Interferon Induction and Its Dependence on the Primary and Secondary Structure of Poly(inosinic acid)·Poly(cytidylic acid)†

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ABSTRACT: The synthetic interferon (IF) inducer $rI_n \cdot rC_n$ was modified by substituting the ribosyl residues with either their corresponding deoxy(dC or dI) or 2′-*O*-methyl analogues (mC or mI). The polynucleotide duplexes of these analogues are inactive as IF inducers. Circular dichroism (CD) studies revealed that, while the deoxy substitution produces significant changes in the conformation of $rI_n \cdot rC_n$, the 2′-*O*-methyl substitution produces no detectable change. Biological competition experiments indicated that the methylated duplexes $mI_n \cdot rC_n$, $rI_n \cdot mC_n$, and $mI_n \cdot mC_n$ all compete with $rI_n \cdot rC_n$ for IF induction, while the deoxy duplexes $dI_n \cdot rC_n$ and $rI_n \cdot dC_n$ do not. These results are consistent with those predicted from the CD data. Copolymer duplexes $(mI,rI)_n \cdot (mC,rC)_n$ of varying degrees of methylation and residue clustering were also evaluated for IF induction in human fibroblasts. The IF-inducing capabilities of these duplexes correlated highly with the presence of clusters containing six or more consecutive ribosyl residues. These combined observations suggest that interaction of $rI_n \cdot rC_n$ with the cell in the induction process may occur in a biphasic manner involving first the topological recognition of a large segment of the RNA to allow for proper binding to the putative cellular receptor, followed by recognition of a much smaller region of the RNA corresponding to 6–12 consecutive ribosyl residues (0.5 to 1 helical turn) which is responsible for the triggering of the induction process.

Interferon (IF[1]) is an antiviral protein whose synthesis is induced after exposure of mammalian cells to viruses or to double-stranded RNAs such as the synthetic polynucleotide complex poly(inosinic acid)·poly(cytidylic acid) ($rI_n \cdot rC_n$). Although the mechanism of IF induction is still unclear, systematic modification of the $rI_n \cdot rC_n$ complex has provided the means to examine the relationship between macromolecular structure and the production of IF. Results from our laboratory (Carter et al., 1972) and from others (DeClercq, 1974) have shown that in order for IF induction by $rI_n \cdot rC_n$ to occur the inducer has to meet certain common structural requirements. These include a sufficiently high molecular weight, intactness of the double-stranded complex, and adequate resistance to nucleases. In addition to these now generally accepted requirements, the backbone configuration of $rI_n \cdot rC_n$ is a major structural requirement. Circular dichroism studies of $rI_n \cdot rC_n$ analogues with 5-pyrimidine and C_7 purine substitutions have suggested that the IF-inducing ability of a nucleic acid is related to its helicity (Bobst et al., 1976).

By determining the extent to which the backbone of $rI_n \cdot rC_n$ must be perturbed in order to abolish its IF-inducing ability, important insights could be gained into the interaction of this molecule with its putative cellular receptor. Based on this premise, we have modified the backbone of $rI_n \cdot rC_n$ in two different ways: (1) by completely substituting the ribosyl residues with either their corresponding deoxy (dC or dI) or 2′-*O*-methyl (mC or mI) analogues and (2) by varying the extent and distribution of the 2′-*O*-methyl substitution. The effect of these modifications on the secondary structure of the modified inducers was studied by CD. In cases where one or the other strand of $rI_n \cdot rC_n$ was only partially substituted by 2′-*O*-methyl residues, the primary structure of that strand was analyzed by alkaline hydrolysis followed by isolation of the resultant alkaline-resistant fragments. Analysis of the hydrolysis data was facilitated by the development of a stochastic model which describes the clustering of residues in copolymers. In this study, we report how these structural parameters are related to the biological activities of the polymers as IF inducers and as competitive inhibitors to $rI_n \cdot rC_n$ for IF induction.

Materials and Methods

Synthesis of Polymers. The starting materials 5′-AMP and 5′-CMP were obtained from Sigma Chemical Co., St. Louis. Preparation of 2′-O-methylated nucleotides and polynucleotide copolymers was according to the procedures of Rottman and Heinlein (1968), and Tazawa et al. (1972).

Nucleotide Ratio Determination. $(mC,rC)_n$ copolymers were degraded to monomers in an incubation mixture containing 222 units/mL *C. atrox* phosphodiesterase (PDase, P-L Biochemicals, Inc.), 1 mg/mL bacterial alkaline phosphatase (BAPase, Worthington Biochemicals), 150 units/mL micrococcal nuclease (Worthington Biochemicals), 0.1 M Tris-Cl (pH 9.0), 5 mM $CaCl_2$, and 10 mM $MgCl_2$. After incubation at 37 °C for 72 h, the mixture was applied to Whatman 1 MM paper and chromatographed in 2-propanol–NH_4OH–H_2O, 7:1:2. Complete separation was achieved after development for 19 h with R_f values of 0.82 and 0.60 for the nucleosides mC and rC, respectively. The nucleosides were eluted with water

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[1] Abbreviations used are: IF, interferon; dC, deoxyribocytosine nucleotide; rC, ribocytosine nucleotide; mC, 2′-*O*-methylribocytosine nucleotide; dI, deoxyriboinosine nucleotide; rI, riboinosine nucleotide; mI, 2′-*O*-methylriboinosine nucleotide; CD, circular dichroism; ICR, $rI_n \cdot rC_n$ receptor for interferon induction.

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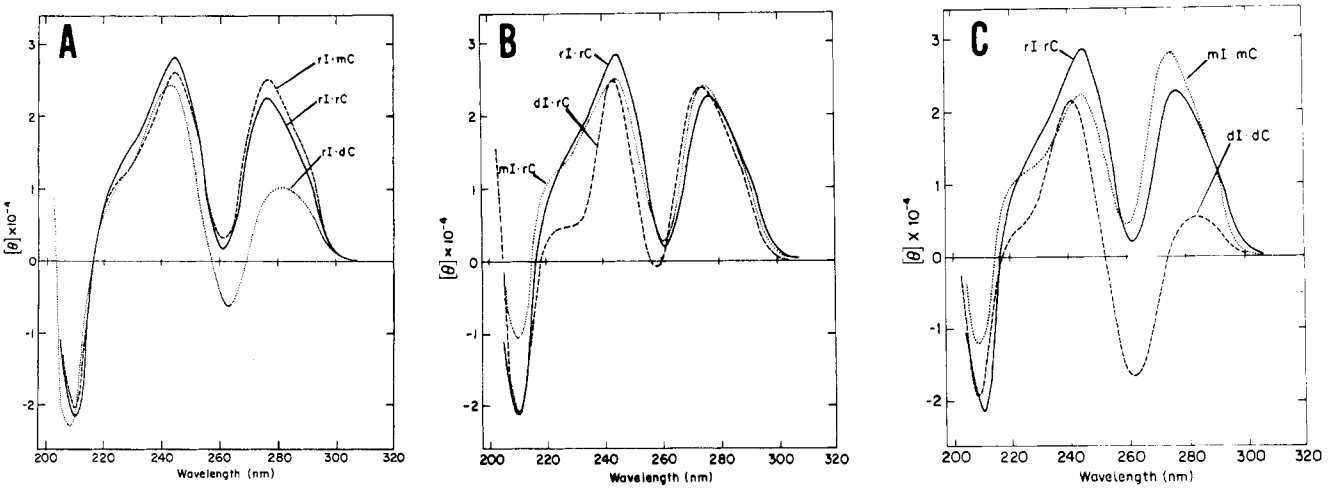


FIGURE 1: CD spectra of $rI_n \cdot rC_n$ and its analogues in 0.15 M NaCl, 0.01 M phosphate, 0.001 M $MgCl_2$ (pH 7.2): (A) effect of C-strand substitution; (B) effect of I-strand substitution; (C) effect of both I- and C-strand substitutions.

and concentrations determined from optical absorbances.

Degradation of $(mI,rI)_n$ was conducted in 0.1 M Tris-Cl (pH 9.0) with 750 units/mL micrococcal nuclease and 2.5 mM $CaCl_2$. After incubation for 72 h at 37 °C, BAPase was added to a final concentration of 1 mg/mL and PDase to a concentration of 65 units/mL and incubated for an additional 24 to 48 h. This mixture was then applied to Whatman 3 MM paper and developed for 20 h in 1-propanol–NH_4OH–H_2O, 55:10:35. R_f values for the nucleosides mI and rI are 0.80 and 0.68, respectively. After chromatography, the nucleosides were eluted with water and their concentrations determined from optical absorbances.

Isolation of mI and mC Clusters. Hydrolysis of the copolymers under alkaline conditions allows for the separation of the alkaline-resistant clusters of 2′-*O*-methyl nucleotides from the alkaline-sensitive clusters of ribosyl nucleotides. $(mI,rI)_n$ was dissolved in 0.1 M KOH and incubated in a sealed glass tube at 100 °C for 30 min. These conditions proved to be too harsh for $(mC,rC)_n$, as evidenced by partial deamination. However, incubation in 0.3 M KOH for 18 h at 37 °C produced satisfactory hydrolysis. Following alkaline hydrolysis, the incubation mixtures were neutralized with HCl, made 0.1 M in Tris-Cl (pH 8.0), and incubated for an additional 90 min with 1 mg/mL BAPase. The reaction products were separated on DEAE-Sephadex A-25 (Pharmacia) using a linear NaCl gradient of 0.05 to 0.6 M. This procedure is summarized in Scheme I. Analysis of this hydrolysis data is given in the Appendix.

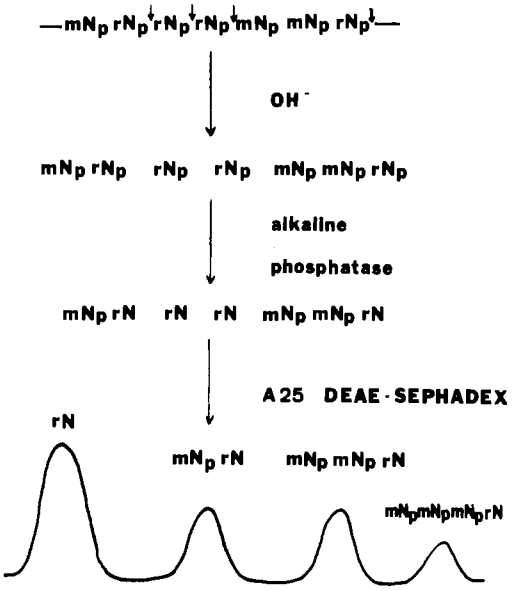
Biological Assay. Human foreskin fibroblasts were exposed to polynucleotide complexes in the physiological salt "buffer A" (0.15 M NaCl, 0.01 M phosphate, 0.001 M $MgCl_2$, pH 7.2) for 1 h at 37 °C and then washed three times before reincubation in fresh maintenance media. After allowing 18 h for the development of the antiviral state, the cells were infected with vesicular stomatitis virus (VSV-Indiana serotype) at a multiplicity of infection of 1 plaque-forming unit/cell.

(1) Intracellular resistance was determined colorimetrically (Finter, 1966; Carter, 1970). Percent intracellular protection is expressed as the arithmetic mean of two or more assays.

(2) Reduction in Virus Yield. Medium harvested from VSV infected cells was diluted serially and titered on mouse L929 cells by the agar-overlay technique. The reduction in virus yield is expressed as the average of at least two titrations.

(3) Interferon Titer. Interferon titers were determined by titration on GM258 cells, human fibroblasts trisomic for chromosome 21.

SCHEME I: Procedure for the Isolation of 2′-*O*-Methyl Nucleotide Clusters.



Results

Circular dichroism (CD) studies of the 2′-O-methylated analogues of $rI_n \cdot rC_n$ along with their corresponding deoxy duplexes were initiated to evaluate the effect of 2′-OH substitution on the conformation of the RNA backbone. Figure 1A shows the effect of C-strand modification on the CD of $rI_n \cdot rC_n$. It can be seen that the CD spectrum of $rI_n \cdot mC_n$ is nearly indistinguishable from that of the parent $rI_n \cdot rC_n$, but both are different from that of $rI_n \cdot dC_n$. Similarly, in the case of I-strand modification, the CD spectra of $mI_n \cdot rC_n$ and $rI_n \cdot rC_n$ are almost identical but differ from that of $dI_n \cdot rC_n$ (Figure 1B). Replacement of both strands by 2′-*O*-methyl polynucleotides $(mI_n \cdot mC_n)$ alters the CD spectrum slightly but clearly not to the extent as replacement by deoxy residues $(dI_n \cdot dC_n$, Figure 1C). These optical studies suggest that, whereas the deoxy substitution results in a profound conformational change, 2′-O-methylation does not greatly alter the general conformation or "topology" of the $rI_n \cdot rC_n$ complex.

Previous studies have shown that the deoxy (Colby et al., 1971) and 2′-*O*-methyl (DeClerq et al., 1972; Merigan and Rottman, 1974) analogues of $rI_n \cdot rC_n$ are inactive as interferon inducers. This result may reflect the inability of these com-

TABLE I: Indirect Competition.

incubation (1 h)		% protection	
1st	2nd	expt A	expt B
buffer A	rI·rC (10^{-4} M)	100	100
buffer A	mI·rC (10^{-4} M)	2	1
buffer A	rI·mC (10^{-4} M)	0	0
buffer A	mI·mC (10^{-4} M)	0	ND
buffer A	dI·rC (10^{-4} M)	0	ND
buffer A	rI·dC (10^{-4} M)	0	ND
rI·rC (10^{-4} M)	rI·rC (10^{-4} M)	92	100
mI·rC (10^{-5} M)	rI·rC (10^{-4} M)	100	100
mI·rC (10^{-4} M)	rI·rC (10^{-4} M)	76	76
mI·rC (10^{-3} M)	rI·rC (10^{-4} M)	36	39
rI·mC (10^{-5} M)	rI·rC (10^{-4} M)	95	97
rI·mC (10^{-4} M)	rI·rC (10^{-4} M)	66	65
rI·mC (10^{-3} M)	rI·rC (10^{-4} M)	30	28
mI·mC (10^{-5} M)	rI·rC (10^{-4} M)	96	
mI·mC (10^{-4} M)	rI·rC (10^{-4} M)	78	
mI·mC (10^{-3} M)	rI·rC (10^{-4} M)	5	
dI·rC (10^{-5} M)	rI·rC (10^{-4} M)	97	
dI·rC (10^{-4} M)	rI·rC (10^{-4} M)	100	
rI·dC (10^{-5} M)	rI·rC (10^{-4} M)	85	
rI·dC (10^{-4} M)	rI·rC (10^{-4} M)	97	
rI·rC (10^{-4} M)	mI·rC (10^{-4} M)	90	
rI·rC (10^{-4} M)	mI·rC (10^{-3} M)	87	
rI·rC (10^{-4} M)	rI·mC (10^{-4} M)	98	
rI·rC (10^{-4} M)	rI·mC (10^{-3} M)	93	
rI·rC (10^{-4} M)	mI·mC (10^{-4} M)	100	
rI·rC (10^{-4} M)	mI·mC (10^{-3} M)	86	

plexes to bind to the putative $rI_n \cdot rC_n$ receptor (ICR) for interferon induction. In order to test this possibility, the ability of these analogues to compete with $rI_n \cdot rC_n$ in the induction of IF was examined. Since the modified complexes induce no IF, successful competition with $rI_n \cdot rC_n$ would result in binding to the ICR without contributing to IF production. Such binding would block the accessibility of the ICR, reducing the effectiveness of $rI_n \cdot rC_n$ to induce IF.

The modified analogues were competed either indirectly or directly with $rI_n \cdot rC_n$. In the indirect competition experiment, human fibroblast cells HF926 were preincubated with the test complexes for 1 h, washed, and then incubated for another hour with 10^{-4} M $rI_n \cdot rC_n$. Table I shows the protection provided by these treatments to HF926 cells against challenge by VSV. The analogues $mI_n \cdot rC_n$, $rI_n \cdot mC_n$, $mI_n \cdot mC_n$, $dI_n \cdot rC_n$, and $rI_n \cdot dC_n$ were all inactive. These results also demonstrate that preexposure of cells to $mI_n \cdot rC_n$, $rI_n \cdot mC_n$, and $mI_n \cdot mC_n$ significantly reduces induction of protection against VSV by $rI_n \cdot rC_n$. Control cultures (i.e., first preexposure to $rI_n \cdot rC_n$ and then a second exposure to $rI_n \cdot rC_n$) as well as cultures pretreated with the deoxy analogue $dI_n \cdot rC_n$ or $rI_n \cdot dC_n$ showed no significant reduction in the effectiveness of the second $rI_n \cdot rC_n$ treatment. The results for the deoxy compounds are based on only one set of data. Due to the shortage of material, this experiment was not repeated.

To ensure that the diminution of $rI_n \cdot rC_n$ activity by pretreatment with 2′-O-methylated analogues was not the result of cytotoxicity induced by the analogues, the order of polymer addition was reversed. The results (Table I) from this experiment show that the second treatment by the 2′-O-methylated complexes had no effect in reducing the induction of protection against virus by $rI_n \cdot rC_n$.

In the direct-competition experiment, $mI_n \cdot rC_n$, $rI_n \cdot mC_n$, and $mI_n \cdot mC_n$ are added to HF926 cells together with $rI_n \cdot rC_n$. Protection against VSV challenge was measured in one experiment (experiment A) and the IF titer and VSV yield reduction in addition to protection against VSV were measured in the other experiment (experiment B). Our results, shown

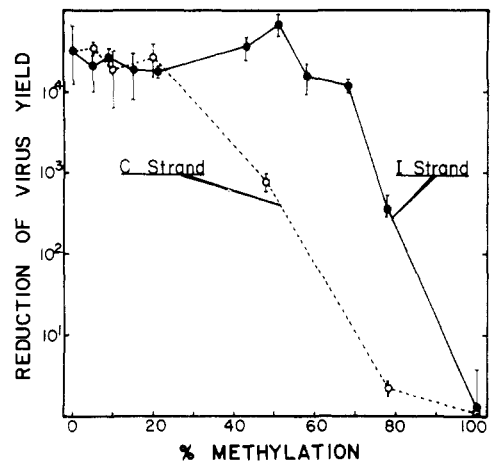


FIGURE 2: Effect of 2′-O-methylation on the antiviral activity of $rI_n \cdot rC_n$. Copolymers containing both ribosyl and 2′-*O*-methyl nucleotides were synthesized in aqueous buffer according to the procedures under Materials and Methods and annealed to their unmodified complementary strands. The resultant duplexes containing the copolymers were tested for their ability to inhibit VSV virus replication in human fibroblast cells. The "error" bars indicate the highest and lowest values. The solid (I strand) and open (C strand) circles are the average values. Virus controls (untreated cultures) gave virus yields of between 2 and 8×10^8 pfu.

in Table II, indicate that all these analogues compete with $rI_n \cdot rC_n$. However, $mI_n \cdot mC_n$ may not be as good a competitor as $mI_n \cdot rC_n$ or $rI_n \cdot mC_n$. These results are all consistent with those predicted from the CD data. Thus, the ability of $rI_n \cdot rC_n$ analogues to compete with $rI_n \cdot rC_n$ for the ICR is apparently determined by the "topological similarity" of the analogues to $rI_n \cdot rC_n$.

While complete substitution of either strand in the $rI_n \cdot rC_n$ duplex by deoxy or 2′-*O*-methyl residues abolishes its biological activity, this does not preclude the possibility that partially substituted polynucleotides are active as IF inducers. Therefore, the biological activities of polynucleotides containing both (rI,mI) or (rC,mC) residues with varying extents of substitution were investigated. The antiviral activity of these copolymer complexes $(rI,mI)_n \cdot rC_n$ and $rI_n \cdot (rC,mC)_n$ were assayed in human fibroblasts by their ability to inhibit VSV virus yield. The results of this assay (Figure 2) show several interesting features. First, increasing the level of methylation in either the I or C strand does not depress their antiviral activity until a certain critical degree of methylation is reached, whereupon the activity rapidly decreases. Secondly, the decline in activity begins to occur at a lower level of methylation in the C strand than in the I strand. These results confirm the previous observations by Merigan and Rottman (1974).

The copolymers used in this study were synthesized in aqueous buffer. It has generally been assumed that the distributions of the nucleotide residues are random. More recently, it has been shown that the action of PNPase is under the influence of the solvent (Rottman and Johnson, 1969) and that the nucleotide distribution is not always random (Chou and Singer, 1971; Simuth et al., 1971; Rottman and Johnson, 1969). However, synthesis of copolymers in 30% (v/v) dimethyl sulfoxide (Me_2SO) increases the degree of randomization in the incorporation of purine nucleotides (Rottman and Johnson, 1967).

Copolymers $(rI,mI)_n$ having similar degrees of methylation as those synthesized in aqueous buffer were synthesized in 30% Me_2SO to determine to what extent the distribution of residues contributes to the IF-inducing ability of an RNA duplex. The results shown in Table III reveal that at high levels of 2′-*O*-methyl substitution (68% or higher) the duplex containing the

TABLE II: Direct Competition Experiment.[a]

treatment (1 h)	expt A, % protect.	expt B		
		% protect.	IF titer (units)	virus reduct.[b]
$rI_n \cdot rC_n$ (10^{-4} M)	100	100	270	1.8×10^9
$rI_n \cdot mC_n$ (10^{-5} M) + $rI_n \cdot rC_n$ (10^{-4} M)	100	98	310	1.5×10^9
$rI_n \cdot mC_n$ (10^{-4} M) + $rI_n \cdot rC_n$ (10^{-4} M)	65	70	75	5.1×10^8
$rI_n \cdot mC_n$ (10^{-3} M) + $rI_n \cdot rC_n$ (10^{-4} M)	8	36	31	7.2×10^8
$mI_n \cdot rC_n$ (10^{-5} M) + $rI_n \cdot rC_n$ (10^{-4} M)	100	82	180	1.5×10^9
$mI_n \cdot rC_n$ (10^{-4} M) + $rI_n \cdot rC_n$ (10^{-4} M)	84	49	120	1.8×10^9
$mI_n \cdot rC_n$ (10^{-3} M) + $rI_n \cdot rC_n$ (10^{-4} M)	33	23	70	4.5×10^8
$mI_n \cdot mC_n$ (10^{-5} M) + $rI_n \cdot rC_n$ (10^{-4} M)	100	100	220	3.6×10^9
$mI_n \cdot mC_n$ (10^{-4} M) + $rI_n \cdot rC_n$ (10^{-4} M)	100	97	150	7.8×10^8
$mI_n \cdot mC_n$ (10^{-3} M) + $rI_n \cdot rC_n$ (10^{-4} M)	68	57	90	6.2×10^8

[a] HF926 cells were exposed to $rI_n \cdot rC_n$ (10^{-4} M) alone or to $rI_n \cdot rC_n$ (10^{-4} M) plus the methylated analogues for 1 h. The biological assays were as described under Material and Methods. [b] The VSV virus yield in the untreated control cultures was 3.6×10^9 plaque-forming units.

TABLE III: Relationship between Residue Clustering and Biological Activity.

polymer composition	% methylation	solvent[a]	$P(\geq n)$[b]			VSV yield reduct.
			$n = 3$	$n = 6$	$n = 9$	
$(rI)_n \cdot (rC)_n$	0	H_2O	1.0	1.0	1.0	3.3×10^4
$(rI_{91}mI_9)_n \cdot (rC)_n$	9	H_2O	ND	ND	ND	2.8×10^4
$(rI_{90}mI_{10})_n \cdot (rC)_n$	10	Me_2SO	ND	ND	ND	5.2×10^4
$(rI_{42}mI_{58})_n \cdot (rC)_n$	58	H_2O	0.592	0.262	0.107	1.6×10^4
$(rI_{41}mI_{59})_n \cdot (rC)_n$	59	Me_2SO	0.408	0.104	0.024	8.6×10^3
$(rI_{33}mI_{67})_n \cdot (rC)_n$	67	Me_2SO	0.265	0.036	0.004	1.6×10^1
$(rI_{32}mI_{68})_n \cdot (rC)_n$	68	H_2O	0.347	0.078	0.014	1.2×10^4
$(rI_{22}mI_{78})_n \cdot (rC)_n$	78	H_2O	0.232	0.049	0.009	3.8×10^2
$(rI_{22}mI_{78})_n \cdot (rC)_n$	78	Me_2SO	0.131	0.004	<0.001	5.0×10^0
$(rI)_n \cdot (rC_{22}mC_{78})_n$	78	H_2O	0.068	0.002	<0.001	2.1×10^0

[a] Solvents in which the polymers were synthesized: H_2O or 30% Me_2SO. [b] Probability of finding clusters of ribonucleotides greater than or equal to n consecutive residues.

copolymer $(rI,mI)_n$ synthesized in aqueous buffer was more active in viral reduction than its corresponding duplex containing the copolymer $(rI,mI)_n$ synthesized in 30% Me_2SO. The $(rI,mI)_n$ copolymers synthesized in Me_2SO have the same composition as those synthesized in water but presumably have different residue distributions.

These observations suggested that the primary structure of the RNA inducer plays a central role in the induction of IF. Meaningful interpretation of the biological data is therefore dependent upon determination of the $(rI,mI)_n$ sequence composition. This was accomplished by the statistical analysis of alkaline hydrolysis data according to the procedures described under Materials and Methods and in the Appendix. The result of this analysis is given in Table III. Nucleotide residues were more clustered in $(rI,mI)_n$ copolymers synthesized in water than in their corresponding copolymers synthesized in 30% Me_2SO. Little clustering of the ribosyl residues was observed in the pyrimidine copolymer $(rC_{22}mC_{78})_n$. These findings are reminiscent of the cooperative clustering in $(rU,mA)_n$, which occurs to a greater extent than in the pyrimidine copolymer $(rU,mC)_n$ (Rottman and Johnson, 1969).

The probability data in Table III also indicate that the presence of long stretches of consecutive ribosyl residues (>12 residues) is not necessary for antiviral activity. However, if the duplex has a low probability for the occurrence of smaller ribosyl clusters (6–12 residues), then its antiviral activity is also low. The antiviral activities of the inducers seem to correlate with the presence of at least six consecutive ribosyl nucleotides in the copolymer strand. This suggests that the recognition of a region comprised of only 6–12 ribosyl base pairs (0.5 to 1 helical turn) in the A or A′ conformation in the RNA duplex is needed to induce IF.

The antiviral activities of the duplexes $(rI_{22}mI_{78})_n \cdot rC_n$ and $rI_n \cdot (rC_{52}mC_{48})_n$, whose copolymers were synthesized in H_2O, support this conclusion. Although both these duplexes have some biological activity, the $(rI_{22}mI_{78})_n \cdot (rC_{52}mC_{48})_n$ duplex has no activity (results not shown). It is conceivable that when the copolymers were annealed to their unmodified complementary strands some stretches of ribosyl base pairs existed. Annealing of the two copolymers to each other greatly reduces the probability of the occurrence of ribosyl helical regions; hence, the abolishment of antiviral activity.

Discussion

That the interaction of $rI_n \cdot rC_n$ with a specific receptor is an obligatory event in IF induction has been inferred from the high degree of specificity in the induction of IF by polynucleotides and also by analogy with many other proteins which are concerned with cell regulation (Colby and Chamberlin, 1969; DeClercq, 1974). Based on a systematic chemical modification of $rI_n \cdot rC_n$, certain structural requirements for IF induction have been determined. However, little is known about how

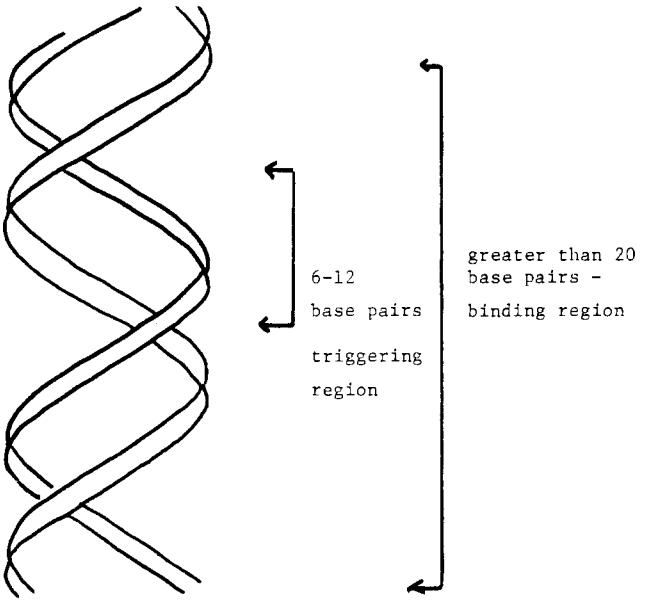


FIGURE 3: Hypothetical recognition regions of $rI_n \cdot rC_n$ in the induction of IF.

these requirements operate at the cellular level. One possibility is that these structural prerequisites determine if the RNA molecule binds to the ICR.

Our CD and biological studies indicate that, while both the deoxy and 2′-*O*-methyl modifications abolish the antiviral activity of $rI_n \cdot rC_n$, the 2′-O-methylation does not appreciably alter the topology of $rI_n \cdot rC_n$. The competition results imply that the 2′-*O*-methyl analogues bind to the ICR and that the deoxy analogues do not. These combined observations suggest that the binding of an analogue to the ICR is dependent upon its topological similarity to $rI_n \cdot rC_n$, but that the ICR binding alone is not the critical event in IF induction—subsequently, a more subtle recognition event must occur.

The indication from the studies with partially substituted 2′-*O*-methyl analogues is that only a relatively small region of the RNA duplex, possibly 6–12 consecutive ribosyl base pairs, is required for IF induction. Such a mechanism would explain the greater sensitivity of C-strand methylation in reducing the biological activity of $rI_n \cdot rC_n$. Copolymerization of rIDP and mIDP with PNPase in H_2O produces greater clustering of similar residues than copolymerization of rCDP and mCDP in H_2O. For the same level of methylation, the $(rI,mI)_n$ copolymers would have greater probabilities of finding ribosyl clusters of six or more residues than their corresponding $(rC,mC)_n$ copolymers.

The participation of only a small region of the RNA duplex in inducing IF appears contrary to the well-established finding that a minimum molecular weight of 2–5S is necessary for IF induction (Lampson et al., 1970; Tytell et al., 1970; Niblack and McCreary, 1971; Morahan et al., 1972; Arimura, 1975). Moreover, an earlier study from our laboratory showed that complexes of oligo(I·rC)$_n$, where the oligomeric length of I was less than 20, were inactive as IF inducers (Carter et al., 1972). These apparent contradictions can be reconciled if the IF-induction process is considered as a biphasic event involving first the general recognition of a large segment of the RNA of the proper topology, thereby permitting proper positioning and binding to the ICR, followed by the recognition of a much smaller region of the RNA duplex corresponding to 6–12 base pairs (0.5–1 helical turn) which triggers the critical induction event (Figure 3).

Appendix: Statistical Analysis

If the occurrence of residues in a sequence is influenced or correlated by the nature of a finite number of neighboring residues, then the probability of an arbitrary specific sequence can be expressed in terms of probabilities that reflect the extent of correlation between neighboring residues. Three cases which represent successively greater degrees of correlation will be considered here: (I) no correlation or random occurrence, (II) nearest-neighbor correlation, and (III) nearest- and next-nearest-neighbor correlation.

For example, designating the probability of the sequence -rNrNmNrNmN- as $P = P_{rrmrm}$ then for the three cases described above, P, the sequence probability, is given by:

$$\begin{aligned} &\text{(I)}\quad P = P_r P_r P_m P_r P_m \\ &\text{(II)}\quad P = P_r P_{(r/r)} P_{(r/m)} P_{(m/r)} P_{(r/m)} \\ &\text{(III)}\quad P = P_{rr} P_{(rr/m)} P_{(rm/r)} P_{(mr/r)} \end{aligned} \tag{1}$$

The probabilities contained in eq 1 are defined as follows:

P_r = a priori probability of the occurrence of rN = mole fraction of rN residues = f_r (2)

P_{rm} = a priori probability of the occurrence of the sequence rNmN = mole fraction of rNmN doublets = f_{rm}

$P_{(r/m)}$ = conditional probability that given rN at site n, mN follows at site $n + 1$

$P_{(rm/r)}$ = conditional probability that given the sequence rNmN at sites n and $n + 1$, rN follows at site $n + 2$, etc.

The probabilities in eq 2 have the following conservation relationships:

$$\begin{aligned} P_r + P_m &= 1 \\ P_{rm} + P_{rr} &= P_r \\ P_{rmr} + P_{rmm} &= P_{rm} \\ P_{(r/m)} + P_{(r/r)} &= 1 \\ P_{(rm/r)} + P_{(rm/m)} &= 1 \end{aligned} \tag{3}$$

and have the symmetry properties:

$$\begin{aligned} P_{rm} &= P_{mr} \\ P_{mrr} &= P_{rrm} \\ &\text{etc.} \end{aligned} \tag{4}$$

From eq 1 the probability of an arbitrary sequence can be calculated if the appropriate conditional probabilities are known. Below we indicate the experimental data required to determine these quantities.

Case I. The occurrence of any sequence is equal to the product of f_r and f_m in the sequence, since

$$P_r = f_r;\ P_m = f_m \tag{5}$$

All that is required is the mole fractions f_r and f_m.

Case II. The conditional probabilities required for the calculation of the sequence probabilities are dependent upon the fraction of -mNmN-, -mNrN-, -rNrN-, and -rNmN- pairs. From eq 3 and 4, we have:

$$f_{rr} + f_{rm} = f_r;\ f_{mm} + f_{mr} = f_m;\ f_{mr} = f_{rm} \tag{6}$$

Knowing only f_r (or f_m) and f_{rm} (or f_{mr}), the nearest-neighbor

conditional probabilities are determined. Using, for example, the following "pair" relation,

$$P_{mr} = P_m P_{(m/r)}$$

gives

$$P_{(m/r)} = P_{mr}/P_m = f_{mr}/f_r$$

The remaining three conditional probabilities $P_{(r/r)}$, $P_{(r/m)}$, and $P_{(m/m)}$ follow from the conservation and symmetry properties. Determination of f_{mr} is based on the occurrence of a -mNrN- pair at every junction of a cluster of mN nucleotides and a cluster of rN nucleotides. The number of -mNrN- junctions is just equal to the number of rN nucleotides that are not hydrolyzed by alkaline treatment and, hence, are attached to an mN cluster. Thus,

$$[\text{-mNrN-}] = [\text{-rN-}]_{total} - [\text{rN-}]_{hydrolyzed} = [\text{-rNmN-}] \quad (7)$$

or

$$f_{mr} = (f_r)_{total} - (f_r)_{hydrolyzed}$$

Therefore, in this treatment, the only experimental data required is the total amount of rN in the polymer and the amount of rN monomers hydrolyzed.

Case III. The dinucleotides and the trinucleotides in the alkaline hydrolysates separated by DEAE-Sephadex chromatography provide a direct determination of the quantities P_{rmr} and P_{rmmr}. Using the following relations:

$$\begin{aligned} P_{rmr} &= P_{rm}P_{(rm/r)} \\ P_{(rm/r)} + P_{(rm/m)} &= 1 \\ P_{rmmr} &= P_{rm}P_{(rm/m)}P_{(mm/r)} \\ P_{(mm/r)} + P_{(mm/m)} &= 1 \end{aligned} \quad (8)$$

$P_{(rm/r)}$, $P_{(rm/m)}$, $P_{(mm/r)}$, and $P_{(mm/m)}$, four of the eight possible conditional properties can be obtained. With these four conditional probabilities, the probability of any sequence of methyl clusters, rm_nr, is given by:

$$P_{rm_nr} = P_{rm}P_{(rm/m)}[P_{(mm/m)}]^{n-2}P_{(mm/r)} \quad (n \geq 2) \quad (9)$$

Similarly, the probability of a general ribosyl cluster, mr_nm, is:

$$P_{mr_nm} = P_{mr}P_{(mr/r)}[P_{(rr/r)}]^{n-2}P_{(rr/m)} \quad (n \geq 2) \quad (10)$$

In order to use eq 10, the remaining four conditional probabilities are needed. These are related through the following three equations:

$$\begin{aligned} P_{(rr/r)} + P_{(rr/m)} &= 1 \\ P_{(mr/m)} + P_{(mr/r)} &= 1 \\ P_{rrm} = P_{rr}P_{(rr/m)} &= P_{mrr} = P_{mr}P_{(mr/r)} \end{aligned} \quad (11)$$

Since the present experiments cannot provide any ribosyl clusters and, hence, P_{rrm}, P_{mrr}, P_{rrr} (from which one of the remaining conditional probabilities can be obtained and from (eq 11) all of the rest), the following approximation is used:

$$\left(\frac{P_{mrm}}{P_{rrr}}\right)_{case\ III} = \left(\frac{P_{mrm}}{P_{rrr}}\right)_{case\ II} \times (\text{correction factor})$$

$$= \left(\frac{P_m P_{(m/r)} P_{(r/m)}}{P_r P_{(r/r)}}\right) K_r \quad (12)$$

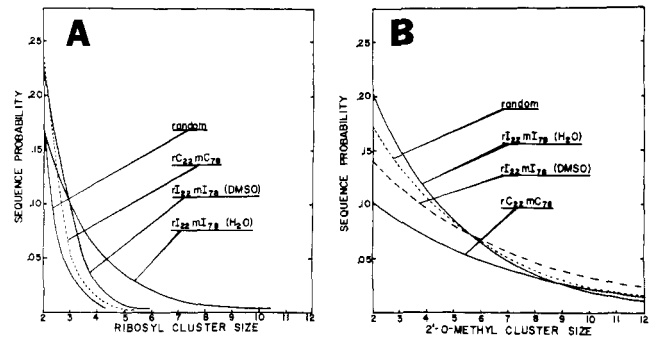


FIGURE 4: Normalized probability profiles for the polymers $(rC_{22}mC_{78})_n$, $(rI_{22}mI_{78})_n$ both synthesized in water, and $(rI_{22}mI_{78})_n$, synthesized in 30% Me_2SO. The probabilities were calculated according to the stochastic model using data obtained from the alkaline hydrolysis: (A) probability profile for the occurrence of ribosyl clusters; (B) probability profile for the occurrence of 2′-*O*-methyl clusters.

This ribosyl cluster correction factor K_r reflects the difference between the treatment based on case II and that based on case III. Assuming that the influence of the next-nearest-neighbor correlation on mN clusters is the same for rN clusters, the experimentally determined ratios for cases II and III for mN clusters can be used:

$K_r \approx K_m$ = methyl correction factor

$$= \left(\frac{P_{rmr}}{P_{mmm}}\right)_{case\ III} \Big/ \left(\frac{P_{rmr}}{P_{mmm}}\right) \quad (13)$$

The case II treatment was used in the analysis of the (rI,mI) hydrolysis data. The case III conditional probabilities are highly dependent upon the ratio of the mNrN and mNmNrN fragments. Small differences in this ratio result in large differences in the conditional probabilities. Since larger fragments often overlapped into the mNmNrN peak in (rI,mI) copolymers, the case III treatment was deemed less desirable for (rI,mI). For the case of the (rC,mC) copolymers, however, the extreme degree of residue nonrandomness (tendency to alternate) necessitates the use of the case III treatment for accurate analysis. Since the residues tend to alternate in (rC,mC), there is little detectable large alkaline-resistant fragments to overlap into the peaks of interest. Figure 4 shows the probability profiles derived from this treatment for the copolymers $(rI_{22}mI_{78})$ synthesized in water and in Me_2SO and also for $(rC_{22}mC_{78})$ synthesized in water.

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Nuclear Magnetic Resonance Investigation of the Interaction of a ^{13}C-Labeled Quinacrine Derivative with DNA†

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ABSTRACT: A quinacrine derivative with [^{13}C]methyl groups on both the aliphatic (side chain) and aromatic (acridine) nitrogens was prepared from quinacrine using [^{13}C]methyl iodide. As expected, the ^{13}C nuclear magnetic resonance spectrum of this compound had two major signals corresponding to the two labeled methyl groups. On adding this compound to sonicated calf thymus DNA at molar ratios of 1:4 (drug to DNA nucleotides) and less (intercalation binding), both methyl signals were dramatically reduced in intensity at low ionic strength. As the ionic strength was increased, the side chain methyl signal became significantly more intense and approached the free solution line width at ionic strength greater than 1.0. Both the ring and side chain methyl signals were also reduced in intensity at low ionic strength with glucosylated T_4D bacteriophage DNA as with calf thymus DNA. These results can be interpreted in terms of a model that involves intercalation and immobilization of the quinacrine aromatic ring even at high ionic strength. The side chain is tightly bound at low ionic strength (presumably through interaction with the DNA phosphate groups) but has a considerably shorter group correlation time at high ionic strength suggesting that the side chain has considerable freedom of movement even though the molecule remains bound to DNA through the acridine ring. These results indicate that a single intercalated molecule has a bimodal interaction with DNA. The acridine ring and side chain groups have different binding constants for DNA and this difference in binding for a *single* molecule can be accentuated by increased salt concentrations. At molar ratios of 1:1 the intercalation sites are saturated but at low ionic strengths the acridine methyl group signal remains at low intensity relative to the unbound compound. The side chain methyl signal, however, has appreciable intensity in this complex. As the ionic strength is increased, both the side chain and aromatic methyl signals increase in intensity suggesting dissociation of the complex. These data are consistent with a binding model involving stacking and immobilization of the acridine ring in a self-association type complex induced by the negatively charged DNA phosphate-deoxyribose backbone. The side chain in this 1:1 complex must possess a considerable degree of rotational freedom to account for its relative short correlation time. Addition of either sodium or magnesium ions disrupts this complex due to competition with DNA phosphate binding sites.

Acridine derivatives display a wide spectrum of biological properties which result from their ability to form complexes with intracellular DNA (Peacocke, 1973). Complexation of the acridine, quinacrine (Figure 1), with DNA in vivo is convincingly demonstrated by its use as a chromosomal fluorescence stain and by its ability to eliminate bacterial plasmids (Lurquin, 1974). In addition, quinacrine and related derivatives possess antimalarial (Henry, 1973) and antineoplastic activity (Cain et al., 1976) which is thought to result from their ability to complex with DNA. Intercalation is the predominant interaction mode of many acridines and related planar aromatic compounds with DNA (Lerman, 1961; Waring, 1970; Blake & Peacocke, 1968). For compounds without cationic substituents, such as proflavin (Lerman, 1964), acridine orange (Blake & Peacocke, 1966), and coralyne (Wilson et al., 1976), intercalation simply involves insertion of the planar aromatic ring between DNA base pairs with local extension and unwinding of the double helix. Very little of the drug aromatic ring system protrudes into either the major or minor groove of DNA. Other compounds such as 2-hydroxystilbamidine (Festy & Daune, 1973), netropsin (Wartell et al., 1974), and

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