Antibody–Nucleic Acid Interactions. Monoclonal Antibodies Define Different Antigenic Domains in 2′,5′-Oligoadenylates†

Margaret I. Johnston,* Jiro Imai,‡ Krystyna Lesiak,‡ Helmut Jacobsen,§ Hiroaki Sawai,∥ and Paul F. Torrence‡

Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4779, Laboratory of Chemistry, National Institutes of Health, Bethesda, Maryland 20205, Institute for Virus Research, Heidelberg, West Germany, and Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-Ku, Tokyo, Japan

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ABSTRACT: To define the epitopes involved in binding anti-oligonucleotide antibodies, several hybridomas producing monoclonal antibodies directed against 2′,5′-oligoadenylate were established. A solid-phase enzyme-linked immunoassay that employed microtiter wells coated with Ficoll–2′,5′-oligoadenylate conjugates proved useful in screening and characterizing hybridoma supernatants. Control experiments demonstrated that the conjugates were irreversibly adsorbed to polystyrene wells under the conditions employed in the assay. Reactivity of monoclonal antibodies with numerous analogues of 2′,5′-oligoadenylate was measured by using a competition assay. Several monoclonal antibodies originating from different mice immunized with the same or different immunogens possessed distinctive fine specificities. At least one 2′,5′-phosphodiester bond was important in forming each epitope, suggesting that the ribose phosphate backbone is a critical element in defining an antigenic domain of an oligonucleotide. The purine bases were also important, and modification of the bases had varied effects on the extent of antibody recognition. The length of the oligonucleotide and the nature of the termini were also of some importance. In several instances the modification created by linkage of 2′,5′-oligoadenylate to carrier protein also contributed to the determinant. The monoclonal antibody most specific for 2′,5′-oligoadenylates was relatively insensitive to ionic strength. In contrast, a monoclonal antibody with a 2′,5′-oligopurine specificity appeared to bind 2′,5′-oligoadenylate through one ion pair, whereas the binding of a monoclonal antibody with a low degree of base specificity appeared to bind through two ion pairs. The results demonstrated that 2′,5′-linked oligoadenylate–protein complexes possess at least three distinct oligonucleotide-related antigenic surfaces that can be recognized with high apparent affinity by monoclonal antibodies. A model for the three epitopes is presented.

Numerous biochemical events involve recognition of nucleic acids by proteins. The features of both the nucleic acid and the protein involved in binding are often difficult to define. Antibodies directed against nucleic acids make excellent model systems for studying protein–nucleic acid interactions. Not only is considerable information on antibody structure available but also antibodies can be obtained in homogeneous form and in large quantity. Well-characterized antibodies are also useful reagents for studying the occurrence and function of nucleic acids (Munns & Liszewski, 1980).

Antibodies that react with specific nucleic acids can be obtained most easily by immunization with nucleic acid–protein conjugates. In the case of long single-stranded, double-stranded, or triple-stranded nucleic acids, electrostatic complexes with methylated bovine serum albumin (mBSA)[1] have been employed to obtain specific antisera (Stollar, 1980). With haptens such as nucleosides, nucleotides, and oligonucleotides, covalent attachment to carrier protein is required (Erlanger, 1980). In many instances attachment to carrier protein has been achieved through periodate oxidation techniques (Erlanger & Beiser, 1964).

Research on the specificity of antibodies that bind helical nucleic acids has demonstrated that their antigenic determinant is predominantly conformational in nature and defined by the ribose phosphate backbone (Stollar, 1975). For example, antibodies directed against poly(A)·poly(U) react very well with poly(I)·poly(C) and naturally occurring double-stranded RNAs (Stollar, 1975). Antibodies to brominated poly(dG-dC)·poly(dG-dC) have been shown to recognize Z-DNA, a conformation distinct from B-DNA (Lafer et al., 1981). Cross-reactivity of anti-nucleotide and anti-nucleoside antibodies is often fairly broad unless a modification such as an

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* Address correspondence to this author at the Uniformed Services University of the Health Sciences.

‡ National Institutes of Health.

§ Institute for Virus Research.

∥ University of Tokyo.

[1] Abbreviations: BSA, bovine serum albumin; mBSA, methylated bovine serum albumin; OA, ovalbumin; ELISA, enzyme-linked immunosorbent assay; F–$(pA)_x$ or Ficoll–$(pA)_x$, $(pA)_x$ conjugated through the 5′-phosphate to AECM-Ficoll; AECM-Ficoll, [*N*-(2-aminoethyl)carbamoyl]methylated Ficoll; BSA–3′-$(pA)_4$, pA2′pA2′pA2′pA conjugated through the 2′(3′) terminus to BSA; mBSA–5′-$(pA)_x$, $(pA)_x$ conjugated through the 5′-phosphate to mBSA; PBS, 0.01 M sodium phosphate, pH 7.4, and 0.14 M NaCl; Tween 20, poly(oxyethylene)sorbitan monolaurate; PBS + T, PBS with 0.05% Tween 20; PBS + T + BSA, PBS + T with 0.2% BSA; PBS + T + OA, PBS + T with 0.2% OA; PBS + BSA, PBS with 0.2% BSA; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); εA, 1,N^6-ethenoadenosine, adenosine modified by addition of an ethenyl bridge between N1 and the 6-amino nitrogen; br^8A, adenosine modified by substitution of bromine for the C8 hydrogen; $p(A2'p)_nAahp$, 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 2-(9-adenyl)-4-hexyl-6-(hydroxymethyl)morpholine; Ficoll, a commercial product consisting of branched copolymers of sucrose and epichlorohydrin; IgG, immunoglobulin G; IgM, immunoglobulin M.

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N7 methyl group is present (Munns & Liszewski, 1980). The nature of the antigenic determinants of oligonucleotides has also been explored to some extent. Antisera directed against 3′,5′-ribotrinucleotides and deoxytrinucleotides have been described (D'Alis & Erlanger, 1974, 1976; Khan & Jacob, 1977). These antibodies displayed some sequence and length specificity. Antisera directed against 2′,5′-oligoadenylates have been shown to possess minimal cross-reactivity with nucleotides and 3′,5′-oligoadenylates (Knight et al., 1980, 1981; Sawai & Shinomiya, 1982; Johnston et al., 1983; Hersh et al., 1984b). Oligoadenylates possessing 2′,5′-phosphodiester linkages are of particular interest in that they have been implicated in the antiviral action of interferon [for review, see Johnston & Torrence (1984)].

Previous studies with rabbit antisera directed against 2′,5′-oligoadenylate identified several features required for recognition by antibody (Johnston et al., 1983). Due to the polyclonal nature of antisera, a single antigenic determinant could not be identified. To more precisely define how antibodies recognize oligonucleotides, specifically 2′,5′-oligoadenylate, we established numerous 2′,5′-oligoadenylate-specific hybridomas and determined the fine specificity of three distinct monoclonal antibodies. Cross-reactivity with over 35 different oligonucleotides, nucleotides, and nucleosides was examined. Each antibody required at least one 2′,5′-phosphodiester bond linking two purine nucleosides. In general, the more specific the interaction between antibody and 2′,5′-oligoadenylate, the less sensitive binding was to ionic strength. We propose that the epitopes, the regions that interact with antibody, encompass only a portion of the oligonucleotide and may or may not include the linkage region between the oligonucleotide and the protein employed as the carrier in the immunogen. On the basis of the fine specificities and the number of ion pairs involved in binding, we present a model defining three distinct epitopes on 2′,5′-oligoadenylate responsible for antibody recognition.

Materials and Methods

Synthesis of Oligonucleotides. Synthesis of 2′,5′-oligoadenylates has been described previously (Imai & Torrence, 1981a,b). Linkage isomers of 2′,5′-oligoadenylate were synthesized as described previously (Lesiak et al., 1983). The brominated and ethenylated analogues have also been described (Lesiak & Torrence, 1983; Lesiak et al., unpublished results). The cytidine, uridine, inosine, and cordycepin analogues have been reported (Sawai et al., 1983; Sawai & Shinomiya, 1982; Sawai et al., 1981; Sawai & Ohno, 1981a,b), and analogues with one hypoxanthine and two adenines were prepared as reported previously (Imai & Torrence, 1985). The 5′-modified derivatives were synthesized as described elsewhere (Imai & Torrence, 1984). The azahexapyranose derivatives made by reaction with *n*-hexylamine (i.e., pA2′pA2′pAahp) have been reported previously (Imai et al., 1982).

Synthesis of 2′,5′-Oligoadenylate–Protein Conjugates. The triethylammonium salt of pA2′pA2′pA2′pA (0.96 μmol) was oxidized in the dark at 0 °C with 1.2 μmol of sodium periodate. After 15 min, BSA (0.9 mg, 0.013 μmol) dissolved in water was added and the pH adjusted to 8.6 with 1 M $NaHCO_3$. After the solution was stirred at 0 °C for 2 h, 5 μmol of sodium cyanoborohydride was added, and the pH of the solution was readjusted to 6.5 with 10% acetic acid. The reaction was incubated at 0 °C for 40 min and then applied to a Sephadex G-100 column (1.3 × 26 cm). The peak eluting at the end of the void volume was pooled. This peak contained 0.013 μmol of BSA as determined by protein assay and 2.3 A_{258} units or 0.055 μmol of pA2′pA2′pA2′pA. The ratio of pA2′pA2′pA2′pA to BSA in this BSA–3′-pA2′pA2′pA2′pA conjugate was about 4.2.

The methylated BSA (mBSA)–5′-pA2′pA and mBSA–5′-pppA2′pA2′pA2′pA conjugates were synthesized by condensing the 5′-imidazolide derivatives of the oligoadenylate with mBSA in dry Me_2SO essentially as described previously for mBSA–5′-pA2′pA2′pA (Johnston et al., 1983). Conjugates were purified by chromatography on Sephadex LH-60 columns eluted with Me_2SO. Dialysis of the eluted samples against 4, 2, 1, 0.5, 0.2, 0.1, and 0.05 M urea was followed by dialysis against PBS buffer. Conjugates that contained 12 mol of pA2′pA or 18 mol of pA2′pA2′pA or 11 mol of pppA2′pA2′pA2′pA per mole of protein were employed as immunogens in the studies described here.

Synthesis of 2′,5′-Oligoadenylate–Ficoll Conjugates. Ficoll (M_r 400 000) was derivatized to [*N*-(2-aminoethyl)carbamoyl]methylated Ficoll (AECM-Ficoll) as described by Inman (1975). Conjugation of pA2′pA2′pA to AECM-Ficoll was described previously, and conjugation of 5′-AMP, pA2′pA, pA2′pA2′pA2′pA, and pppA2′pA2′pA2′pA was achieved by the same procedures (Johnston et al., 1983). Moles of hapten attached per mole of Ficoll were 47, 27, 44, 36, and 15 for pA, pA2′pA, pA2′pA2′pA, pA2′pA2′pA2′pA, and pppA2′pA2′pA2′pA, respectively.

Production of Hybridomas. A total of 26 mice were immunized with protein–2′,5′-oligoadenylate conjugates as follows: mBSA–5′-pA2′pA, 10; mBSA–5′-pA2′pA2′pA, 5; mBSA–5′-pppA2′pA2′pA2′pA, 9; BSA–3′-pA2′pA2′pA2′pA, 2. Exact immunization schedules varied from mouse to mouse. In general, mice were immunized intraperitoneally with 100–150 μg of protein conjugate in complete Freund's adjuvant. After 8–22 days, a subsequent injection (or weekly injections) of 100–150 μg of conjugate in incomplete Freund's adjuvant was administered. After 1–5 months, a boost of 30–400 μg of conjugate in saline was administered intraperitoneally. In a few cases boosts were given each of 4 days prior to sacrifice. In other cases mice were sacrificed on the fourth day after a saline boost. Titers were defined as the inverse of the dilution that resulted in an absorbance reading of 1.0 after 15-min incubation with substrate in the ELISA (described below) and were extrapolated from graphs of absorbance vs. dilution.

Hybridomas were produced by previously developed methods (Köhler & Milstein, 1976; Galfre et al., 1977; Gefter et al., 1977). The sp2/0 cells were maintained in medium containing 0.13 mM azaguanine. Spleen cells were isolated by passage through a sterile steel screen. After the cells were washed with Hank's balanced salt solution, they were washed, counted, mixed with washed mouse myeloma sp2/0 cells at a ratio of 6:1 (spleen:tumor), and pelleted. Fusion was accomplished by gentle dispersion of the pellet in 1 mL of 45% (w/v) poly(ethylene glycol) (M_r 1000) prewarmed to 37 °C and added dropwise over a period of 1 min. After another 1 min of gentle mixing, 10 mL of Dulbecco's modified Eagle medium (DMEM) was added dropwise over a period of 5 min. The cells were pelleted and then gently suspended at a concentration of 2×10^6 cells/mL in hybridoma medium consisting of DMEM with 4.5 gm of glucose/L, 10% fetal calf serum, 10% NCTC-101 (M. A. Bioproducts), 2–6 mM glutamine, 50 units/mL penicillin, 50 mg/mL streptomycin, 0.1 mM hypoxanthine, 16 μM thymine, and 50 μM mercaptoethanol. After incubation overnight at 37 °C, with 7.5% CO_2, aminopterin was added to a final concentration of 40 μM. Approximately 2×10^5 cells/well were distributed into microtiter wells and allowed to grow 10–14 days before the

supernatants were screened. The cells were fed 1–2 drops of fresh medium when necessary. Positive supernatants were grown in 1-mL cultures and then cloned by limiting dilution in the presence of fresh thymocytes at 2×10^6 cells/mL. Each hybridoma was cloned a minimum of 3 times.

Screening Hybridomas. Hybridoma supernatants were screened for 2′,5′-oligoadenylate-specific antibody by binding to Ficoll–2′,5′-oligoadenylate-coated polystyrene microtiter wells (Immunolon I, Dynatech Laboratories, Alexandria, VA). The Ficoll–hapten conjugate employed in screening contained the same hapten used in immunization. For initial screening, binding to Ficoll–5′-AMP-coated wells was determined also. Hybridoma media (usually 60 μL), undiluted or diluted 1:2, 1:10, or 1:100 with PBS supplemented with 0.2% BSA and 0.05% Tween 20 (PBS + BSA + T), were added to the wells and allowed to react at room temperature for 30–90 min. After the wells were washed with PBS supplemented with 0.05% Tween 20 (PBS + T), 25–60 ng of affinity-purified, peroxidase-labeled rabbit anti-mouse IgG (or anti-mouse IgG + IgM) (Kirkegaard-Perry Laboratories, Gaithersburg, MD) diluted in PBS containing 0.2% BSA (PBS + BSA) was added to the wells and allowed to react for 30–90 min at room temperature. After the wells were washed with PBS + T, peroxidase activity was determined by addition of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide (Engvall, 1980). Positive supernatants were identified visually. On rare occasions hybridomas were obtained that gave a strong reaction with Ficoll–5′-AMP-coated wells. Only those that reacted strongly with Ficoll–2′,5′-oligoadenylate and not Ficoll–AMP were grown and cloned.

Competition ELISA Assay. Competitition assays were performed as described previously (Johnston et al., 1983). Briefly, serial dilutions of competing oligonucleotide (diluted in PBS + T) were added in duplicate or triplicate to wells coated with a set amount of Ficoll–2′,5′-oligoadenylate just prior to addition of a set dilution of hybridoma medium [diluted in PBS + T supplemented with 0.2% ovalbumin (P + T + OA)]. Optimal amounts of Ficoll coating and antibody were determined by the checkerboard method as described by Engvall (1980). The total volume was usually 60 μL although volumes as low as 20 μL were employed in instances where the amount of competing oligonucleotide was limited. The volume had no effect on the percentage inhibition of antibody binding. Ficoll–pA2′pA [F–$(pA)_2$], Ficoll–pA2′pA2′pA [F–$(pA)_3$], Ficoll–pA2′pA2′pA2′pA[F–$(pA)_4$], and Ficoll–pppA2′pA2′pA2′pA [F–$pp(pA)_4$] were employed in assays for antibodies directed against pA2′pA, pA2′pA2′pA, pA2′pA2′pA2′pA, and pppA2′pA2′pA2′pA, respectively. After incubation at 30 °C for 90 min, the plate was washed with PBS + T. Affinity-purified peroxidase-conjugated rabbit anti-mouse IgG diluted in PBS + BSA was added and allowed to react for 90 min at 30 °C. The plate was washed again prior to addition of peroxidase substrate. Absorbance readings at 414 nm were taken with a Flow Multiscan when the positive controls, antibody incubated with buffer instead of inhibitor, gave a reading of 1.0–1.2. Negative controls included control medium and buffer and, to measure nonspecific binding of the peroxidase-labeled antibody, buffer alone in the first incubation. These negative controls were usually around 0.05 A_{414} unit. Replicative dilutions were generally within 10% of each other. The absorbance for 50% antibody binding was taken as the midway point between the mean of the positive control wells and the mean of the wells containing control medium. The mean absorbance reading for each oligonucleotide concentration was calculated and plotted as a function of the final molar concentration of oligonucleotide. The concentration of oligonucleotide required to inhibit antibody binding by 50% (IC_{50}) was extrapolated from these graphs. Each oligonucleotide was assayed with each antibody a minimum of 3 times, and most were assayed 3–5 times. Mean IC_{50} values were calculated. The standard deviation of the IC_{50} determinations was about 40%.

The relative reactivities of the oligonucleotides with a given antibody were determined by comparison of their indexes of dissimilarity (ID's) (Prager & Wilson, 1971). Each ID was calculated by dividing the mean IC_{50} of the oligonucleotide by the mean IC_{50} of the 2′,5′-oligoadenylate used in the immunogen. For example, under the conditions employed, the concentrations of pA2′pA, pA2′pA2′pA, and pA2′pA2′pA2′pA required to inhibit Hy24-4F3C binding by 50% were extrapolated to be 122, 12, and 3.5 nM, respectively. Since pA2′pA2′pA2′pA was the hapten used in the immunogen for this mouse, the ID's were 35, 3.4, and 1.0, respectively. This means that 35 times more pA2′pA relative to pA2′pA2′pA2′pA was required to achieve the same degree of inhibition of antibody binding. The higher the ID, the less reactive was the oligonucleotide with the antibody.

Salt Dependency of Antibody Binding. Hybridoma media were diluted in PBS + T + OA with ≤0.15 M NaCl or supplemented with additional NaCl to give the desired final salt concentration. In one set of experiments, one antibody dilution was tested for binding at a wide range of salt concentrations. Absorbance readings were taken 15 min after addition of peroxidase substrate. The maximum absorbance reading for each antibody was set at 100% antibody binding, and the percentage antibody binding at each salt concentration was calculated by dividing the absorbance by the maximum absorbance. The percentage antibody binding was plotted as a function of the salt concentration. In other experiments, antibody was serially diluted at several salt concentrations and tested for binding in the ELISA. The antibody dilution required to give an absorbance of 1.0 in a set time (usually 20 min) was determined from graphs of absorbance vs. antibody dilution at each salt concentration. The log of this value was plotted against the log of the salt concentration, and the slope of the line was determined by a least-squares linear regression.

Determination of Antibody Subclass. Microtiter plates coated with subclass-specific antibody and blocked with ovalbumin were kindly provided by Christel Augl (USUHS). Hybridoma medium diluted 1:5 was added to the wells and incubated for 2 h at room temperature. After the wells were washed with PBS + T, peroxidase-labeled antibody was added. After 2–3 h at room temperature the plate was washed and peroxidase substrate added. Readings were taken visually.

Results

To obtain hapten–macromolecule conjugates, pA2′pA, pA2′pA2′pA, and pppA2′pA2′pA2′pA were attached through a 5′-phosphoramide bond formed from the 5′-phosphate of the oligonucleotide or a free amino group on derivatized Ficoll or on methylated BSA (mBSA). Tetramer monophosphate, pA2′pA2′pA2′pA, was attached through the 2′(3′)-terminal ribose to BSA and through the 5′-phosphate to Ficoll. For use as a control, 5′-AMP was attached to Ficoll through the 5′-phosphate. In general, the 5′-coupling procedure was significantly more efficient than the 2′(3′)-coupling procedure (30–50% vs. 5%) and resulted in a greater degree of substitution (11–18 vs. 4–7 mol of hapten per mole of protein). The degree of substitution of derivatized Ficoll was significantly higher than for protein (15–47 vs. 11–18 mol per mole of protein). The degree of substitution of mBSA showed no

Table I: Screening of Anti-2′,5′-Oligoadenylate Hybridoma Supernatants

MAb	immunogen	plate coating[a]	titer[a]	subclass[b]
34C3-6	mBSA–5′-$(pA)_3$	0.025 μg/mL F–$(pA)_3$	3300	γ2b
21-3AC9	mBSA–5′-$(pA)_3$	0.10 μg/mL F–$(pA)_3$	20000	γ1
23-3A1A	mBSA–5′-$(pA)_3$	0.10 μg/mL F–$(pA)_3$	10000	γ1
24-4F3C	BSA–3′-$(pA)_4$	0.05 μg/mL F–$(pA)_4$	17000	γ1
36FF4-5A	mBSA–5′-$pp(pA)_4$	0.20 μg/mL F–$pp(pA)_4$	5000	γ1

[a] Concentration of Ficoll–hapten employed and hybridoma medium titer were determined by checkerboard titration as outlined under Materials and Methods and described in the text. [b] Determined as described under Materials and Methods.

correlation with the titer of the resulting antisera (results not shown). Serum titers ranged from 1/100 to 1/300 000, but a high serum titer did not assure a productive fusion. Serum titers at the time of fusion ranged from 1/300 to 1/13 000 in the instances where positive hybridomas were obtained.

Hybridomas secreting an antibody directed against 2′,5′-oligoadenylates were detected by using an enzyme-linked immunosorbent assay as described under Materials and Methods. The specificity and subclass of the monoclonal antibodies produced by hybridomas derived from the same spleen were often identical. These hybridomas may have arisen from the same lymphocyte clone or by somatic mutation. Although dozens of hybridomas were obtained, for simplicity and because our results suggest that many may secrete the same protein, the hybridomas described here are those that arose from fusions of spleen cells of different mice.

Ten mice were immunized with pA2′pA–protein conjugates, but disappointingly, in no instance were stable clones obtained (results not shown). The substitution of pA2′pA onto protein was within the range of the other oligoadenylate–protein conjugates, and the titers of circulating antibody elicited in the mice were comparable to those for other mice. Our inability to obtain a stable hybridoma secreting antibody directed against pA2′pA cannot be explained.

Media from stable positive hybridomas were subjected to checkerboard titration against Ficoll–hapten containing the hapten used in immunization. The concentration of Ficoll–hapten chosen for competition assays was an amount less than saturating but high enough to give an absorbance reading greater than 1.0 for a range of antibody dilutions (Figure 1B). These concentrations were 0.025–0.2 μg of Ficoll/mL, which corresponds to 2–14 ng of Ficoll added per well. The chosen antibody dilution gave an absorbance of 1.0–1.2 in 15–30 min and was in the linear portion of the titration curve (Figure 1A).

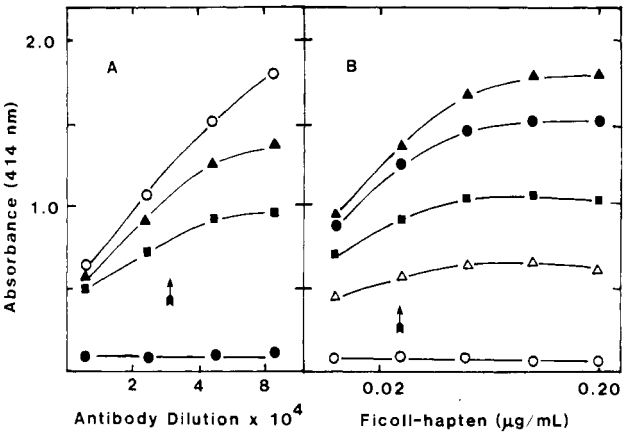

FIGURE 1: Checkerboard titration of Hy34C3-6. Microtiter plates were coated with Ficoll–pA2′pA2′pA at varying concentrations and tested with serial dilutions of Hy34C3-6 medium as described under Materials and Methods (Engvall, 1980). The absorbance of all controls was ≤0.06. Arrows designate the concentrations selected for competition assays. (A) Absorbance is plotted as a function of antibody dilution. Ficoll–pA2′pA2′pA concentrations were 0.2 μg/mL, 0.1 μg/mL, and 0.05 μg/mL (○), 0.025 μg/mL (▲), 0.0125 μg/mL (■), and 0.2 μg/mL with control medium (●). (B) Absorbance is plotted as a function of Ficoll–hapten concentration employed. Hybridoma dilutions were 9.2×10^{-4} (▲), 4.6×10^{-4} (●), 2.3×10^{-4} (■), 1.2×10^{-4} (△), and 1×10^{-3} with control medium (○).

The optimal amounts of Ficoll–hapten conjugate and hybridoma dilution were ascertained in this manner for each hybridoma supernatant (Table I).

To ascertain antibody specificities, the ability of oligonucleotides and nucleotides to inhibit the binding of antibody to Ficoll–hapten-coated plates was investigated. The concentration of each oligonucleotide required to achieve a 50% inhibition of antibody binding (IC_{50}) was determined as described under Materials and Methods.

Initial experiments revealed that monoclonal antibodies of three of the five established hybridoma lines, Hy21-3AC9, Hy34C3-6, and Hy24-4F3C, were inhibited by 3–15 nM pA2′pA2′pA (Table II). In sharp contrast, two monoclonal antibodies, Hy23-A1A and Hy36FF4-5A, were not inhibited by pA2′pA2′pA or pA2′pA2′pA2′pA at concentrations as high as 1–10 μM. However, these hybridomas were inhibited by pA2′pA2′pA attached to Ficoll. This indicates that the determinant probably included the phosphoramidate linkage between the oligonucleotide and the carrier protein. Since Ficoll–pA2′pA was not efficient in inhibiting Hy23-3A1A or Hy36FF4-5A, those epitopes must include pA2′pA2′pA as well as the phosphoramidate bond. Each monoclonal antibody that was inhibited by nanomolar concentrations of free pA2′pA2′pA was also inhibited effectively by Ficoll–pA2′pA2′pA.

Table II: Comparison of Antibody Specificities

	monoclonal antibody[a]				
inhibitor	Hy21-3AC9 [mBSA–5′-$(pA)_3$]	Hy23-3A1A [mBSA–5′-$(pA)_3$]	Hy24-4F3C [BSA–3′-$(pA)_4$]	Hy34C3-6 [mBSA–5′-$(pA)_3$]	Hy36FF4-5A [mBSA–5′-pp-$(pA)_4$]
pA2′pA2′pA	3	20000	12	15	nd[d]
pA2′pA2′pA2′pA	22	>10000	3.5	5	>1000
pA2′pA2′pAahp[b]	40000	>10000	0.7	3	nd[d]
pA2′pA2′pA2′pAahp[b]	1200	nd[d]	0.2	1.8	nd[d]
F–pA2′pA2′pA[c]	2.2	13	2.4	0.3	200
F–pA2′pA[c]	>200	>1200	>340	27	>340
F–5′-AMP[c]	nd[d]	nd[d]	nd[d]	>350	nd[d]

[a] Concentrations (nanomolar) required to achieve a 50% inhibition of antibody binding in a competition ELISA (IC_{50}'s) are given. See Materials and Methods. The immunogen is designated in brackets under the antibody name. [b] The suffix "ahp" designates azahexapyranose as the 2′(3′)-terminal sugar. See Materials and Methods. [c] For Ficoll–hapten conjugates, values given are the final concentrations of hapten (nanomolar), calculated by assuming the degree of substitution reported under Materials and Methods. [d] nd = not determined.

Table III: Reactivity of Monoclonal Antibodies with 2′,5′-Oligonucleotides and Adenosine Nucleotides

inhibitor	monoclonal antibody[a] Hy21-3AC9	Hy24-4F3C	Hy34C3-6
cores			
A2′pA	10000	>2900	>670
A2′pA2′pA	15	20	>670
A2′pA2′pA2′pA	18	2.4	330
5′-monophosphates			
pA2′pA	170	35	17
pA2′pA2′pA	1.0[b]	3.4	1.0[b]
pA2′pA2′pA2′pA	7.3	1.0[b]	0.3
pA2′pA2′pA2′pA2′pA	8.0	0.5	0.3
5′-triphosphates			
pppA2′pA	nd	14	13
pppA2′pA2′pA	1	6.6	3.3
pppA2′pA2′pA2′pA	5.3	0.8	1.6
adenosine and nucleotides			
adenosine	$>3 \times 10^5$	$>1.4 \times 10^6$	6.7×10^4
5′-AMP	$\geq 5 \times 10^5$	4.6×10^5	1.3×10^4
2′-AMP	$>3 \times 10^4$	5.7×10^5	1.2×10^4
5′-ADP	nd[c]	4.6×10^5	3.0×10^4
5′-ATP	2.8×10^5	2.3×10^6	1.9×10^4
2′,5′-ADP	1×10^6	2300	8.0×10^3
linkage isomers and deoxyribose analogues			
pA3′pA2′pA	1.7	57	130
pA2′pA3′pA	800	33	3.3
pA2′pA3′pA2′pA	10	13	1.5
pA3′pA2′pA2′pA	6.7	4.6	7.3
pA3′pA3′pA	>1000	100	>170
p(3′dA)2′p(3′dA)2′p(3′dA)	15	21	12
pA2′pA2′p(2′dA)	1700	2.6	1.6
base analogues			
p(br^{8}A)2′p(br^{8}A)2′p(br^{8}A)	310	24	120
p(ϵA)2′p(ϵA)2′p(ϵA)	910	13	2.3
pI2′pI2′pI	1500	20	>670
pA2′pA2′pI	33	3.4	1.7
pA2′pI2′pA	40	4.0	0.3
pI2′pA2′pA	27	8.0	13.0
pC2′pC2′pC	3700	8600	>670
pU2′pU2′pU	1100	830	nd[c]
5′-terminal additions			
Man6pp5′A2′pA2′pA	2.5	8.3	18
A5′pppp5′A2′pA2′pA	0.7	3.7	2.7
3′-terminal additions			
pA2′pA2′pAahp	13000	0.15	0.20
pA2′pA2′pA2′pAahp	>1700	0.04	0.12
pppA2′pA2′pA2′pAahp	>1700	0.05	nd[c]

[a] Numbers given are the index of dissimilarity (ID), calculated by using a competition ELISA as described under Materials and Methods. The larger the ID, the poorer was the reaction with antibody. [b] Hapten used in the immunization, set at 1.0. [c] nd = not determined.

Preliminary comparisons also revealed that the monoclonal antibodies possessed distinct specificities, even if obtained from different mice immunized with the same hapten–protein conjugate. Hy21-3AC9 was poorly reactive with 2′,5′-oligoadenylates modified with a terminal azahexapyranose (ahp), whereas both Hy24-4F3C and Hy34C3-6 were very reactive with those analogues (Table II). Mice 21 and 34 were immunized with the same conjugate, mBSA–5′-pA2′pA2′pA, whereas mouse 24 was immunized with BSA–3′-pA2′pA2′pA2′pA. Because Hy23-3A1A and Hy36FF4-5A reacted poorly with free 2′,5′-oligoadenylate, their specificities were not examined further.

To clearly define the epitopes recognized by three monoclonal antibodies, Hy21-3AC9, Hy24-4F3C, and Hy34C3-6, the ability of various oligonucleotides and nucleotides to inhibit antibody binding was examined (Table III). The three antibodies were distinct in their fine specificities. While all displayed a preference for 2′,5′-oligoadenylates with a 5′-monophosphate moiety, only reactivity of Hy34C3-6 depended strongly on a 5′-phosphate. All preferred trimers to dimers, but the relative dependence on length varied. Hy21-3AC9 reacted best with trimer monophosphate, and increasing the length decreased reactivity slightly. In contrast, Hy24-4F3C and Hy34C3-6 reacted slightly better with tetramers and pentamers relative to trimers. Addition of phosphates to form 5′-triphosphates had little affect on antibody recognition. Hy34C3-6 reacted with adenosine and adenosine nucleotides in the range of 0.12 mM, and Hy21-3AC9 and Hy24-4F3C reacted with adenosine and adenosine nucleotides in the millimolar range.

The effect of alteration of the ribose and ribose phosphate backbone of oligoadenylates on their immunoreactivity was examined (Table III). Each antibody required the presence of at least one 2′,5′-phosphodiester bond. Two of the three antibodies displayed a strong preference for which phosphodiester bond it required to be 2′→5′. Hy21-3AC9 preferred pA3′pA2′pA over pA2′pA3′pA, whereas Hy34C3-6 reacted better with the latter. Hy24-4F3C reacted equally well with both of those trimer linkage isomers and was only 100-fold less reactive with pA3′pA3′pA than pA2′pA2′pA. In contrast, reactivity of the other two monoclonals with 2.5–3.0 μM 3′,5′-linked oligoadenylate was not detected. In the tetramer category of linkage isomers, all antibodies reacted well with pA3′pA2′pA2′pA and slightly less well with pA2′pA3′pA2′pA. The 3′-hydroxyl was not critical to the epitope; the cordecypin analogue reacted well with each monoclonal antibody. How-

ever, the 2′-OH was required for recognition by Hy21-3AC9.

To ascertain the importance of the base moieties in forming the epitopes, several base analogues of 2′,5′-oligoadenylate were employed in competition assays (Table III). Their relative reactivities with the three antibodies varied. Hy24-4F3C reacted well with all purine analogues but reacted poorly with pyrimidine analogues. Hy34C3-6 reacted only with the purine analogues that retained two unmodified C6 amino groups and the C8 hydrogens. Further, Hy34C3-6 was slightly more sensitive to modification of the base nearest the 5′ terminus. Hy21-3AC9 was the most base-specific antibody, reacting significantly only with analogues containing two unmodified adenosines.

Since certain antibodies appeared to be more sensitive to changes at one end of the molecule or the other, the effect of additions to the 5′ or 2′(3′) terminus was investigated (Table III). In only one instance was antibody recognition decreased by an alteration at the terminus; Hy21-3AC9 was very sensitive to modification of the 2′-terminal ribose, but the other two antibodies reacted well with the azapyranose-modified oligonucleotides. None of the three antibodies was significantly affected by modification at the 5′ terminus beyond the 5′-phosphate.

Experiments designed to determine the effect of salt concentration on antibody binding revealed further differences in the three antibodies. In control experiments to determine the effect of salt on Ficoll–hapten-coated wells, Ficoll–pA2′pA2′pA-coated wells were filled with buffer containing 0.05–2.5 M NaCl and incubated at 30 °C for 90 min. The wells were then washed and incubated with antibody diluted in physiological saline with added Tween 20 and ovalbumin. The results suggested that the amount of Ficoll–hapten coating was not altered by incubation in high salt buffers under the conditions of these assays (Figure 2A and results not shown). When one dilution of each antibody was tested over a range of NaCl concentrations, substantial salt-dependent inhibition of binding of all but one of the antibodies was observed; binding of Hy21-3AC9 was relatively insensitive to the salt concentration (Figure 2A and results not shown).

Theoretically, the number of ion pairs involved in binding antibody to a polyanion can be calculated from plots of log K vs. log (ionic strength) (Lee et al., 1982). For single-stranded DNA, the slope of the line = 0.7 (number of ion pairs) (Record et al., 1976). Since in the case of oligonucleotides at physiological pH the only ions present are the phosphate ions of the ribose phosphate backbone, the number of ion pairs involved in binding would be the number of phosphates that interact with the antibody combining site. We did not measure antibody affinity directly. The dilution of antibody required to achieve a given amount of antibody binding at several salt concentrations was ascertained (see Materials and Methods). Plots of log (antibody dilution) vs. log (NaCl concentration) were linear. The slopes of these lines were 1.56 for Hy24-4F3C, 0.82 for Hy23-3A1A, 0.79 for Hy36FF4-5A, 0.65 for Hy34C3-6, and ≤0.40 for Hy21-3AC9 (Figure 2B and data not shown). Assuming that antibody binding in ELISA is proportional to antibody affinity, these results imply that the number of phosphates involved in antibody binding is 2 for Hy24-4F3C and 1 for Hy34C3-6, Hy36FF4-5A, and Hy23-3A1A. The results for Hy21-3AC9 are less clear but suggest that no phosphates are directly involved in antibody binding.

Discussion

An efficient method for linking nucleotides and oligonucleotides to macromolecules has been described. Utilization

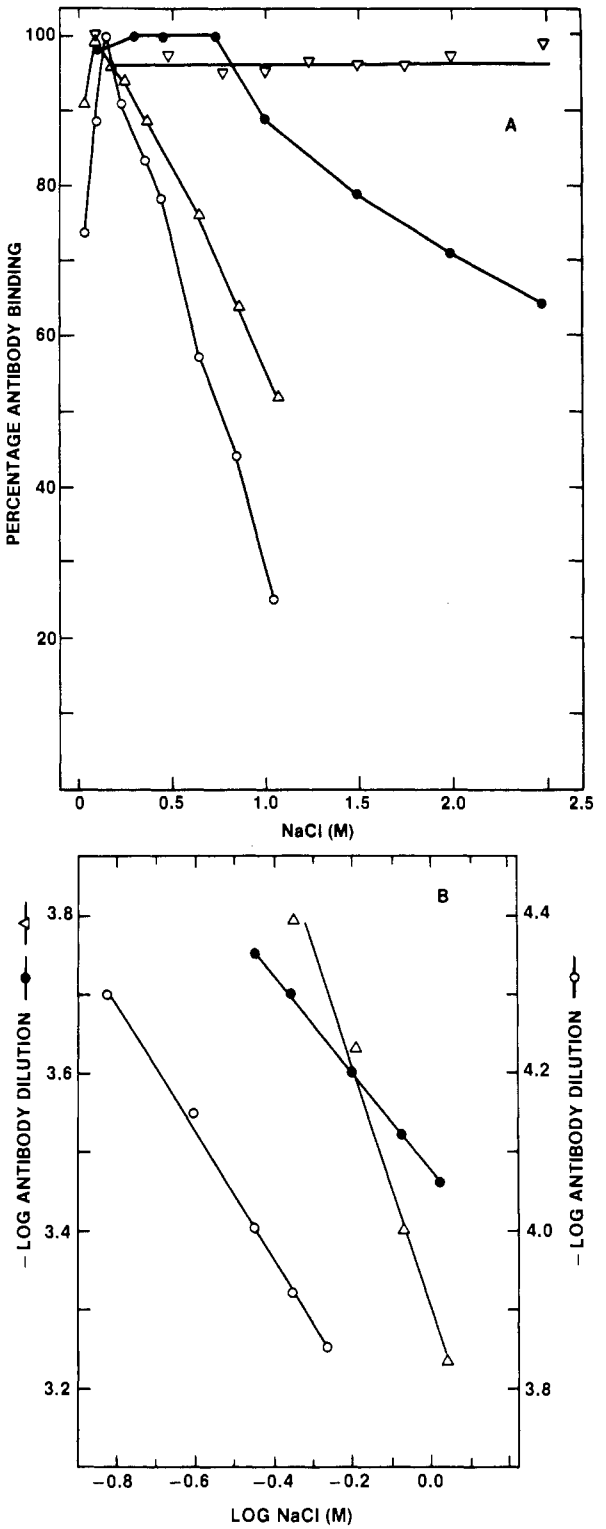

FIGURE 2: Effect of salt concentration on antibody binding. (A) Monoclonal antibodies Hy24-4F3C, Hy34C3-6, and Hy21-3AC9 were diluted to 5×10^{-5}, 3×10^{-4}, and 5×10^{-5}, respectively, in a phosphate buffer with 0.05% Tween 20, 0.2% ovalbumin, and 0.05–2.5 M NaCl. After incubation in Ficoll–pA2′pA2′pA-coated wells, antibody binding was determined as indicated under Materials and Methods. Control medium at a 10^{-3} dilution showed <5% binding at all concentrations of salt tested. In control experiments, wells were filled with buffer containing 0.05–2.5 M NaCl and incubated at 30 °C for 90 min. The wells were then washed and incubated with antibody diluted in PBS of normal salinity. The control shown here is Hy21-3AC9. Symbols: Hy21-3AC9 control (▽); Hy21-3AC9 (●); Hy34C3-6 (△); Hy24-4F3C (○). (B) The log of the antibody dilution required to achieve a set degree of binding at each salt concentration, determined as described under Materials and Methods, is plotted against the log of the salt concentration for three of the five antibodies assayed. Symbols: Hy23-3A1A (○); Hy34C3-6 (●); Hy24-4F3C (△).

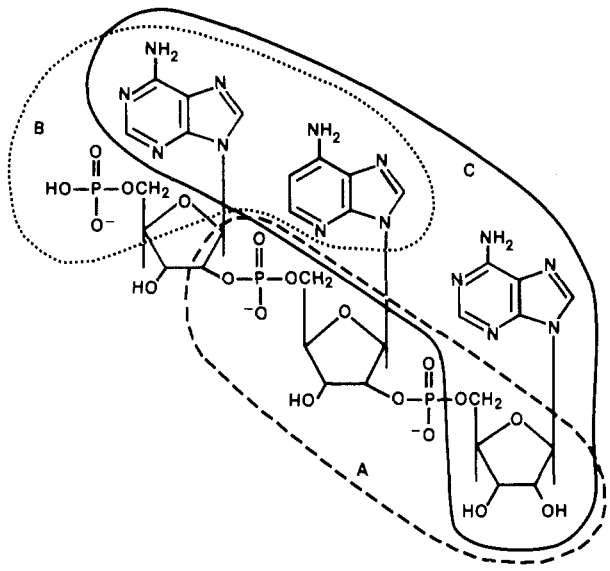

FIGURE 3: Schematic models of epitopes defined by three monoclonal antibodies: (A) Hy24-4F3C; (B) Hy34C3-6; (C) Hy21-3AC9.

of the imidazolide derivative of 2′,5′-oligoadenylates resulted in a greater efficiency of coupling to protein than possible by carbodiimide-mediated attachment or by periodate oxidation techniques. Methylated BSA was more soluble in Me_2SO than BSA so mBSA was employed in these coupling reactions. This procedure may be particularly useful for coupling oligonucleotides such as 2′,5′-oligoadenylate that are available in limited quantity.

The imidazolide derivatives of oligonucleotides also reacted efficiently with AECM-Ficoll. Ficoll–hapten conjugates have been employed previously to study B lymphocyte activation. The number of free amino groups that result from the Ficoll derivatization procedures employed here has been estimated to be as high as 60–80, and coupling of as many as 60 mol of hapten per mole of Ficoll has been reported (Inman, 1975). We found that Ficoll also makes an excellent hapten carrier in solid-phase immunoassays. Ficoll is readily available, can be derivatized with a variety of haptens, is amenable to reaction in organic solvent, adsorbs tightly to polystyrene plates, and would not be expected to cross-react with antibodies directed against carrier protein. Further, the linking arm probably allows the hapten ready accessibility to the solvent.

Three distinct monoclonal antibodies directed against 2′,5′-oligoadenylate were characterized. This work contrasts previous reports in that we evaluated the specificity of anti-oligonucleotide monoclonal antibodies in considerably more detail than has been accomplished previously. Cross-reactivity studies in combination with salt-sensitivity observations provided valuable information on the features of 2′,5′-oligoadenylate required for antibody binding. Each antibody displayed a unique fine specificity and appeared to recognize different substructures of 2′,5′-oligoadenylate. We have identified the areas most important for binding each antibody and present schematic models for three epitopes identified by these antibodies. Measurements of space-filled molecular oligoadenylate models suggest that these epitopes could fit within the dimensions of known antibody combining sites (results not shown) (Segal et al., 1974). More exact definition of the epitopes will be possible when the three-dimensional structure of 2′,5′-oligoadenylate is known.

Hy24-4F3C was the least base specific and the most charge dependent of the three monoclonal antibodies (Figure 3, area A). Increasing lengths displayed increased apparent affinity probably due to their increased charge. The 5′-phosphate did not appear to be a prerequisite for binding; A2′pA2′pA2′pA reacted as well as did pA2′pA2′pA. However, two internucleotide phosphates were required for strong antibody binding. The phosphodiester bonds were of equal importance. Alteration of one or two bonds to a 3′,5′-linkage had a less drastic effect than changing the bases to pyrimidines. This result underlines the importance of the bases in defining an epitope. Although not depicted in the diagram, the purine bases may be involved directly in binding antibody. Alternatively, whether the base is a purine or a pyrimidine may affect binding by affecting the ribose phosphate backbone. It is difficult to distinguish these two possibilities without knowing the effect of each base modification on the conformational parameters and flexibility of the oligonucleotide backbone. However, since purine modifications that resulted in altered reactivity with other antibodies had no significant effect on recognition by Hy24-4F3C, we propose that the bases play an indirect role in forming the epitope recognized by Hy24-4F3C. That the base moieties may help define a ribose phosphate epitope is consistent with previous observations on nucleotides and polynucleotides. For example, poly(G)·poly(C) is immunochemically distinct from poly(I)·poly(C) and poly(A)·poly(U) (Schwartz & Stollar, 1969), and more recently, an antibody population reactive with poly(I)·poly(C) but not poly(A)·poly(U) has been reported (Kitagawa & Okuhara, 1980). The increased reactivity with pA2′pA2′pAahp relative to pA2′pA2′pA (23-fold) suggests that the azapyranose linkage region contributed directly to the epitope. Since neither the azahexapyranose modification or increasing chain length decreased reactivity substantially, we propose that 2′,5′-oligoadenylate binds to a groove rather than in a pocket in the Hy24-4F3C antigen binding site.

Hy34C3-6 also preferred trimer and longer 2′,5′-oligoadenylates, but in this case the 5′-phosphate was critical to the epitope (Figure 3, area B). Tetramer core, A2′pA2′pA2′pA, was substantially less reactive than even dimer monophosphate, pA2′pA. One phosphate, probably the 5′-phosphate, is involved directly in binding. Since the first phosphodiester bond was more critical than the second, and since the 5′-terminal base was more important than the others, an epitope encompassing the 5′-end is proposed. Purines with two C6 extracyclic amino groups and C8 hydrogens were preferred. Hy34C3-6 showed slightly increased (2–5-fold) reactivity with 3′-modified azahexapyranose derivatives. This may be due to hydrophobic interactions at or near the antibody binding site since the azapyranose was not the mode of linkage to carrier protein and since all other evidence suggests that the major epitope is at the 5′ area of the molecule. Like Hy24-4F3C, because increased chain length was an acceptable modification, we propose that the binding site is a groove rather than a pocket.

Hy21-3AC9 was the most specific antibody obtained and preferred the 2′,5′-trimer monophosphate pA2′pA2′pA (Figure 3, area C). The 5′-phosphate did have a minor effect on the epitope since all monophosphate forms reacted slightly better than their corresponding core forms. Most base modifications resulted in a considerable change in antibody recognition; only substitution of one adenine to hypoxanthine was tolerated appreciably. The second phosphodiester linkage was more important in forming the determinant than the first, and an unmodified 2′(3′) terminus was required. We propose that this epitope includes the bases and the 2′(3′)-terminal hydroxyl, but no phosphates. Since this antibody was somewhat length specific and very sensitive to 2′(3′) modification, we propose that 2′,5′-oligoadenylate binds to a pocket in the antibody

binding site and that the analogues with 5′-terminal additions bind well by virtue of the flexibility inherent in pyrophosphate linkages (Saenger et al., 1977).

Most of the monoclonal antibodies were inhibited more effectively by Ficoll-conjugated pA2′pA2′pA than by free pA2′pA2′pA (Table II). This enhanced inhibitory capacity may be due in part to the multivalent nature of Ficoll conjugates. If the oligonucleotide is evenly distributed over the length of the Ficoll, there would be one oligonucleotide for every 50–150 sugars. It is unlikely that the oligonucleotides are close enough in primary sequence to bind the same antibody, although folding may allow oligonucleotides to come into close proximity. Alternatively, the increased inhibitory capacity of Ficoll–pA2′pA2′pA relative to free pA2′pA2′pA may be due to a contribution to the determinant by the phosphoramidate linkage of the former. This is probably the case for Hy34C3-6, Hy23-3A1A, and Hy36FF4-5A.

Antibodies directed against double-stranded RNA have been shown previously to be drastically affected by alteration of the ribose phosphate backbone (Johnston et al., 1975; Johnston & Stollar, 1978). Similarly, methylation of the 2′-position of 5′-GMP resulted in a considerable alteration in immunoreactivity (Vold, 1981). In this study, the ribose phosphate backbone of oligonucleotides was also shown to be critical in forming the antigenic domains. One or more 2′→5′ bonds were required even in the case where the phosphates were probably not in contact with the antibody (Hy21-3AC9), as well as in instances where the phosphoramidate bond played a role in defining the determinant (Hy23-3A1A and Hy36FF4-5A). The presence of a purine was essential for reactivity, although in one case, Hy24-4F3C, all purine modifications tested were acceptable. These results imply that the general nature of the base moiety is also important in forming the epitope. The purine bases may provide contact points for the antibody, or they may force the 2′,5′-oligoadenylate backbone into a conformation that is distinct from that of a 2′,5′-oligopyrimidine. Certain heterologous rabbit antisera directed against 2′,5′-oligoadenylate do react with 2′,5′-oligopyrimidines (Johnston et al., 1983).

Recently, Munns and co-workers investigated the features of nucleosides required for recognition by specific antibody (Munns et al., 1984). By measuring reactivity of antibodies with a variety of nucleoside–protein conjugates in a solid-phase enzyme-linked assay, they surmised that antibody recognition depends on the presence of certain atoms in the nucleoside and that the immunodominant atoms may be different in different nucleosides. Our study suggests that the epitope of oligonucleotides may also consist of only a portion of the molecule and that different antibodies may bind different epitopes.

A monoclonal antibody directed against A2′p5′A has been described previously (Cailla et al., 1982). This antibody reacted strongly with $A(2'p5'A)_n$, where $n = 1–4$. In contrast to the antibodies described here, addition of phosphates to the 5′ terminus reduced antibody reactivity considerably. Cross-reactivity with various nucleotides was observed at very high concentrations, as observed in this report.

Monoclonal antibodies can provide discriminatory reagents. Other studies demonstrated that monoclonal antibodies recognize different epitopes of Z-DNA (Möller et al., 1982). Monoclonal antibodies that recognize different structures of poly(adenosine diphosphate ribose) have also been reported (Kawamitsu et al., 1984). The monoclonal antibodies described here appear to recognize different portions of 2′,5′-oligoadenylate. These monoclonal antibodies may facilitate the isolation and identification of 2′,5′-oligoadenylates from cells or tissues. Unusual 2′,5′-oligoadenylate-like material has been isolated from interferon-treated, SV-40-infected (Hersh et al., 1984a) or Herpes-infected cells (Cayley et al., 1984) and from normal mouse tissues (Laurence et al., 1984). These products were reactive in radioimmune assays and/or radiobinding assays and had the nuclease resistance and sensitivity patterns expected of 2′,5′-linked nucleotides but were not biologically active pppA2′pA2′pA as judged by their high-performance liquid chromatography retention times and/or their ability to activate the 2′,5′-oligoadenylate-specific endonuclease. A panel of well-defined monoclonal antibodies that recognize substructural aspects of 2′,5′-oligoadenylate may be useful in determining the nature of the modification present in such material.

The only naturally occurring protein that has been described that binds 2′,5′-oligoadenylate is an M_r 80 000 endonuclease (Wreschner et al., 1981; Floyd-Smith et al., 1982). These observations were based on binding studies that employed a 2′(3′)-modified radiolabeled derivative of 2′,5′-oligoadenylate, pppA2′pA2′pA2′pA3′[^{32}P]pC. A possibility to be considered is whether naturally occurring proteins can also bind specifically to different portions of 2′,5′-oligoadenylate. Proteins that do not bind 2′(3′)-modified 2′,5′-oligoadenylate may have been overlooked.

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Registry No. 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; pA2′pA2′pA, 61172-40-5; pA2′pA2′pA2′pA, 66048-58-6; pA2′pA2′pAahp, 83807-28-7; pA2′pA2′pA2′pAahp, 83807-29-8; 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′pA2′pA2′pA, 66048-59-7; pppA2′pA, 65954-94-1; pppA2′pA2′pA, 65954-93-0; pppA2′pA2′pA2′pA, 65954-95-2; 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; pA3′pA3′pA, 1684-34-0; p(3′dA)2′p(3′dA)2′p(3′dA), 84311-63-7; pA2′pA2′p(2′dA), 95314-26-4; p(br^{8}A)2′p(br^{8}A)2′p(br^{8}A), 84311-64-8; p(ϵA)2′p(ϵA)2′p(ϵA), 84877-05-4; pI2′pI2′pI, 66048-63-3; pA2′pA2′pI, 95314-29-7; pA2′pI2′pA, 95314-28-6; pI2′pA2′pA, 95314-27-5; pC2′pC2′pC, 84311-66-0; pU2′pU2′pU, 66048-60-0; Man6pp5′A2′pA2′pA, 84311-67-1; A5′pppp5′A2′pA2′pA, 77063-72-0; pppA2′pA2′pA2′pAahp, 83807-30-1; adenosine, 58-61-7; 5′-adenylic acid homopolymer, 24937-83-5.

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