Immunochemical Analysis of the Structure of 2′,5′-Oligoadenylate†

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ABSTRACT: Rabbit antibodies specific for 2′,5′-oligoadenylate were induced by immunization with pA2′pA2′pA conjugated through the 5′-terminal phosphate to methylated bovine serum albumin, or through the 2′(3′)-terminal ribose to bovine serum albumin. A competition enzyme-linked immunosorbent assay was devised to explore the specificity of the antisera. The oligonucleotides employed in cross-reactivity studies included 2′,5′-oligoadenylates, mixed linkage isomers with both 2′,5′- and 3′,5′-phosphodiester bonds, 3′,5′-oligoadenylates, base-modified 2′,5′-oligonucleotides, 2′,5′-oligo(3′-deoxyadenylate), and 2′,5′-oligoadenylates modified at the 5′ or 2′(3′) terminus. When the immunogen was pA2′pA2′pA conjugated to carrier protein through the 5′-phosphate, the resulting antibodies recognized determinants specified by the 2′,5′-phosphodiester backbone, the 5′-phosphate, and the bases. When conjugation to carrier was through the 2′(3′)-ribose, the resulting antiserum lacked base specificity but maintained a strong preference for 2′,5′-linked oligonucleotides. The strong preference of both antisera for 2′,5′-phosphodiester bonds may reflect a unique three-dimensional conformation of the ribose–phosphate backbone of 2′,5′-oligonucleotides. Unlike other alterations, modification at the 5′ terminus did not substantially alter the features of 2′,5′-oligoadenylate recognized by either antiserum.

Research on the mechanism of the antiviral action of interferon led to the discovery of 2′,5′-oligoadenylate synthetase [for review, see Lengyel (1982)]. This interferon-induced enzyme is activated in vitro by synthetic double-stranded RNA (Kerr & Brown, 1978; Baglioni, 1979) by a process that depends on the size and nature of the dsRNA[1] (Minks et al., 1979; Torrence et al., 1981b). The product, 2′,5′-oligoadenylate, or 2′,5′-oligo(A), is polymerized from ATP. The occurrence of 2′,5′-oligo(A) in some interferon-treated, virus-infected cells has been reported (Williams et al., 1979; Nilsen et al., 1981a; Silverman et al., 1982). Activation of the 2′,5′-oligo(A) synthetase in vivo may be mediated by dsRNA in viral replicative intermediates (Nilsen et al., 1980, 1981b). In cell extracts, 2′,5′-oligo(A) appears to bind preferentially to one protein, which is probably the 2′,5′-oligo(A) specific endonuclease (Cayley et al., 1982; Floyd-Smith et al., 1982). Binding of 2′,5′-oligo(A) to the latent endonuclease results in the degradation of RNA (Clemens & Williams, 1978; Ratner et al., 1978; Williams et al., 1978; Eppstein & Samuel, 1978; Chernajovsky et al., 1979) with a preference for cleavage after UpA or UpU sequences (Wreschner et al., 1981b; Floyd-Smith et al., 1981). This degradation may play a role in the antiviral action of interferon. A preferential degradation of viral RNA in extracts of interferon-treated cells (Nilsen & Baglioni, 1979) and a 2′,5′-oligoadenylate-mediated cleavage of ribosomal RNA in interferon-treated virus-infected cells have been demonstrated (Wreschner et al., 1981a). A role for 2′,5′-oligo(A) in the inhibition of viral mRNA cap formation (Goswami et al., 1982) and in cellular regulation and/or differentiation (Stark et al., 1979; Krishnan & Baglioni, 1980; Besancon et al., 1981; Kimchi et al., 1979) has also been suggested. When deliberately introduced into cells, 2′,5′-oligo(A) results in an inhibition of protein synthesis and of viral replication (Hovanessian & Wood, 1980; Williams & Kerr, 1978).

These observations suggest that 2′,5′-oligo(A) or its congeners may be useful in studying the mechanism of interferon action, or as chemotherapeutic agents. Since certain forms, namely, the 5′-monophosphorylated species, p5′A2′(p5′A)$_n$, where $n \geq 2$, block the activation of the endonuclease by 2′,5′-oligo(A) in extracts of mouse L cells (Torrence et al., 1981a), development of antagonists of 2′,5′-oligo(A) is also feasible. On the basis of the success of others in obtaining oligonucleotide specific antibodies, we investigated the interaction of 2′,5′-oligoadenylate with antibodies to further define the antigenic determinants of nucleic acids and to identify unique features of 2′,5′-oligoadenylate that may be required for its specific recognition by the endonuclease.

Materials and Methods

Reagents. Methylated bovine serum albumin (mBSA) and poly(oxyethylene) monolaurate sorbitan (Tween 20) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) was purchased from Miles Laboratories (Elkhart, IN), and poly(I)·poly(C) was obtained from P-L Biochemicals (Milwaukee, WI). Ficoll 400, Sephadex G-100, Sephadex LH-60, and DEAE-Sephadex were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Affinity-pu-

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[1] Abbreviations: 2′,5′-oligo(A), pppA2′p(A2′p)$_n$A where n = 1 to about 10; dsRNA, double-stranded RNA; ELISA, enzyme-linked immunosorbent assay; Ficoll–(pA)$_3$, pA2′pA2′pA conjugated through the 5′-phosphate to AECM-Ficoll; AECM, [N-(2-aminoethyl)carbamoyl]-methylated; BSA, bovine serum albumin; BSA–2′(3′)-(pA)$_3$, pA2′pA2′pA conjugated through the 2′(3′) terminus to BSA; mBSA, methylated bovine serum albumin; mBSA–5′-(pA)$_3$, pA2′pA2′pA conjugated through the 5′-terminal phosphate to mBSA; PBS, 0.01 M sodium phosphate, pH 7.4, and 0.14 M NaCl; Tween 20, poly(oxyethylene) monolaurate sorbitan; PBS–T, PBS with 0.05% Tween 20; NADP, nicotinamide adenine dinucleotide phosphate; TEAB, triethylammonium bicarbonate; Im(pA)$_3$, 5′-imidazolide of pA2′pA2′pA; ϵA, 1,N^6-ethenoadenosine, adenosine modified by addition of an ethenyl bridge between N1 and the 6-amino nitrogen; br^{8}A, adenosine modified by substitution of bromine for the C8 hydrogen; p(A2′p)$_n$Aahp, oligoadenylate modified by periodate oxidation and Schiff base formation with n-hexylamine followed by cyanoborohydride reduction, i.e., the 2′(3′)-terminal ribose was converted to azahexapyranose to give to 2-(9-adenyl)-4-hexyl-6-(hydroxymethyl)morpholine; Ficoll, a commercial product consisting of branched copolymers of sucrose and epichlorohydrin.

rified peroxidase-labeled goat anti-rabbit IgG was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Poly(vinyl chloride) plates were obtained from Dynatech Laboratories (South Windhad, ME). The 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) and A3'pA3'pA were purchased from Boehringer Mannheim (Indianopolis, IN). The p(2'dA)3'p(2'dA)3'p(2'dA) was obtained from P-L Biochemicals (Milwaukee, WI).

Preparation of Oligoadenylates. The preparation of 5'-phosphoryladenylyl(2'→5')adenylyl(2'→5')adenosine (pA2'pA2'pA) has been described elsewhere (Imai & Torrence, 1981b). Other 2',5'-linked oligonucleotides and mixed linkage isomers were synthesized and purified from reaction mixtures essentially as described by Sawai et al. (1981). In brief, the oligoadenylates prepared by Pb^{2+} ion-catalyzed polymerization of phosphoroimidazolides were separated by chromatography on DEAE-cellulose and then identified by thin-layer chromatography of the products obtained after treatment with bacterial alkaline phosphatase, nuclease P_1, snake venom phosphodiesterase, or alkali. Further purification was accomplished by high-performance liquid chromatography on a Beckman ODS C18 column eluted with a gradient of 50 mM ammonium phosphate and methanol/water (1/1). 5'-Triphosphorylated oligoadenylates were prepared as described previously (Imai & Torrence, 1981a) or purchased from P-L Biochemicals. Preparation of the 2'(3')-modified pA2'pA2'pAahp and similar compounds has been described (Imai et al., 1982). Synthesis of other oligonucleotides, including pA3'pA3'pA (K. Lesiak, J. Imai, and P. F. Torrence, unpublished data), A5'pppp5'A2'pA2'pA (J. Imai and P. F. Torrence, unpublished data), and mannose 6pp5'A2'pA2'pA (K. Lesiak and P. F. Torrence, unpublished data), will be published elsewhere. The base-substituted oligonucleotides pI2'pI2'pI, pC2'pC2'pC, and pU2'pU2'pU and the cordycepin analogue p(3'dA)2'p(3'dA)2'p(3'dA) were the generous gifts of Dr. Hiroaki Sawai, University of Tokyo [see Sawai et al. (1983); Sawai & Shinomiya, 1982; Sawai et al., 1981; Sawai & Ohno, 1981a,b]. Cordycepin trimer core was provided by Dr. Wolfgang Pfleiderer (University of Konstanz) and Dr. Robert Glazer (NCI).

Thin-Layer Chromatography. Analytical thin-layer chromatography was done on Analtech silica gel GF and Merck poly(ethylenimine)–cellulose F plates with the following solvent systems: (A) 1-butanol/ethanol/water/concentrated ammonium hydroxide, 60/20/20/1; (B) 0.1 M ammonium bicarbonate.

Preparation of Imidazolide of pA2'pA2'pA. pA2'pA2'pA (triethylammonium salt, 233 A_{260} units, 6.74 μmol) was dissolved in dry pyridine and dried by two consecutive additions and evaporations of dry pyridine, followed by two consecutive additions and evaporations of dry benzene. The residue was dissolved in a mixture of dry dimethyl sulfoxide (Me_2SO) (130 μL), triethylamine (5 μL, 36 μmol), and tri-*n*-octylamine (2.5 μL, 5.7 μmol). Carbonyldiimidazole (5.5 mg, 33.8 μmol) was added, and the reaction mixture was stirred at room temperature for 40 min. After completion of the reaction had been ascertained by silica gel thin-layer chromatography (system A), the reaction mixture was poured into a solution of NaI (10.0 mg, 67.5 μmol) in dry acetone (2.5 mL) on a vortex mixer. After a few minutes of mixing, the resultant white precipitate was pelleted by centrifugation at 1500 rpm for 5 min. The precipitate was washed twice with dry acetone and then dissolved in dry methanol for absorbance measurements. The yield of imidazolide of pA2'pA2'pA [(Im(pA_3)] was 98% (228 A_{260} units, 6.61 μmol). The methanol solution was evaporated to dryness, and the residue was dried over P_2O_5 for a few hours before further reaction.

Conjugation to Ficoll. [N-(2-Aminoethyl)carbamoyl]-methylated-Ficoll (AECM-Ficoll) was prepared by the method of Inman (1975). To a mixture of AECM-Ficoll (10.0 mg, 25 nmol) and dry formamide (100 μL) was added Im($pA)_3$ (228 A_{260} units, 6.61 μmol). The reaction mixture was heated at 55 °C in a silicone oil bath for 2 h. After cooling, the reaction mixture was diluted with 0.1 M triethylammonium bicarbonate (TEAB) buffer, pH 7.5, and dialyzed against TEAB buffer at 5 °C for 18 h. The sample was then applied to a Sephadex G-100 column (1.7 cm × 26 cm) equilibrated with distilled water. The column was eluted with water at a speed of 12 mL/h. Fractions of 1.0 mL were collected and monitored by UV absorbance at 280 nm. Two distinct peaks were obtained at fractions 10–28 (void volume) and fractions 31–46. Fractions of the first peak were pooled (17 mL), and the absorbance was determined. The yield was 45.6 A_{260} units (1.32 μmol of pA2'pA2'pA). Lyophilization of the solution gave a residue of 9.3 mg (23.2 nmol of AECM-Ficoll). The ratio of moles of pA2'pA2'pA attached to Ficoll through the nucleotide 5'-phosphate per mole of Ficoll was estimated to be 57. This conjugate is referred to as Ficoll–($pA)_3$ and has the following putative structure: Ficoll–$OCH_2C(O)NH(CH_2)_2NHPO_2$-5'A2'p5'A2'p5'A.

Conjugation to Methylated BSA. To a solution of mBSA (16.2 mg, 246 nmol) in dry dimethyl sulfoxide (350 μL) was added Im($pA)_3$ (280 A_{260} units, 8.11 μmol) in dry Me_2SO (150 μL). The reaction mixture was stirred at 70 °C for 1 h and then applied to a Sephadex LH-60 column (1.7 cm × 80 cm) equilibriated with Me_2SO. The column was eluted with Me_2SO at a speed of 18 mL/h. Fractions of 3.0 mL were collected and monitored by UV absorbance at 280 nm. Two distinct peaks at fractions 14–20 and 28–36 were obtained. Fractions of the first peak, the void volume, were pooled, and the yield was determined to be 205 A_{260} units (5.94 μmol of pA2'pA2'pA). The sample was dialyzed against 8 M urea overnight. Half of the dialysate was discarded and replaced with distilled water every 3 h. This procedure was continued until the urea concentration of the dialysate was about 0.01 M to prevent precipitation of the protein. Finally, the sample was dialyzed against distilled water. The final yield was determined to be 4.52 μmol (156 A_{260} units) of pA2'pA2'pA, and the molar ratio of triadenylate to mBSA was 18. This conjugate is referred to a mBSA–5'-($pA)_3$.

Conjugation to BSA. pA2'pA2'pA was linked to BSA by the periodate oxidation method of Erlanger & Beiser (1964) essentially as described by Knight et al. (1981). The molar ratio of pA2'pA2'pA to BSA determined spectrophotometrically was 7. This conjugate is referred to as BSA–2'(3')-($pA)_3$.

Immunization and Treatment of Antisera. Three New Zealand White rabbits (80–016, 80–017 and 80–018) were immunized with 1 mg of mBSA–5'-($pA)_3$ emulsified in complete Freund's adjuvant and injected at multiple intradermal and intramuscular sites. Subsequent injections of 1 mg of conjugate in incomplete Freund's adjuvant were administered by subcutaneous and multiple intradermal injections on days 8, 19, and 64 following the initial injection. Sera were collected on days 27 ("a" bleed) and 74 ("b" bleed). Further boosts and bleedings were done at monthly intervals (bleeds "c", "d", etc.). Two New Zealand White rabbits (80–019, 80–020) were immunized with 650 μg of BSA–2'(3')-($pA)_3$ emulsified in complete Freund's adjuvant and injected as above. Subsequent boosts of conjugate in incomplete Freund's adjuvant were given

on days 23 and 57. Bleeds a and b were obtained on days 33 and 65, respectively. These rabbits were also further boosted and bled at monthly intervals. Unless noted otherwise, all experiments were performed with b bleeds. All sera were heated at 56 °C for 30 min prior to storage at −20 °C or at 4 °C with added 0.1% sodium azide. The antibodies to BSA that were present in serum 80–019b were removed by precipitation with BSA in slight antigen excess as determined by quantitative precipitation analysis (Williams & Chase, 1971). Protein was measured by the method of Bradford (1976) with reagents purchased from Bio-Rad Laboratories (Rockville Centre, NY).

Enzyme-Linked Immunosorbent Assay (ELISA). Polyvinyl 96-well microtiter plates rather than polystyrene plates were employed because initial experiments showed that they bound Ficoll–$(pA)_3$ more efficiently. Careful studies to determine the optimal concentrations of coating [Ficoll–$(pA)_3$] and antisera were determined by using checkerboard titrations as outlined by Engvall (1980). Polyvinyl plates were incubated with 0.2 mL per well of 0.02 μg/mL Ficoll–$(pA)_3$ at 37 °C for 3 h and then overnight at room temperature. The plates were washed once with PBS to which Tween 20 had been added to 0.05% (PBS–T) and stored at −20 °C. Each plate was warmed to room temperature and washed 3 times with PBS–T prior to use. Antiserum diluted with PBS–T or PBS–T with 0.2% BSA was added, and the plate was incubated for 3 h at room temperature or for 90 min at 30 °C. After the plates were washed 3 times with PBS–T, allowing the plates to soak for several minutes in each wash, peroxidase-labeled goat anti-rabbit IgG (40–200 ng/mL) in PBS with BSA (0.2–0.5%) was added. The plates were then incubated for 3 h at room temperature or for 90 min at 30 °C. After the plates were washed 3 times with PBS–T, substrate solution was added to the wells. This solution, as described by Engvall (1980), was 1 mg/mL 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in 0.1 M KH_2PO_4–citrate (pH 4.0) containing fresh H_2O_2 (0.003%). The absorbance in each well was read against a substrate blank after 15–30 min at room temperature by scanning the plate in a Titertek Multiscan (Flow Laboratories, McLean, VA), or a MR50 Microelisa Reader (Dynatech Laboratories). In competition assays, the test oligonucleotide diluted in PBS–T was added to the well prior to the diluted rabbit antiserum. The concentration of inhibitor required to achieve 50% inhibition of antibody binding was read from graphs of inhibitor concentration vs. absorption and was termed the IC_{50}. The final reaction volumes were usually 30–50 μL but could be varied from 20 to 200 μL. All data represent the average of several assays performed on duplicates or triplicates of each dilution.

Results

Antisera Screening. Initial experiments showed that the optimal concentration of Ficoll–$(pA)_3$ for coating poly(vinyl chloride) plates was 0.02 μg/mL (data not shown). Subsequent coating with human serum albumin or ovalbumin did not effect the binding or inhibition curves (data not shown). All rabbits immunized with either mBSA–5′-$(pA)_3$ or BSA–2′(3′)-$(pA)_3$ produced antibodies that bound to the Ficoll–$(pA)_3$-coated microtiter wells (Figure 1). The absorbance readings obtained in the ELISA were inversely proportional to the dilution of antiserum applied to the wells. The reactivity of various sera with pA2′pA2′pA and A2′pA2′pA were compared in competition assays (Table I). Sera from rabbits immunized with mBSA–5′-$(pA)_3$ (80-016, 80-017, and 80-018) showed a strong preference for the hapten used in the immunization, pA2′pA2′pA. Serum from one animal immunized with BSA–2′(3′)-$(pA)_3$ (80-019) reacted more strongly with A2′pA2′pA than with pA2′pA2′pA, whereas serum from another (80-020) showed a slight preference for pA2′pA2′pA over A2′pA2′pA. Inhibition was unaltered if the wells were coated with BSA–2′(3′)-$(pA)_3$ instead of Ficoll–5′-$(pA)_3$, indicating that inhibition was not dependent on the type of linkage in the coating material (data not shown). Wells coated with unconjugated AECM-Ficoll showed no significant reactivity with antiserum in the presence or absence of competitor, illustrating that no detectable binding of competitor–antibody complexes occurred and that the binding of the rabbit antibodies was to the pA2′pA2′pA portion of the Ficoll–$(pA)_3$ (data not shown). Antisera 80-017b and 80-019b were selected for further study on the basis of their sensitivities and differing specificities.

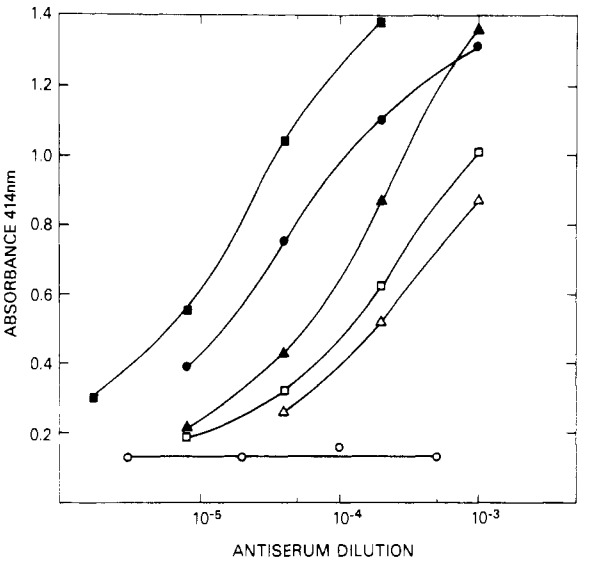

FIGURE 1: Screening of rabbit antisera for antibodies directed against p5′A2′p5′A2′p5′A. Rabbit antisera were diluted in PBS–T and incubated in microtiter wells precoated with Ficoll–$(pA)_3$. The binding of rabbit IgG to the wells was quantitated by binding of peroxidase-labeled anti-rabbit IgG antibody followed by addition of peroxidase substrate as described under Materials and Methods. (●) 80-017a; (■) 80-017b; (△) 80-019a; (▲) 80-019b; (□) 80-020b; (○) nonimmune rabbit serum.

Table I: Screening of Antisera to 2′,5′-Oligo(A)

antiserum	dilution[a]	concentration for 50% inhibition (μM)[b] pA2′pA2′pA	A2′pA2′pA
80-016a	2×10^{-3}	0.50	>30
80-016d	2×10^{-5}	0.05	5
80-017a	1×10^{-4}	0.15	>10
80-017b	1×10^{-5}	0.04	3
80-019a	1×10^{-3}	0.30	0.15
80-019b	2×10^{-4}	0.008	0.003
80-020a	1×10^{-3}	0.60	6

[a] Antisera dilutions were chosen from graphs as in Figure 1 so that the absorbance reading after 15–30 min would be 0.9–1.2. [b] Antisera diluted in PBS-T were added to Ficoll–$(pA)_3$-coated microtiter wells containing varied dilutions of either pA2′pA2′pA or A2′pA2′pA also diluted in PBS-T. The determination of the 50% inhibition points were from graphs of inhibitor concentration vs. absorbance (such as Figure 2).

Specificity of 80-017b. We determined the concentration of various nucleic acids required to inhibit the binding of the antiserum to immobilized Ficoll–$(pA)_3$ by 50% (Figure 2). Inhibition of the anti-mBSA–5′-$(pA)_3$ serum (80-017b) was most effective with the immunizing hapten, pA2′pA2′pA (Table II). Only slightly less reactive were the oligo-

Table II: Reactivity of Antisera 80-017b and 80-019b

	80-017b		80-019b	
	IC_{50}[a]	ID[b]	IC_{50}[a]	ID[b]
A2'pA	1000	25 000	0.14	18
A2'pA2'pA	3	75	0.003	0.4
A2'pA2'pA2'pA	1	25	0.0005	0.06
pA2'pA	25	625	0.30	38
pA2'pA2'pA	0.04	1.0	0.008	1.0
pA2'pA2'pA2'pA	0.15	3.8	0.004	0.5
pA2'pA2'pA2'pA2'pA	0.12	3.0	0.002	0.25
ppA2'pA	4	100	0.20	25
pppA2'pA	7	175	0.30	38
pppA2'pA2'pA	0.08	2.0	0.020	2.5
pppA2'pA2'pA2'pA	0.13	3.3	0.005	0.63
adenosine	≥5000	≥125 000	≥5000	≥625 000
5'-AMP	≥6000	≥150 000	2000	250 000
2'-AMP	≥1000	≥25 000	2000	250 000
5'-ADP	≥6000	≥150 000	500	62 500
5'-ATP	>4000	≥100 000	2000	250 000
2',5'-ADP	>1000	>25 000	700	87 500
NADP	>1000	≥25 000	40	5 000

[a] IC_{50} is the concentration (μM) required for 50% inhibition of the antiserum. [b] ID is the index of dissimilarity that is calculated by dividing the IC_{50} of the sample by the IC_{50} for the immunizing hapten pA2'pA2'pA (Prager & Wilson, 1971).

Table III: Reactivity of Rabbit Antisera with Linkage Isomers of $pA(2'pA)_n$

	80-017b		80-019b	
	IC_{50}[a]	ID[a]	IC_{50}[a]	ID[a]
pA2'pA2'pA	0.04	1.0	0.008	1.0
pA3'pA2'pA	10	250	0.2	25
pA2'pA3'pA	8	200	1.6	200
pA2'pA3'pA2'pA	8	200	0.2	25
pA3'pA2'pA2'pA	0.2	5	0.01	1.3
A3'pA3'pA	>100	>2500	300	37500
pA3'pA3'pA	>10	>250	15	1875
p(2'dA)3'p(2'dA)3'p(2'dA)	nd[b]	nd[b]	>1000	>125000
(3'dA)2'p(3'dA)2'p(3'dA)	>20	>500	>10	>1250
p(3'dA)2'p(3'dA)2'p(3'dA)	>20	>500	14	1700
(3'dA)2'p(3'dA)2'p(3'dA)2'p(3'dA)	20	500	4	500
p(3'dA)2'p(3'dA)2'p(3'dA)2'p(3'dA)	2	50	4	500

[a] IC_{50} and ID are defined in footnotes *a* and *b* of Table II. [b] Not determined.

nucleotides that contained the pA2'pA2'pA sequence. Dimers, particularly core (A2'pA), reacted poorly. Cross-reaction with longer oligomers was substantial, particularly if a 5'-phosphate was present. Reaction with ATP was barely detectable and no reactivity with adenosine, 5'-AMP, 5'-ADP, 2',5'-ADP, or NADP was detected even at the highest concentrations tested.

Less reactive than trimer and tetramer cores were the monophosphorylated mixed-linkage isomers containing one 3',5'-phosphodiester bond and one or two 2',5' bonds (Table III). In most cases the concentration of mixed-linkage isomer required to attain 50% inhibition of 80-017b was substantially greater than the amount of corresponding 2',5'-linked oligomer required to achieve the same degree of inhibition. The exception was the tetramer mixed-linkage isomer pA3'pA2'pA2'pA which reacted slightly less than pA2'pA2'pA. The 3',5'-linked oligoadenylates were unreactive at the highest concentrations tested, and if the 3'-OH groups were absent, as the cordycepin 5'-monophosphate analogue, p(3'dA)2'p(3'dA)2'p(3'dA), antibody reactivity was decreased drastically. When the adenylate moiety contained an ethenyl bridge between N1 and the 6-amino nitrogen [p(ϵA)2'p(ϵA,)2'p(ϵA)] or when the base was replaced by hypoxanthine, cytosine, or uracil, no reactivity was observed at the highest concentrations tested (10 μM) (Table IV). Inhibition by the analogue containing bromine in place of the adenylate C8 hydrogen was detectable, but the inhibition curve was substantially flatter than those observed for all other compounds

Table IV: Reactivity of Rabbit Antisera with Base-Modified Analogues

	80-017b		80-019b	
	IC_{50}[a]	ID[a]	IC_{50}[a]	ID[a]
pA2'pA2'pA	0.04	1.0	0.008	1.0
p(br^{8}A)2'p(br^{8}A)2'p(br^{8}A)	nd[b]	nd[b]	0.70	90
p(ϵA)2'p(ϵA)2'p(ϵA)	>100	>2500	0.004	0.5
pI2'pI2'pI	>10	>250	0.009	1.1
pC2'pC2'pC	>10	>250	0.15	19
pU2'pU2'pU	>10	>250	0.20	25

[a] IC_{50} and ID are defined in footnotes *a* and *b* of Table II. [b] Not determined. See text for explanation.

Table V: Reactivity of Rabbit Antisera with Terminally Modified Analogues

	80-017b		80-019b	
	ID_{50}[a]	ID[a]	IC_{50}[a]	ID[a]
pA2'pA2'pA	0.04	1.0	0.008	1.0
A5'pppp5'A2'pA2'pA	0.10	2.5	0.015	1.9
mannose 6pp5'A2'pA2'pA	0.14	3.5	0.006	0.8
pA2'pAahp[b]	200	5000	0.50	63
pA2'pA2'pAahp[b]	1.0	25	0.005	0.6
pA2'pA2'pA2'pAahp[b]	2.0	50	0.001	0.1
pppA2'pA2'pAahp[b]	2.0	50	0.001	0.1

[a] IC_{50} and ID are defined in footnotes *a* and *b* of Table II. [b] The 2'(3')-terminal ribose was modified to an N-substituted morpholine (azahexapyranose) by sequential reactivity with periodate, hexylamine, and $NaBH_3CN$ (Imai et al., 1982).

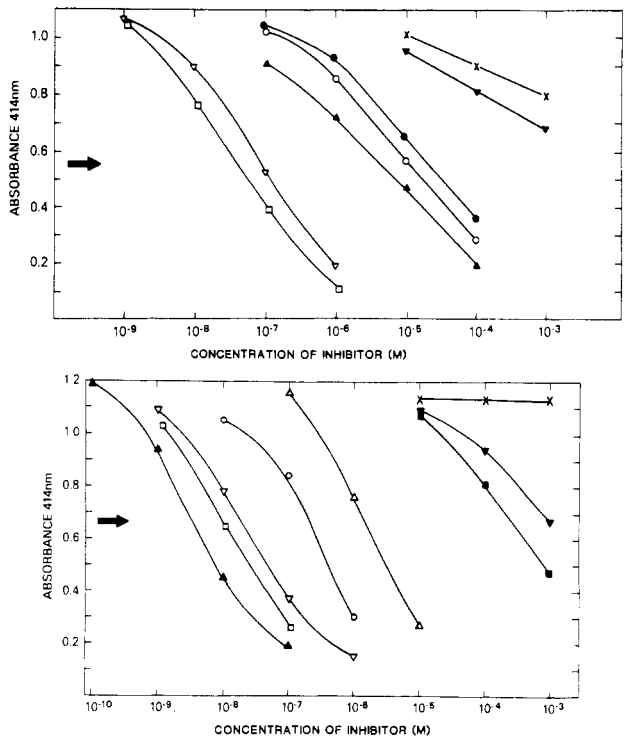

FIGURE 2: Representative competitive IEMA assays. Dilutions of various nucleic acids were incubated with antiserum 80-017b (top panel) at 1×10^{-5} final dilution or with 80-019b (bottom panel) at 2×10^{-4} final dilution in microtiter wells precoated with Ficoll–$(pA)_3$. Antibody binding, as reflected by the absorbance at 414 nm, was determined as described under Materials and Methods. The arrow designates the 50% point. (▲) A2′pA2′pA; (□) pA2′pA2′pA; (▽) pppA2′pA2′pA; (○) pA2′pA3′pA2′pA; (●) pA3′pA2′pA; (△) pA2′pA3′pA; (■) ADP; (▼) ATP; (×) adenosine. The arrow indicates the 50% inhibition point.

(Figure 2), making assessment of the IC_{50} point impossible. When the trimer was modified at the 5′ terminus to A5′pppp5′A2′pA2′pA or mannose 6pp5′A2′pA2′pA, recognition was not altered substantially (Table V). Degradation of these analogues to pA2′pA2′pA was not detected (data not shown). When the ribose of the 2′(3′)-terminal nucleotide was transformed to an N-substituted morpholine (azahexapyranose) (Imai et al., 1982), reactivity was decreased moderately.

Specificity of 80-019b. In contrast to 80-017b, the anti-BSA–2′(3′)-$(pA)_3$ serum (80-019b) showed a slight preference for the core oligoadenylates over the corresponding monophosphorylated oligoadenylates (Table II). The strongest competitor tested, A2′pA2′pA2′pA, inhibited 80-019b by 50% at a concentration of 0.5 nM. Inhibition (25%) was also detectable with as little as 0.5 nM (10 fmol) trimer, A2′pA2′pA. The biologically active pppA2′pA2′pA was only slightly less reactive than the hapten used for immunization. Reactivity of 80-019b with NADP AMP, ADP, and ATP was barely detectable, whereas no reactivity with adenosine was observed.

The mixed-linkage isomers displayed varied reactivity with 80-019b (Table III). As with 80-017b described above, the strongest cross-reacting mixed-linkage isomer was the tetramer pA3′pA2′pA2′pA, and the least reactive was pA2′pA3′pA. The 3′,5′-linked trimer core, A3′pA3′pA, and the trimer monophosphate, pA3′pA3′pA, were over 1000 times less reactive than the corresponding 2′,5′-linked oligoadenylates. No reaction with 3′,5′-oligo(2′-deoxyadenylates) at millimolar concentrations was detected. However, reactivity with the cordecypin analogue was observed, albeit at concentrations substantially higher than required for 2′,5′-oligoadenylates containing 3′-OH groups. In contrast to that observed with 80-017b, 80-019b reacted surprisingly well with all base-modified 2′,5′-oligonucleotides (Table IV). The pyrimidine analogues were slightly less reactive than the purine analogues. The 8-bromo analogue of adenosine resulted in the most drastic base alteration. As with 80-017b, the least drastic modifications were the additions to either end of the molecule. The 5′-modified analogues were potent inhibitors of 80-019b, and several of the 2′(3′)-terminally modified analogues were the most potent modified inhibitors of 80-019b tested (Table V).

Discussion

Antibodies specific for various conformations of large helical nucleic acids [reviewed by Stollar (1975)] as well as antibodies reactive with modified nucleosides [reviewed by Munns & Liszewski (1980)] have been employed in numerous areas of research. The structural features of oligonucleotides and the nature of their antigenic determinants have been explored by using antisera directed against 3′,5′-linked triribonucleotides (D'Alisa & Erlanger, 1974, 1976; Shinomiya et al., 1978) and trideoxyribonucleotides (Khan & Jacob, 1977). In cross-reactivity studies, the least drastic alteration was usually a change in oligonucleotide length of one to two nucleotides. Oligonucleotides of altered sequence showed weaker cross-reactivity, demonstrating that antibodies can recognize trinucleotide sequences but not with absolute specificity.

In our studies, antisera were elicited by immunization of rabbits with two different conjugates of pA2′pA2′pA. Detailed cross-reactivity studies of two antisera with numerous modified trinucleotides suggested that the following features contribute to the antigenic determinant(s) of oligonucleotides.

(1) Nature of the Phosphodiester Backbone Including the 3′-OH. The results demonstrate that the ribose-phosphate backbone formed the major antigenic determinant and that 2′,5′ linkages are substantially different than 3′,5′ linkages. Alteration of one 2′,5′ linkage was tolerated to varying degrees. The most reactive mixed linkage isomer was the pA3′pA2′pA2′pA, suggesting that the antibodies may recognize the unmodified trimer sequence pA2′pA2′pA within the tetramer molecule. This is similar to the results obtained with 3′,5′-oligodeoxynucleotides by Khan & Jacob (1977). The mixed-linkage isomer least reactive with 80-019b was pA2′pA3′pA, indicating that this antiserum was more sensitive to backbone changes nearer the 2′(3′) terminus, the point of attachment in the immunogen. Removal of the 3′-OH moieties decreased the inhibitory capacity substantially when tested with both antisera, but from these results it is not possible to determine if the hydroxyl groups participated directly to the binding site, or if they affected reactivity by affecting the conformational features of the ribose–phosphate backbone.

(2) Nature of the Base. The contribution of the bases differed substantially with the two sera analyzed. With 80-019b, most base modifications resulted in little alteration in antibody recognition; only one containing a bromine at C8 was substantially reduced in reactivity. This modification may result in a change in the orientation of the base about the glycosidic bond from anti to syn and in the degree of base stacking (Howard et al., 1969). With serum 80-017b, the IC_{50} for the base-modified and cordycepin oligonucleotides could not be determined due to limited availability of those compounds. Thus, it was not possible to compare the relative contribution of the bases and the 3′-hydroxyl groups. However, the bases played a major role in recognition by 80-017b. The base moieties may participate directly in the antigenic determinant, and the decreased reactivity of the oligopurines with

80-019b compared to the oligopyrimidines may have resulted from steric hindrance afforded by the larger purines. Alternatively, the bases may participate indirectly by affecting the conformation of the ribose–phosphate backbone.

(*3*) *5′-Terminal Phosphate If Present in the Immunogen.* Serum from rabbit 80-019b reacted better with A2′pA2′pA than pA2′pA2′pA. Since linkage of pA2′pA2′pA to protein was through the 2′(3′)-terminal ribose in this instance, removal of the 5′-phosphate may have occurred to some extent in vivo, resulting in the presentation of A2′pA2′pA2′(3′)–BSA and production of antibodies specific for the trimer "core" molecule. In contrast, removal of the 5′-terminal phosphate of pA2′pA2′pA resulted in a substantial decrease in reactivity with 80-017b antiserum from a rabbit immunized with mBSA–5′pA2′pA2′pA. Tetramer core reacted better than trimer core probably because it contains the pA2′pA2′pA sequence, which the antiserum apparently recognized, albeit not as well as if the phosphate were at the 5′ terminus. Addition of β- and γ-phosphates had a minor effect on recognition by both antisera, suggesting that addition of these phosphates produced little if any effect on the overall features of the 2′,5′ backbone.

(*4*) *Length of the Oligonucleotide.* Size appeared to play a role in determining antibody reactivity. Although decreasing a length to dimer reduced reactivity with 80-019b, increasing the length increased reactivity slightly for reasons that are not readily apparent. A similar increase in antibody reactivity with increasing oligonucleotide length was reported by Cailla et al. (1982) for the ppp(A2′p)$_n$A series. In contrast, three nucleotides were preferred by 80-017b. Decreasing the length to dimer decreased reactivity substantially whereas increasing the length resulted in small decreases in reactivity. This is in contrast to the results obtained with serum 80-019b but in agreement with the reports of others (D'Alisa & Erlanger, 1974; Khan & Jacob, 1977).

(*5*) *Nature of the 2′ Terminus.* Conversion of the 2′-(3′)-terminal ribose to an azahexapyranose by methodology employed in the synthesis of the immunogen, BSA–2′(3′)-pA2′pA2′pA (Imai et al., 1982), resulted in increased reactivity by 80-019b probably because the substituted morpholine contributed to the antigenic determinant. The presence of an azahexapyranose resulted in a moderate decrease in reactivity with 80-017b. Furthermore, converted tetramer was the poorest among the tetramers in inhibiting 80-017b even though it contained many of the features required for the antigenic determinant, i.e., a pA2′pA2′pA sequence. This result reinforces the importance of total length and implies a minor role for the 2′(3′) terminus in the formation of the antigenic determinant.

(*6*) *Nature of the 5′ Terminus beyond the α-Phosphate.* With 80-017b, evidence that 5′ modification (providing the α-phosphate remained) is slightly less drastic than 2′(3′) modification comes through comparison of two tetramers, pA3′pA2′pA2′pA and pA2′pA2′pA2′pAahp. The first, which can be considered a 5′-modified trimer, was less reactive than pA2′pA2′pA but more reactive than the latter, a 2′(3′)-modified trimer; i.e., the antibodies appear to recognize pA2′pA2′pA within pA3′pA2′pA2′pA better than in pA2′pA2′pA2′Aahp. The 5′ modifications had little effect on 80-019b.

Comparison of our results with those reported previously (Knight et al., 1980, 1981; Cailla et al., 1982; Sawai & Shinomiya, 1982) illustrates several variables in factors that contribute to the antigenic determinants of 2′,5′-oligo(A) including the presence of 5′-phosphates and the oligonucleotide length. After completion of this work, Sawai & Shinomiya (1982) described antisera raised by immunization of rabbits with a A2′pA2′pA–BSA conjugate made by the method of Erlanger & Beiser (1964). Although their assay was less sensitive, their cross-reactivity results were similar to those reported here with a few exceptions. In general, their antibodies displayed broader cross-reactivity than did ours, reacting better with the cordycepin analogues and the mixed-linkage isomers. They reported weak inhibition by the inosine analogue but no detectable inhibition by the uracil and cytosine analogues, whereas we observed that the base analogues reacted well with one antiserum (80-019b) but not to any detectable degree with another (80-017b) (Table IV).

The different antibody specificities may have resulted from variability in the individual rabbit responses and/or the degree of heterogeneity of the antibodies studied. Sawai & Shinomiya (1982) used total serum IgG at several milligrams per milliliter before and after absorption on AMP–Sepharose or RNA–Sepharose; absorption increased the specificity of the antibodies somewhat. We employed whole antiserum diluted either 5000-fold (80-019b) or 100 000-fold (80-017b). Unless the antisera consisted of a very small number of high-affinity antibodies and a very large number of low-affinity antibodies, our measurements probably reflect the specificity of only a few populations of antibodies. It is possible that the more narrow specificity of 80-017b resulted from a less heterogeneous population of antibodies than present in 80-019b. Alternatively, the different modes of attachment of the hapten in the immunogen may have contributed to these differences. Preliminary results of the analysis of other antisera suggest that the importance of the base moieties may be determined by the type of hapten to protein linkage employed in the immunogen (M. I. Johnston, unpublished results). Shinomiya et al. (1978) suggested that the nature of the 2′(3′)-terminal base nearest the protein carrier was of negligible importance for antibody reactivity, whereas D'Alisa & Erlanger (1974) observed that this was the most important base for recognition by anti-trinucleotide antibodies. We have not estimated the relative importance of each of the base moieties in a 2′,5′-linked trimer oligonucleotide. However, our results with various 5′- and 2′(3′)-modified oligoadenylates and mixed-linkage isomers are consistent with the theory that the area of hapten through which conjugation to the carrier protein was accomplished contributes to the antigenic determinant (D'Alisa & Erlanger, 1974; Wallace et al., 1971).

Our results confirm and extend those reported previously in demonstrating that the nature of the phosphodiester backbone was consistently a critical factor in antibody recognition. This may reflect a unique three-dimensional conformation of the phosphodiester backbone of 2′,5′-oligonucleotides. Furthermore, studies of serum 80-017b demonstrated that 2′,5′-oligo(A) possesses features that distinguish it from other 2′,5′-oligonucleotides. Conformational analyses of 2′,5′- and 3′,5′-oligonucleotides have shown differences in their tendency to stack, although there is some disagreement as to which is more stacked (Doornbos et al., 1981; Brahms et al., 1967; Ts'o et al., 1969). Nuclear magnetic resonance studies have suggested that the intramolecular base stacking of core A2′pA2′pA differs from that of pppA2′pA2′pA but that the environments of the internucleoside phosphate groups are not significantly affected by phosphorylation at the 5′ terminus (Martin et al., 1979).

Other studies have shown that the features of a nucleic acid required for recognition by antibodies may parallel those required for biological activity (Johnston et al., 1975). The

results obtained to date suggest that specificity of 80-017b closely approximates that required for binding to the endonuclease (P. F. Torrence, K. L. Lesiak, J. Imai, M. I. Johnston, and H. Sawai, unpublished results). Immunochemical observations may contribute to the design of modified derivatives of 2′,5′-oligo(A) that retain their ability to bind to the 2′,5′-oligo(A)-specific endonuclease and that may be useful as potential antiviral agents, or as antagonists of the action of 2′,5′-oligo(A). For example, the cordycepin analogue was less reactive than 2′,5′-oligo(A) but more reactive than 3′,5′-oligo(A). Likewise, the cordycepin analogue binds to but does not activate the 2′,5′-oligo(A)-dependent endonuclease in mouse L cell extracts (Sawai et al., 1982) whereas the 3′,5′-isomer binds very poorly and also does not activate the enzyme (Knight et al., 1980; L. Lesiak, J. Imai, G. Floyd-Smith, P. F. Torrence, unpublished results). Conversion of the 2′(3′)-terminal ribose to an azahexapyranose resulted in a structure that was very reactive with antibody (Table V) and that activated the endonuclease (Imai et al., 1982). Results with both antisera, but particularly 80-017b, demonstrated that in contrast to backbone or base changes, modification at the 5′ terminus beyond the α-phosphate had a minor effect on antibody recognition and even less of an effect than did modification at the 2′(3′) terminus. This comparison has not been made previously and suggests that 5′-terminal modification may have a smaller effect than 2′(3′) modification on the conformation of 2′,5′-oligo(A) and its recognition by the endonuclease.

Acknowledgments

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Registry No. A2′pA, 2273-76-9; A2′pA2′pA, 70062-83-8; A2′pA2′pA2′pA, 73853-00-6; pA2′pA, 20307-28-2; pA2′pA2′pA, 61172-40-5; pA2′pA2′pA2′pA, 66048-58-6; pA2′pA2′pA2′pA2′pA, 66048-59-7; ppA2′pA, 81410-37-9; pppA2′pA, 65954-94-1; pppA2′pA2′pA, 65954-93-0; pppA2′pA2′pA2′pA, 65954-95-2; adenosine, 5682-25-7; 5′-AMP, 61-19-8; 2′-AMP, 130-49-4; 5′-ADP, 58-64-0; 5′-ATP, 56-65-5; 2′,5′-ADP, 3805-37-6; NADP, 53-59-8; pA3′pA2′pA, 78983-51-4; pA2′pA3′pA, 78983-50-3; pA2′pA3′pA2′pA, 78983-52-5; pA3′pA2′pA2′pA, 85883-00-7; A3′pA3′pA, 917-44-2; pA3′pA3′pA, 1684-34-0; p(2′dA)3′p(2′dA)-3′p(2′dA), 14258-27-6; (3′dA)2′p(3′dA)2′p(3′dA), 76885-99-9; p-(3′dA)2′p(3′dA)2′p(3′dA), 84311-63-7; (3′dA)2′p(3′dA)2′p(3′dA)-2′p(3′dA), 84651-05-8; p(3′dA)2′p(3′dA)2′p(3′dA)2′p(3′dA), 85060-15-7; A5′pppp5′A2′pA2′pA, 77063-72-0; mannose 6pp5′A2′pA2′pA, 84311-67-1; pA2′pAahp, 83807-27-6; pA2′pA2′pAahp, 83807-28-7; pA2′pA2′pA2′pAahp, 83807-29-8; pppA2′pA2′pAahp, 85883-01-8; p(br^8A)2′p(br^8A)2′p(br^8A), 84311-64-8; p(εA)2′p(εA)2′p(εA), 84877-05-4; pI2′pI2′pI, 66048-63-3; pC2′pC2′pC, 84311-66-0; pU2′pU2′pU, 66048-60-0; $Im(pA)_3$, 85883-02-9; pA2′pA2′pA·Et_3N, 85883-03-0; carbonyldiimidazole, 530-62-1.

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Quantitative Analysis of Resonance Raman Spectra of Purple Membrane from *Halobacterium halobium*: L550 Intermediate†

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ABSTRACT: The bacteriorhodopsin photocycle consists of a series of intermediates, each characterized by a different visible absorption. We have examined one of these intermediates, L550, by *quantitatively* analyzing the resonance Raman spectra of purple membrane recorded under different conditions. A rotating cell was used to control the contribution of the early intermediates in the resonance Raman spectra. These spectra were then fitted to a superposition of Voigtian line shapes to obtain the position, width, and integrated intensity of each band. We find evidence for the appearance within 5 μs of two bands at 1539 and 1551 cm^{-1}. By correlating the intensity changes in these bands with the protonated Schiff base band, we are able to establish that the intermediates giving rise to them are protonated. It is thus concluded that M412 is the first deprotonated intermediate in the bateriorhodopsin photocycle. Because the ratio of the integrated intensity of 1539- and 1551-cm^{-1} bands remains constant for various exposure times and laser power, the possibility is discussed that they both arise from the L550 intermediate rather than from L550 and a second "X" intermediate as previously proposed. In addition, it is concluded that a conformational change in the chromophore has occurred in L550 relative to that in the light-adapted bR570. A possible linear relation between C=N stretching frequency and wavelength maximum for visible absorption is discussed.

Much interest has focused on bacteriorhodpsin, the light-transducing proton pump of the purple membrane (PM)[1] in *Halobacterium halobium* [for a review see Stoeckenius et al. (1979)]. In contrast to other membrane transport proteins, considerable information is available about bacteriorhodopsin including its amino acid sequence (Ovchinnikov et al., 1979; Khorana et al., 1979) and secondary structure (Henderson & Unwin, 1975; Engelman et al., 1980). However, the basic mechanism of bacteriorhodopsin proton translocation and energy transduction still remains unelucidated.

In order for further progress to be made, it will be necessary to elucidate in detail the molecular changes occurring in both the protein, bacterioopsin, and the chromophore, retinal, subsequent to light absorption. Resonance Raman spectroscopy (RRS) offers a selective method of probing the chromophore during the bacteriorhodopsin proton pump cycle. Information obtained by using this method thus far indicates the following: (i) A linear correlation exists between the C=C stretching frequency of the isoprene chain of the retinylidene chromophore (1500-1570-cm^{-1} range) and the absorption maximum of the spectral intermediates (Heyde et al., 1971; Aton et al., 1977; Doukas et al., 1978). This correlation can be used to identify the presence of different intermediates in the RRS of PM. (ii) The bands at 1642 cm^{-1} in the light-

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[1] Abbreviations: BR, bacteriorhodopsin; PM, purple membrane; RRS, resonance Raman spectrum.

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