) J. Exp. Pathol. 38, 551-562
2. Friedman, R. M. (1977) Bacteriol. Rev. 41, 543-567
3. Torrence, P. P., and De Clercq, E. (1977) Pharmacol. Ther. 2, 1-88
4. Kerr, I. M., Brown, R. E., and Ball, L. A. (1974) Nature 250, 57-59
5. Kerr, I. M., Brown, R. E., Clemens, M. J., and Gilbert, C. S. (1976) Eur. J. Biochem. 69, 551-561
6. Roberts, W. K., Hovanessian, A., Brown, R. E., Clemens, M. J., and Kerr, I. M. (1976) Nature 264, 477-480
7. Lewis, J. A., Falcoff, E., and Falcoff, R. (1978) Eur. J. Biochem. 86, 497-509
8. Baglioni, C. (1979) Cell 17, 255-264
9. Baglioni, C., Minks, M. A., and Maroney, P. A. (1978) Nature 273, 684-687
10. Clemens, M. J., and Williams, B. R. G. (1978) Cell 13, 565-572
11. De Clercq, E. (1974) Mol. Biol. Rep. 52, 173-208
12. Greene, J. J., Alderfer, J. L., Tazawa, I., Tazawa, S., Ts'o, P. O., O'Malley, J., and Carter, W. A. (1978) Biochemistry 17, 4214-4220
13. Minks, M. A., West, D. K., Benvin, S., and Baglioni, C. (1979) J. Biol. Chem. 254, 10180-10183
14. Minks, M. A., Benvin, S., Maroney, P. A., and Baglioni, C. (1979) J. Biol. Chem. 254, 5068-5084
15. Lenz, J. R., and Baglioni, C. (1978) J. Biol. Chem. 253, 4219-4223
16. West, D. K., and Baglioni, C. (1979) Eur. J. Biochem. 101, 461-468
17. Rottman, F., and Heinlein, K. (1968) Biochemistry 7, 2634-2641
18. Tazawa, I., Tazawa, S., Alder, J., and Ts'o, P. O. P. (1972) Biochemistry 11, 4931-4937
19. Baglioni, C., Maroney, P. A., and West, D. K. (1979) Biochemistry 18, 1765-1770
20. Merigan, T. C., and Rottman, F. (1974) Virology 60, 297-301
21. Sen, G. C., Taira, H., and Lengyel, P. (1978) J. Biol. Chem. 253, 5915-5921
22. De Clercq, E., Torrence, P. F., and Witkop, B. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 182-186
23. De Clercq, E., Torrence, P. F., Stollar, B. D., Hobbs, J., Fukui, T., Kakiuchi, N., and Ikehara, M. (1978) Eur. J. Biochem. 88, 341-349
Protein Kinase and 2',5'-Oligo(A) Polymerase

ACTIVATION OF 2',5'-OLIGOADENYLATE AND PROTEIN KINASES BY INTERFERON-TREATED Hela Cells by 2',5'-Oligoadenylate Acid-Methyloligoadenylate ACIDS: CORRELATION WITH INTERFERON-INDUCING ACTIVITY

Michael A. Mattei, Deborah M. West, Susan Kirch, James J. Garrett, Paul O.R. Tev's and Howard Meyler

MATERIALS AND METHODS

Hela cells were purchased from New England Nuclear. Hela cells were grown in subculture medium (18) and treated with 100 units/ml of human interferon of known activity (1 x 10^6 units/ml) obtained from the Interferon Working Group, National Cancer Institute, NIH for 17 hr prior to harvest. Cell extracts were prepared as previously described (18). The interferon-dependent protein kinase was assayed by monitoring the phosphorylation of a polylysine of Mr > 12,000 as previously described (16, 18). Synthesis of 2'-5'-oligo(A) was determined by incubating for 1 hr at 37°C as previously described (18). 0.1% of cell extract (about 10 mg/ml of protein) 5.0 ml of 10 mM Tris-Cl, 0.2 mM MgCl2, and 1.5 ml of a final solution of 0.1 M K3PO4 unless otherwise indicated. The reaction products were separated by 15% polyacrylamide gel electrophoresis at 200 v for 2 hr, stained with Coomassie blue, and photographed. The reaction was initiated by the addition of the 2'-5'-oligo(A) and was terminated by the addition of 0.1 M HClO4. The reaction was stopped by the addition of 0.1 M HClO4.

Table 1

Comparison between poly(1-lysine) and 2',5'-oligo(A) on 2'-5'-oligo(A) polymerase

| Polymer | Proteinase activity | 2',5'-oligo(A) |
|---------|---------------------|----------------|
| (a) | (b) | (c) |
| Poly(1-lysine) | 100 | 50 |
| Poly(2',5'-oligo(A)) | 30 | 10 |

Table 2

Relationship between nucleic acid polymerase activity of
the 2',5'-oligoadenylate polymerase

| Polymer composition | Solvent | Relative activity | n = 6 |
|---------------------|---------|------------------|-------|
| (a) Poly(1-lysine) | H2O | 100 | 100 |
| (b) Poly(2',5'-oligo(A)) | 0.4 M NaCl | 100 | 100 |
| (c) Poly(1-lysine) | 0.05 M MgCl2 | 100 | 100 |
| (d) Poly(2',5'-oligo(A)) | 0.05 M MgCl2 | 100 | 100 |

The activity of the indicated polymers was determined as described. The activity of the indicated polymers on 2',5'-oligo(A) was determined as described (12). The activity of the indicated polymers on 2',5'-oligo(A) was determined as described (12). The activity of the indicated polymers on 2',5'-oligo(A) was determined as described (12). The activity of the indicated polymers on 2',5'-oligo(A) was determined as described (12).