Antibody–nucleic acid interactions. Antibodies to psoralen-modified RNA as probes of RNA structure

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ABSTRACT

Antisera elicited by immunization of rabbits with 4'-aminomethyl-trioxsalen (AMT)-modified poly(A,U) complexed with methylated bovine serum albumin was characterized in competition radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA). AMT-poly(A,U) was over 10,000-fold more reactive than unmodified poly(A,U) or AMT alone. The antiserum cross-reacted to varying extents with AMT-modified-RNA's and -DNA's. The presence of AMT-uridine usually assured strong reactivity. The amino group of AMT contributed to antibody binding to a small degree. Binding was not significantly affected by high ionic strength, suggesting that binding does not involve ion pair formation. Murine encephalomyocarditis virus replicative intermediates, as well as cellular RNA and DNA were modified by psoralen in intact cells, suggesting that EMCV RNA and cellular RNA's in intact cells possess detectable stretches of base pairs. The antibodies described here will be useful in studying the secondary and tertiary structure of RNA's *in vitro* and in intact cells.

INTRODUCTION

Psoralens are a class of compounds that have become useful tools for examining the secondary and tertiary structures of large RNA's (reviewed, 1). Photoaddition of psoralens to RNA's is dependent on the conformation of the RNA (2). Psoralens intercalate within a double-helical region of a nucleic acid and form stable, but photoreversible, cycloaddition products predominantly with pyrimidine bases upon irradiation with long wavelength ultraviolet light (reviewed, 1). Single-stranded RNA's devoid of secondary structure are not susceptible to psoralen modification under physiological conditions (2). The adducts formed by reaction of 4'-(hydroxymethyl)-4,5',8'-trimethylpsoralen (4'-hydroxymethyltrioxsalen, HMT), with DNA and with natural and synthetic RNA's occur almost exclusively at thymidine (3) and uridine (4, 5) residues. Pyrimidines on adjacent strands can be cross-linked by formation of a diadduct (reviewed, 1).

The effect of psoralen modification on nucleic acid conformation remains somewhat controversial. Psoralen-modified DNA exhibits altered electrophoretic mobility in alkaline agarose gels (6). Molecular models of psoralen cross-linked DNA based on the crystal structure of unmodified DNA and energy minimization calculations suggest that substantial kinking and unwinding occur at sites of psoralen damage (7). However, analysis by differential

decay of birefringence indicated that psoralen cross-links do not introduce any appreciable stable bend in a double-stranded DNA molecule (8).

Antibodies that bind polynucleotides provide models for studying structural differences among nucleic acids (reviewed, 9). Antibodies that recognize double-stranded RNA (dsRNA) recognize conformational features of the ribose-phosphate backbone rather than individual nucleotides (10). For example, antibodies raised against poly(A)-poly(U) react well with naturally occurring double-stranded RNA's, but not DNA or RNA-DNA hybrids (10). Modification of the bases of synthetic dsRNA's generally result in less alteration in reactivity with anti-dsRNA antibodies than do changes in the ribose moieties (11). In contrast to the recognition of predominantly conformational determinants of helical RNA's, antisera raised against modified nucleotides by immunization with either a modified single-stranded polynucleotide, or a nucleotide-protein conjugate, are usually specific for the modified nucleotide (reviewed, 12).

Characterization of the effects of psoralen modification on immunoreactivity will help to define the nature of the antigenic determinants of nucleic acids. Antibody reagents specific for psoralen-modified RNA are immediately useful for detecting and localizing psoralen adducts in RNA, and might be important reagents for examining the secondary and tertiary structure of RNA's. We observed that psoralen modification of base-paired RNA altered its recognition by anti-dsRNA antibodies, and we have produced and characterized antibodies directed against psoralen-modified RNA. We believe this to be the first report of antibodies that bind psoralen-modified RNA.

MATERIALS AND METHODS

Reagents.

Methylated bovine serum albumin (mBSA), poly(oxyethylene) monolaurate sorbitan (Tween 20) and chicken egg albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) and polylysine (mw 1500-8000) were purchased from Miles Laboratories (Elkhart, IN). Synthetic polynucleotides were obtained from P-L Biochemicals (Milwaukee, WI). Immunolon I microtiter plates were obtained from Dynatech Laboratories (Alexandria, VA). The peroxidase substrate, 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and affinity-purified peroxidase-labeled goat anti-rabbit IgG were obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Double-stranded RNA's from *Penicillium chrysogenum* and from the S7 killer strain of *Saccharomyces cerevisiae* were generously provided by Drs. Paul Torrence (NIH) and Reed Wickner (NIH), respectively. Fixed *Staphylococcus aureus* cells of the Cowan I strain (Pansorbin^R) was obtained from Calbiochem (San Diego, CA). Tritiated AMT, (³H)-AMT, and tritiated HMT, (³H)-HMT, were obtained from HRI Associates (Emeryville, CA) and had specific activities of 4.3 Ci/mmole and 14.5 Ci/mmole, respectively.

AMT modification and RNA purification.

Nucleic acids were dissolved in a buffer appropriate for obtaining psoralen addition (2). This was usually 0.01 M sodium citrate, pH 8.0, 0.15 M NaCl, 1 mM EDTA, (SSCE), for base-paired nucleic acids, and 10 mM Tris, pH 7.5, 5 mM MgCl₂, 5 mM NaCl (low salt TNM) for single-stranded polymers such as poly(U). Nucleic acids were mixed with AMT and placed in an ice-water bath or left at room temperature with stirring. Samples were irradiated at a distance of 7 - 9 cm with a Blak-Ray Model B-100A ultraviolet lamp with peak intensity at 366 nm. Ultraviolet dosage rates measured at 365 nm with an Ultraviolet Products Model UVX-36 radiometer were 10-20 mW/cm². The total energy of irradiation was usually 1400-2400 J/m². Controls included untreated RNA, RNA irradiated in the absence of AMT, and RNA treated with AMT but not irradiated. Modification with HMT was accomplished as described for AMT.

The immunoreactivity and melting characteristics of the RNA's were examined after purification by extraction twice with phenol/chloroform-/isoamyl alcohol (50:45:5) and once with ether, followed by precipitation with ethanol. Purified RNA was collected by centrifugation at 10,000 x g for 30 min., dried under vacuum, and dissolved in SSCE or in 0.01 M Tris HCl, pH 7.5, 0.01 M KCl, 1 mM EDTA, (TKE), or in sterile distilled water.

A known amount of tritiated psoralen derivative was mixed with unlabelled psoralen and nucleic acid prior to irradiation in some experiments. The degree of psoralen modification was determined by scintillation counting of either purified RNA samples or trichloroacetic acid precipitates of crude reaction mixtures.

Preparation of (³H)-AMT-poly(A,U) for radioimmunoassays.

Reaction mixtures consisting of 0.2-1.0 mL 0.25 mM poly(A,U), 0.05 mM (³H)-AMT in SSCE were irradiated with long wavelength UV light (366 nm, 15-20 mW/cm²) with stirring at room temperature for 5-20 minutes. One fifth of the AMT was added at 0, 4, 8, 12 and 16 minutes during a 20 minute irradiation of larger reaction mixtures. (³H)-AMT-modified poly(A,U), (³H)-AMT-poly(A,U)), was separated from unincorporated (³H)-AMT by extraction with phenol and ethanol precipitation, as described above.

Immunization and absorption procedures.

Rabbits were immunized with poly(A)-poly(U), or AMT-poly(A,U), complexed with methylated BSA (mBSA) and emulsified in either complete Freund's adjuvant for the first injection, or incomplete Freund's adjuvant for subsequent boosts. Serum Ra80-015b was obtained in week 12 from a rabbit that had received 6 injections of 350 ug poly(A)-poly(U). Ra85-074b was obtained in week 15 after 4 injections of 100 ug AMT-poly(A,U). Complement in both antisera was inactivated by heating at 56° C for 30 min.

Ra85-074b was absorbed with AMT-poly(U)-cellulose as follows. Poly(U)-cellulose was prepared and analyzed essentially as described for poly(I)-poly(C)-cellulose (14). (³H)-AMT was diluted in low salt TNM buffer with unlabeled AMT to a final specific activity of 8.2 Ci/mole. Poly(U)-cellulose was mixed with (³H)-AMT at a ratio of 1 AMT per 2 nucleotides on

ice with stirring. Samples were irradiated for 20 min. at 14.1 mW/cm², and then washed with 2X SSC buffer (20 mM sodium citrate, pH 7.5, 0.30 M NaCl, 2 mM EDTA). Small portions of the washed cellulose were counted in 10 mL Aquassure or subjected to base hydrolysis for spectrophotometric estimation of the moles of nucleotide present. The resin was estimated to contain 1 AMT per 65 nucleotides. Serum 85-074b (0.75mL) was mixed with packed cellulose (0.75 mL) containing 5.1×10^{-7} moles uridine. After incubation at 37° C for 2 hr and overnight at 4° C, the slurry was centrifuged. The resin was washed once with SSCE buffer and the wash combined with the first supernatant.

Enzyme-linked immunosorbant assay.

Optimal amounts of nucleic acid used in coating microtiter plates and of antiserum were determined by checkerboard titration (13). Poly(I)·poly(C) was mixed with polylysine at a 20:1 molar ratio. Soluble complexes of poly(I)·poly(C)·polylysine, (ICL), were diluted to 20 ng/mL and distributed to the wells of polystyrene microtiter plates for assay of the anti-dsRNA antibody (80-015b). AMT-modified calf thymus DNA (AMT-ctDNA) at 0.5 µg/mL was used to coat preirradiated microtiter plates (15) for assay of the anti-AMT-poly(A,U) antiserum (85-074b). Plates were incubated with the appropriate nucleic acid coating at 37° C for 3-4 hours and overnight at room temperature and then stored at -20° C. Plates were incubated with 1% ovalbumin at room temperature for 30-60 min. prior to use. Antiserum was diluted in PBS with 0.02% Tween 20 and 0.20 % ovalbumin, (PTO) to a final dilution of 2×10^{-5} for Ra80-015b and 5×10^{-5} for Ra85-074b. Antibody was incubated in the presence or absence of soluble RNA's at 30° C for 90 minute in ligand-coated wells. Plates were washed three times with PBS containing 0.02% Tween 20, (PT), and incubated with peroxidase-labelled goat anti-rabbit IgG in PTO buffer for 90 min. at 30° C. Plates were washed prior to addition of peroxidase substrate, (ABTS), and H₂O₂ and read in 15-30 min when the absorbance at 414 nm of the uninhibited controls was 1.0 - 1.2. The mean absorbance of duplicates or triplicates was plotted as a function of the molar concentration of inhibitor. The concentration of inhibitor required to achieve 50% inhibition of antibody binding, (IC₅₀), was extrapolated from these graphs. Relative reactivities were determined by dividing the IC₅₀ of the sample by the IC₅₀ for AMT-poly(A,U) determined in the same assay (16). The relative reactivities presented are the mean of 2-4 determinations

Radioimmunoassay.

Lyophilized, fixed *Staphylococcus aureus* was suspended in distilled water to a final concentration of 10%. Fixed *S. aureus* was washed twice with buffer containing 0.01 M sodium phosphate, pH 7.5, 0.15 M NaCl, 0.25% NP40, and 1 mM EDTA, (PBS/NE), and once or twice with PBS/NE containing 0.5 mg/ml ovalbumin, (PBS/NEO) before final resuspension in PBS/NEO (17).

Bentonite that pelleted at 8500 rpm but not at 2500 rpm in a GSA rotor was thoroughly washed once with distilled water, once with 0.1 M EDTA, pH 7.0, and three times with distilled

water as described by Fraenkel-Conrat et al. (18). The washed bentonite was autoclaved and suspended in distilled water to a final stock concentration of about 4.5 mg/ml.

For antibody titration experiments, 100 μ L washed *S. aureus* was incubated with the equivalent of 0.025 - 5.0 μ L antiserum in a final volume of 100-150 μ L for 30-60 min. on ice. The *S. aureus*-antibody complex was washed twice with PBS/NE and once with PBS/NEO prior to suspension in PBS/NEO. *S. aureus*-antibody complex (100 μ L) was mixed with 50 uL bentonite stock, 50 uL 2X PBS/NEO and 100 uL (3 H)-AMT-poly(A,U) (about 20,000 cpm) in PBS/NEO. Incubations were for 1 h at 37 °C followed by 2-64 h at 4 °C. Precipitates were collected by filtration on nitrocellulose filters or by centrifugation at 10,000 x g for 3 min., and washed four times with PBS/NE. Filters were counted in 5 ml of Aquassure (Amersham) or Ready Solv (Beckman). Pellets were resuspended in PBS/NEO and counted in Aquassure.

For competition assays, a large batch of *S. aureus*-antibody complex was prepared by incubating antiserum and *S. aureus* for 30-60 min. on ice. The complex was washed twice with PBS/NE, once with PBS/NEO, and then suspended in PBS/NEO. The competition assay consisted of 50-100 uL *S. aureus*-antibody complex, 50 uL bentonite stock, 50-75 uL competitor in SSCE, 50 uL 2X concentrated PBS/NEO, and 25-50 uL (3 H)-AMT-poly(A,U), containing about 20,000 cpm in PBS/NEO. Incubations were as described above. Relative reactivities were calculated as described above for ELISA's.

Melting.

Absorbance values and melting curves were obtained with a Gilson spectrophotometer equipped with a thermoprogrammer and chart recorder. Poly(A,U) in SSCE or natural dsRNA's in TKE were melted at 0.5° C/min or 1.0° C/min from 25° C to 99° C. The ability of the RNA's to reanneal after melting was determined by rapid cooling to 4° C, incubation at room temperature for 15-60 min., and determination of the remaining hyperchromicity.

Base hydrolysis

RNA samples purified and dried as described above were dissolved in water and brought to 0.5 M KOH by addition of 5 M KOH. After incubation at 37° C for 24-36 hr, 1M NaH₂PO₄, pH 4.0, was added to a final concentration of 100 mM. The pH was adjusted to 7.0 by addition of 6 N HCl. Sodium phosphate and KCl were added to control samples to achieve the same final concentrations as in the hydrolyzed samples.

Preparation of radiolabelled RNA from mouse L cells infected with encephalomyocarditis virus (EMCV).

Mouse L cells were grown in suspension culture as described previously (19). Crude EMCV was the generous gift of Dr. Paul F. Torrence (NIH) and was purified and assayed as described previously (19). Cells were concentrated to 1.2×10^6 cells/mL and infected at a multiplicity of infection of 10 in the presence 20 μ Ci/mL (3 H)-uridine. AMT was added three hours after the addition of virus to a final concentration of 20 μ g/mL. Cells were kept at 4 °C and irradiated for 5 min. as described above, or were maintained under red light without UV

irradiation. Cells were washed twice and lysed by freeze-thawing in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 1 mM aurintricarboxylic acid, 10 mM vanadyl ribonucleoside complex, and 1% SDS. The 10,000 x g supernatant was treated with pronase at 500 ug/mL for 2 hours at room temperature and extracted with buffer-saturated phenol. RNA was precipitated with ethanol and then with 2M LiCl (20). The RNA was fractionated further by chromatography on Sepharose 2B (21). Binding of Sepharose 2B fractions diluted 1:5 to RA85-074b was determined by competition ELISA as described above.

RESULTS

Characterization of psoralen-modified RNA's.

Psoralens form both monoaddition products and cross-links (reviewed,1). The synthetic homopolymer, poly(A,U), and two naturally-occurring double-stranded RNA's, *P. chrysogenum* (*Pc*) dsRNA and dsRNA from the S7 killer strain of *Saccharomyces cerevisiae* were modified with AMT, melted, and tested for their ability to snap-back, as described under Materials and Methods. This analysis was done to determine if cross-linking occurred under the treatment and irradiation conditions employed. Controls included RNA treated with AMT but not irradiated, and RNA irradiated in the absence of AMT. The melting temperature, (*T_m*), of poly(A,U) increased from 40° C to 49° C after treatment with AMT and UV light, and the *T_m* for *Pc* dsRNA increased from 85.5° C to 90° C. RNA's subjected only to AMT or to UV light showed no alteration in *T_m*. The percent hyperchromicity observed upon melting poly(A,U) decreased from 42-45% for controls to 31% after treatment with both AMT and UV light. All poly(A,U) samples reannealed to within 2% of their original absorbance values, as expected for an RNA of low complexity that readily forms intrastrand hydrogen bonds. The percent hyperchromicity observed upon melting of *Pc* dsRNA decreased from 29-30% in controls to 25% after treatment with both AMT and UV light. Control *Pc* dsRNA's did not reanneal significantly, whereas *Pc* dsRNA treated with AMT and UV light reannealed to within 9% of its original absorbance value, demonstrating that cross-linking probably had occurred. Similar results were obtained for dsRNA from killer yeast (results not shown).

Effect of psoralen modification of RNA's on recognition by anti-dsRNA antibodies.

RNA was treated with AMT and irradiated with long wavelength UV light as described under Materials and Methods. Purified and crude psoralen-modified and control RNA's were tested for reactivity with antibodies specific for unmodified double-stranded RNA. Dilutions of crude reaction mixtures gave results identical to dilutions of purified RNA's (Figure 1A,B, and data not shown), demonstrating that free AMT does not interfere with antibody binding. Poly(A,U) subjected to both AMT and UV light was 10-15-fold less reactive than control poly(A,U) samples (Fig 1A,B). Natural double-stranded RNA's consisting of an uninterrupted double-helix, as opposed to the probable loops, hairpins and helices of poly(A,U), were also affected by psoralen modification. Treatment of these dsRNA's with AMT and UV light reduced

Figure 1: Reaction of AMT-modified RNA's with anti-poly(A).poly(U) antiserum Ra80-015b in a competition ELISA. The wells of microtiter plates were coated with poly(I).poly(C)-polylysine complexes and incubated with 2×10^{-4} dilution of Ra80-015b and varying concentrations of nucleic acid. Antibody binding was determined with a peroxidase-labeled second antibody as described under Materials and Methods. Each point represents the mean of duplicate determinations. RNA's were untreated (○), or treated with AMT only (△), UV light only (□), or AMT and UV light (●), as described under Materials and Methods. The AMT to nucleotide ratio employed in the reaction mixtures was 1.0. A. poly(A,U), diluted directly from reaction mixtures. B. poly(A,U), purified from reaction mixtures prior to the ELISA. C. *P. chrysogenum* dsRNA, purified prior to assay.

their reactivity with anti-dsRNA antibody by 10-15-fold (Figure 1C). These experiments were performed with unlabeled AMT so it was not possible to estimate the extent of substitution. However, approximately 1-2% of the nucleotides were psoralen-modified under identical reaction conditions when (^3H)-AMT was added. These results demonstrate that recognition of double-stranded RNA by anti-dsRNA antibodies was affected by covalent modification of the dsRNA with AMT. We then sought to determine if an antibody specific for psoralen-modified RNA could be obtained.

Production of anti-AMT-poly(A,U) antiserum.

Rabbits were immunized with psoralen-modified poly(A,U) complexed with methylated BSA. Poly(A,U), a random sequence heteropolymer containing equal amounts of adenosine and uridine, was chosen because of the variety of conformations that might be present, as expected for naturally-occurring RNA's. These may include stretches of single-stranded RNA, dsRNA,

Figure 2. Effect of AMT substitution of poly(A,U) on reactivity with Ra85-074b. Poly(A,U) was reacted with varying amounts of AMT and then tested for its ability to bind RA85-074b in a competition RIA. Input (^3H)-AMT-poly(A,U) and RA85-074b was 15,900 cpm (9,000 acid-precipitable cpm, 32.6 ng) and 0.07 μL , respectively. The ratio of nucleotide to AMT in the reaction mixtures was as follows: (●), 2.5; (△), 5; (□), 10; (▲), 20; (○), 40; (■), 80; (×), none.

loops and hairpin turns. The degree of AMT substitution in the immunogen was approximately 1 AMT per 54 nucleotides. One rabbit produced antibodies that bound poly(A,U) modified with (^3H)-AMT, (^3H)-AMT-poly(A,U), after four doses of immunogen and is the subject of this characterization. Increasing amounts of this antiserum, RA85-074b, bound increasing amounts of (^3H)-AMT-poly(A,U) but preimmune serum did not bind (^3H)-AMT-poly(A,U) (results not shown).

In competition radioimmunoassays, antibody binding to (^3H)-AMT-poly(A,U) was inhibited by unlabeled AMT-poly(A,U) but not by unmodified poly(A,U) or AMT at concentrations up to 10,000-fold higher than the concentration of AMT-poly(A,U) required to achieve 50% inhibition of antibody binding. Control reaction mixtures containing RNA's mixed with AMT but not subjected to irradiation also did not inhibit antibody binding (results not shown).

AMT modification of poly(A,U) was accomplished using differing ratios of unlabelled AMT to nucleotide. Tritiated AMT was included in identically-treated reaction mixtures to estimate the extent of psoralen modification. Increasing the amount of AMT in the reaction mixture increased the degree of AMT substitution (results not shown). Increasing the degree of AMT substitution on poly(A,U) increased its ability to inhibit antibody binding to (^3H)-AMT-poly(A,U) (Figure 2), providing further evidence that the antibody was directed against an AMT-induced alteration of the RNA.

Figure 3. Representative competition immunoassays. Nucleic acids were modified with AMT and tested for their ability to inhibit the binding of Ra85-074b to (^3H) -AMT-poly(A,U) in a RIA or an ELISA as described under Materials and Methods. Each point represents the mean of duplicate determinations. The degree of AMT modification of each nucleic acid is given in Table I. (A) Radioimmune assay. Positive control binding was 6,030 cpm and binding by preimmune serum was 450 cpm. (B) ELISA. Positive and negative control binding was 1.2 and 0.15, respectively (■) AMT-poly(A,U); (□), AMT-calf thymus DNA; (△), AMT-yeast soluble RNA.

Cross-reactivity of anti-AMT-poly(A,U) antiserum.

A variety of nucleic acids were modified with AMT as described under Materials and Methods. Dilutions of each reaction mixture were tested for their ability to inhibit antibody binding to (^3H) -AMT-poly(A,U) in a competition RIA (Figure 3A and results not shown), or to AMT-calf thymus DNA (AMT-ctDNA) in an ELISA (Figure 3B and results not shown). Since AMT-poly(A,U) bound inconsistently to microtiter plates, AMT-ctDNA was used as the coating

Table I. Reactivity of AMT-modified nucleic acids with RA85-074b

Nucleic Acid	Nucleotide/AMT ^a	Relative reactivity ^b		
		RIA	ELISA	ELISA/Abs ^c
poly(A,U)	80	1.0	1.0	1.0
poly(A)·poly(U)	34	1.0	37	65
poly(A-U)	26	2.0	40	20
poly(I)·poly(C)	30	670	10 ⁵	10 ⁵
poly(rA)·poly(dT)	18	14	34	270
poly(dA)·poly(dT)	35	11	66	65
poly(dA-dT)	15	5.4	12	12
calf thymus DNA	26	15	85	54
E. coli tRNA	34	0.2	2.2	2.5
yeast sRNA	45	0.4	3.6	2.8
HMT-poly(A,U) ^d	12	5.4	5.2	2.5
(U) _n	34	3.7	7.4	40
(dT) _n	29	15	31	27

^a The nucleotide to psoralen ratio was calculated by measuring incorporation of tritiated AMT into an identically-treated sample, as described under Materials and Methods. ^b Relative reactivity is the ratio of the concentration of antigen required to achieve 50% inhibition of antibody binding to the concentration of AMT-poly(A,U) required to achieve 50% inhibition of antibody binding. The higher the number, the lower the reactivity with antibody. ^c These values were determined with Ra85-074b antiserum absorbed with AMT-poly(U). ^d Poly(A,U) modified with 4'-hydroxymethyltrioxsalen (HMT).

antigen in ELISA's. The concentration of AMT-poly(A,U) required to achieve 50% inhibition of antibody binding was 200 nM in most RIA experiments and 30 nM in ELISA's. The reactivities of other AMT-modified nucleic acids relative to AMT-poly(A,U) varied somewhat depending on the assay employed and whether the antiserum had been absorbed with AMT-poly(U) (Table I). Differing populations of antibody probably react with AMT-poly(A,U) in the RIA compared to those that react with AMT-ctDNA in the ELISA. Similarly, removal of certain antibody populations by absorption would be expected to alter the reactivity pattern.

HMT-poly(A,U) was 3-5-fold less reactive with Ra85-074b than was AMT-poly(A,U) even though the amount of HMT substitution in this sample was higher than achieved for AMT-poly(A,U) (Table I). This result suggests that the 4'-amino group of AMT plays a small role in forming the epitope. Most AMT-modified large RNA's displayed considerable antibody reactivity (Table I). Poly(I)·poly(C) modified with AMT was the exception. AMT-modified DNA's were 5-85-fold less reactive with unabsorbed antiserum than was AMT-poly(A,U). When the extent of

Table II. Reactivity of AMT-modified nucleic acids with RA85-074b

Nucleic Acid	Relative reactivity ^a		
	RIA	ELISA	ELISA/Abs ^b
poly(A,U)	1.0	1.0	1.0
(U) _n	3.7	7.4	4.0
(U) ₆	2.0	2.4	8.5
(U) ₂	n.d. ^c	6.4	290
(dT) _n	1.5	3.1	2.7
(dT) ₂₅	n.d. ^c	1.4	3.6
(dT) ₆	4.7	1.7	2.9
(dT) ₄	n.d. ^c	4.4	7.5
(dT) ₂	n.d. ^c	9.2	41.5
(dA-dT) _n	5.4	1.2	1.2
(dA-dT) ₅	1.2	3.3	4.9
(dA-dT) ₂	n.d. ^c	4.5	10.8
poly(A,U)-KOH ^d	1.0	1.1	1.1
poly(A,U)+KOH ^d	7.3	1.6	1.3
poly(U)-KOH ^d	n.d. ^c	6.1	2.1
poly(U)+KOH ^d	n.d. ^c	1.9	10.3

a,b See footnotes b and c, Table I. ^c n.d. = not determined. ^d Samples were hydrolyzed with KOH (+KOH) prior to assay. Control samples (-KOH) were prepared as described under Materials and Methods.

AMT substitution was taken into account in calculating relative reactivities, all DNA's were greater than 25-fold less reactive than AMT-poly(A,U). Absorption of the antiserum with AMT-poly(U) removed antibodies that were highly reactive with AMT-poly(U). Antibody binding to AMT-dsDNA was not significantly affected by absorption. The slightly increased reactivity of AMT-poly(A-U) with absorbed antiserum is not readily explained. Binding of RA85-074b to AMT-modified oligonucleotides increased with increased length of the oligomer (Table II). This may reflect a greater apparent avidity resulting from bivalent binding to longer and more highly substituted nucleic acids.

The specificity of antiserum RA85-074b was investigated further by testing its reactivity with AMT-poly(A,U) and AMT-poly(U) after hydrolysis with KOH. The hydrolysis procedure resulted in a complete loss of acid precipitable counts and no residual oligonucleotides

Figure 4. Effect of base hydrolysis of AMT-poly(A,U) on binding to RA85-074b. AMT-poly(A,U) subjected to base hydrolysis and unhydrolyzed control RNA's were prepared as described under Materials and Methods. Binding to Ra85-074b was determined by competition RIA (A) and competition ELISA (B). The points represent the range and mean of duplicate determinations. (■), control poly(A,U); (□), hydrolyzed poly(A,U).

were detected by reverse phase HPLC (results not shown). Reactivity with AMT-poly(A,U) decreased 7-fold in the RIA and less than 2-fold in ELISA (Fig. 4 & Table II). Reactivity of AMT-poly(U) similarly decreased 3- to 5-fold after hydrolysis (Table II). This slight alteration in antibody reactivity demonstrates that the epitope recognized by Ra85-074b was

primarily AMT-uridine. However, the slope of the inhibition curve for AMT-poly(A,U) in the RIA was altered after hydrolysis (Fig. 4A). Virtually identical results were obtained with hydrolyzed AMT-poly(U). This observation, in addition to the observation that the reactivity with antibody was also slightly altered by hydrolysis, suggests that a portion of the antibodies that react in the RIA but not the ELISA may prefer an epitope that is larger than a modified nucleotide.

Effect of ionic strength on antibody binding.

The effect of increased ionic strength on antibody binding was investigated to determine if ionic interactions were important in antibody binding. Control experiments indicated that increasing the ionic strength had no significant effect on binding of the antibody by fixed *S. aureus*. RA85-074b was bound to *S. aureus* at 0.15 M NaCl and incubated with (^3H)-AMT-poly(A,U) at concentrations of NaCl ranging from 0 to 1.0 M. Binding of (^3H)-AMT-poly(A,U) was optimal at 0.05-0.15 M NaCl. 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) (22). For single-stranded DNA, the slope of the line = $-0.7 \times$ (number of ion pairs) (23). Phosphate ions are the only ions present in nucleic acids at physiological pH, so the number of ion pairs involved in binding would be the number of phosphates involved in binding. The log of antibody binding to (^3H)-AMT-poly(A,U) was plotted as a function of the log of the sodium chloride concentration. A linear plot with a slope of -0.16 was obtained. This result suggests that ion pair formation between antibody and the ribose-phosphate backbone of AMT-poly(A,U) is negligible, assuming that antibody binding in the RIA is proportional to antibody affinity.

Antibody binding of RNA extracted from virus-infected cells treated with AMT and UV light.

Binding of RA85-074b to RNA extracted from cells treated with AMT and UV light was investigated. Mouse L cells were infected with encephalomyocarditis virus, (EMCV), a ssRNA virus that is believed to form double-stranded RNA during replication (24). Cells were treated with AMT four hours after infection and exposed to long wavelength UV light. (^3H)-Uridine-labeled RNA was purified as described under Materials and Methods and tested for binding to anti-AMT-poly(A,U) antibodies in an RIA. Antibodies bound total RNA from cells infected with EMCV and treated with AMT and UV light but did not bind RNA from control cells treated with AMT only. Viral replicative intermediates were purified as described under Materials and Methods. In the final stage of purification, these intermediates elute in the void volume of a Sepharose 2B column whereas cellular RNA's elute later (20, 21). Fractions from the Sepharose 2B column were diluted and tested for their ability to bind Ra85-074b in a competition ELISA.

Each RNA containing fraction from cells treated with AMT and UV light bound antibody, whereas RNA from cells treated with AMT only did not bind antibody (Figure 5). RNA from uninfected cells treated with AMT and UV light were similarly purified. The profile of binding obtained with Sepharose 2B fractions of these preparations was virtually identical to that seen with infected cells, except that no reactive RNA eluted in the void volume. RNA from cells treated

Figure 5. Binding of RA85-074b to Sepharose 2B fractions containing RNA extracted from EMCV-infected mouse L cells. Cells were infected with EMCV and labeled with ^3H -uridine as described under Materials and Methods. At three hours post infection, cells were divided into two groups. One was treated with AMT only, and the other was treated with AMT and irradiated with long wavelength UV light. RNA was extracted and chromatographed as described under Materials and Methods. Fractions at a 1:10 final dilution were tested for binding to RA85-074b in an ELISA. Each point represents the mean of duplicate determinations. (□), RNA from EMCV-infected cells treated with AMT only; (■), RNA from EMCV-infected cells treated with AMT and UV light.

only with UV light did not bind the antibody (results not shown). DNA extracted from control and from EMCV-infected cells treated with AMT and UV light also reacted with Ra85-074b (results not shown). We have not explored the effect of treating cells with psoralen and UV light on the efficiency of RNA and DNA extractions, or on the extent of cross-contamination of RNA and DNA preparations.

DISCUSSION

Techniques for detecting psoralen-modified nucleic acids have included ribonuclease resistance analysis, electron microscopy, gel electrophoresis, and sucrose and glycerol gradient centrifugation. Base pairing in RNA's from Rous Sarcoma Virus (25) and poliovirus (26), and between heteronuclear RNA and small nuclear RNA (27, 28) has been determined by psoralen cross-linking techniques.

Mapping the positions of psoralen cross-links in a nucleic acid is a more formidable task than is detection. Mapping has been achieved by electron microscopy (30, 31), and by RNase digestion followed by electrophoretic characterization (5, 32, 33). Psoralen adducts block reverse transcriptase and two such psoralen-induced stops have been localized in 16S RNA

primed with a 3'-proximal restriction fragment (34). General use of this latter method would require synthesis of oligonucleotides complementary to about every 200 nucleotides of the RNA. Residues near the site of psoralen cross-links were identified recently in samples hybridized to end-labeled cDNA and subsequently treated with mung bean nuclease (35). This nuclease nicked the DNA at or near bases complementary to cross-linked residues in the RNA strand and the location of those nicks were discernable on a sequencing gel. Mapping techniques have provided information on the positions of base pairs in precursor rRNA's (36), as well as in 16S rRNA (31, 37), 23S rRNA (33), and 5S rRNA (32) of *Escherichia coli*, and tRNA^{Phe} from yeast (38) and *E. coli* (5). Psoralens have clearly shown to be useful as probes for detecting and mapping base pairs in purified RNA's and in RNA-protein complexes (28, 39). Cells are permeable to psoralens which opens possibilities for investigating the conformation of RNA's in intact cells (29, 40).

Model studies suggest that psoralen cross-linking of DNA produces an unusual kinking of the backbone (7). Antibodies that bind nucleic acids are valuable probes of nucleic acid structure (9). Our result that modification of dsRNA reduced reactivity with anti-dsRNA antibodies (Fig.1) supports the postulate that psoralen modification alters the ribose-phosphate backbone of RNA's. While this work was in progress, Santella and coworkers (41) reported production of antibodies specific for psoralen-modified DNA, suggesting that psoralen-modification altered the antigenic determinant of DNA. Binding of these antibodies to psoralen-modified RNA was not investigated.

This is the first report of antibodies directed against a psoralen-modified RNA. Salt sensitivity studies suggested that the phosphates of the backbone were probably not involved directly in antibody binding. AMT or poly(A,U) alone did not react with these antibodies and base hydrolysis of the RNA resulted in a slight alteration in antibody recognition. The nature of the epitope appeared to involve AMT-uridine, predominantly, although a portion of the antibodies may be directed toward a structure that is more complex than a nucleotide adduct. Psoralen-modified DNA's were generally less reactive than psoralen-modified RNA's, suggesting that the furanose played a role in forming the determinant. The observation that all AMT-uridine- and AMT-thymidine-containing nucleic acids reacted substantially better than did AMT-poly(I)·poly(C) suggests that binding involves interaction with the pyrimidine C4 oxy group.

The ability of psoralens to form both monoadducts and diadducts complicates analysis of antibody specificity. The immunogen employed in these studies probably contained some intra- or inter-strand cross-linking, although the extent to which the epitope is a monoadduct or a cross-link is not yet known.

Encephalomyocarditis virus is believed to form dsRNA during replication (24) and this dsRNA is believed to be the moiety responsible for induction of interferon and activation of interferon-induced enzymes. (reviewed, 42). Indirect evidence for AMT cross-linking of RNA

in cells infected with EMCV has been reported previously (29). However, others reported that AMT does not modify RNA formed during infection with a similar picornavirus, poliovirus (26). Antiserum RA85-074b, directed against AMT-poly(A,U), bound RNA from EMCV-infected cells treated with both AMT and UV light and did not bind RNA from infected cells treated with AMT only (Fig. 5). Fractionation of the RNA from infected and uninfected cells demonstrated that both viral and cellular RNA populations reacted with anti-AMT-poly(A,U) antibodies (Fig. 5 and results not shown). Reactivity per microgram of RNA of EMCV replicative intermediates (fractions 23-25, Fig. 5) and cellular tRNA (fractions 71-74, Fig. 5) was significantly higher than for ribosomal RNA in the ELISA. More AMT per μg RNA may be incorporated into replicative intermediates and tRNA than into ribosomal RNA (M. O'Connor and M. I. Johnston, unpublished results). Increased AMT incorporation may increase the apparent avidity and explain, at least in part, the increased binding of viral RNA and tRNA.

The results presented here demonstrate that both cellular and EMCV RNA were covalently modified with AMT in intact cells and suggests that viral and cellular RNA's in intact cells possess the base-pairing/secondary structure required for AMT intercalation and photoaddition. Further, formation of RNA photoadducts may play a role in the inhibition of cellular proliferation that follows treatment of cells with psoralen and long wavelength UV light (43, 44).

In conclusion, the antibodies described in this report will be useful probes of RNA structure, both in detecting the presence of psoralen adducts, and perhaps also in mapping the position of psoralen-modified nucleotides. Such information is critical in understanding the structure-function relationship of RNA's and in exploring the conformation of cellular and viral RNA's in intact cells.

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REFERENCES

1. Cimino, G. D., Gamper H. B., Isaacs, S. T., and Hearst, J. E. (1985) *Ann. Rev. Biochem.* **54**, 1151-1193.
2. Thompson, J. F., Bachelierie, J.-P., Hall, K. and Hearst, J. E. (1982) *Biochemistry* **21**, 1363-1368.
3. Kanne, D., Straub, K., Hearst, J. E. and Rapoport, H. (1982) *J. Am. Chem. Soc.* **104**, 6754-6764.
4. Bachelierie, J.-P., Thompson, J. F., Wegnez, M. R., and Hearst, J. E. (1981) *Nucleic Acids Res.* **9**, 2207-2222.

5. Bachellerie, J.-P., and Hearst, J. E. (1982) *Biochemistry* **21**, 1357-1363.
6. Cech, T. R. (1981) *Biochemistry* **20**, 1431-1437.
7. Pearlman, D. A., Holbrook, S. R., Pirkir, D. H. and Kim, S.-H. (1985) *Science* **227**, 1304-1308.
8. Sinden, R. R. and Hagerman, P. J. (1984) *Biochemistry* **23**, 6299-6303.
9. Calvet, J. P. and Pederson, T. (1981) *Cell* **26**, 363-370.
10. Stollar, B. D. (1975) *CRC Crit. Rev. Biochem.* **3**, 45-69.
11. Stollar, B. D. (1970) *Science* **169**, 609-611.
12. Johnston, M. I. and Stollar, B. D. (1978) *Biochemistry* **17**, 1959-1964.
13. Munns, T. W. and Liszewski, M. K. (1980) *Prog. Nucleic Acid Res. Molec. Biol.* **24**, 109-165.
14. Engvall, E. (1980) *Methods Enzymol.* **70**, 419-439.
15. Wells, J. A., Swyryd, E. A. and Stark, G. R. (1984) *J. Biol. Chem.* **259**, 1363-1370.
16. Zouali, M. and Stollar, B. D. (1986) *J. Immunol. Methods* **90**, 105-110.
17. Prager, E. M. and Wilson, C. A. (1971) *J. Biol. Chem.* **246**, 5978-5989.
18. Irarie, R. D. and Jones, P. P. (1979) *Anal. Biochem.* **97**, 24-35.
19. Fraenkel-Conrat, H., Singer, B., Tsugita, A. (1961) *Virology* **14**, 54-58.
20. Torrence, P. F. T. and Friedman, R. M. (1979) *J. Biol. Chem.* **254**, 1259-1267.
21. Richards, O. C. and Ehrenfeld, E. (1980) *Virology* **36**, 387-394.
22. Spector, D. H. and Baltimore, D. (1975) *J. Virology* **15**, 1418-1431.
23. Lee, J. S., Dombrowski, D. F. and Mosmann, T. R. (1986) *Biochemistry* **21**, 4940-4945.
24. Record, M. T., Jr., Lohman, T. M., and deHaseth, P. (1976) *J. Molec. Biol.* **107**, 145-158.
25. Rekosh, D. M. K. (1977) *The Molecular Biology of Animal Viruses* (Nayak, D. P., Ed.) pp 64-110, Marcel Dekker, New York.
26. Swanstrom, R., Hallick, L. M., Jackson, J., Hearst, J. E. and Bishop, J. M. (1981) *Virology* **113**, 613-622.
27. Richards, O. C., Martin, S. C., Jense, H. G. and Ehrenfeld, E. (1984) *J. Mol. Biol.* **173**, 325-340.
28. Calvet, J. P. and Pederson, T. (1981) *Cell* **26**, 363-370.
29. Setyono, B. and Pederson, T. (1984) *J. Mol. Biol.* **174**, 285-295.
30. Nilsen, T. W., Wood, D. L. and Baglioni, C. (1981) *Virology* **109**, 82-93.
31. Peterlin, B. M., Sullivan, M., Westphal, H. and Maizel, J., Jr. (1978) *Virology* **86**, 91-97.
32. Wollenzien, P., Hearst, J. E., Thammana, P. and Cantor, C. R. (1979) *J. Mol. Biol.* **135**, 255-269.
33. Thompson, J. F., Wegnez, M. R. and Hearst, J. E. (1981) *J. Molec. Biol.* **147**, 417-436.
34. Turner, S., and Noller, H. F. (1983) *Biochemistry* **22**, 4159-4164.
35. Youvan, D. C. and Hearst, J. E. (1982) *Anal. Biochem.* **119**, 86-89.
36. Hui, C.-F., and Cantor, C. R. (1984) *Proc. Natl. Acad. Sci. (U. S. A.)* **82**, 1381-1385.
37. Wollenzein, P. L., Cantor, C. R., Grant, D. M. and Lambowitz, A. M. (1983) *Cell* **32**, 397-407.
38. Thompson, J. F. and Hearst, J. E. (1983) *Cell* **33**, 9-24.
39. Garrett-Wheeler, E., Lockard, R. E. and Kumar, A (1984) *Nucleic Acids Res.* **12**, 3405-3423.
40. Shen, C.-K. J., and Hearst, J. E. (1978) *Nucleic Acids Res.* **5**, 1429-1441.
41. Revet, B. and Benichou, D. (1981) *Virology* **114**, 60-70.
42. Santella, R. M., Dharmaraja, N., Gasparro, F. P. and Edelson, R. L. (1985) *Nucleic Acids Res.* **13**, 2533-2544.