Characterization of the effects on ovalbumin mRNA of aminomethyltrimethylpsoralen photoreaction with hen oviduct mRNA

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ABSTRACT

We have described the reaction of 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT) with hen oviduct mRNA and have investigated the specific effects of AMT photoaddition on ovalbumin mRNA which constitutes 60-70% of oviduct mRNA. The photoreaction of AMT with hen oviduct mRNA appeared to occur in two phases - a rapid monoaddition followed by a slower conversion of monoadducts to diadducts (i.e. crosslinking). Both nondenaturing and denaturing gel electrophoresis revealed a photoreaction time dependent increase in ovalbumin mRNA electrophoretic mobility indicating the formation of a progressively more compact molecular structure. Identical analysis of photoreacted rabbit globin mRNA revealed no change in electrophoretic mobility suggesting that AMT was stabilizing the AU rich secondary structure of ovalbumin mRNA but having no similar effect on the relatively GC rich secondary structure of globin mRNA. Ovalbumin-specific DNA primer extension was used to demonstrate the selective and secondary structure specific photoaddition of AMT to uracil bases at the 5' end of ovalbumin mRNA.

INTRODUCTION

Psoralens are a group of bifunctional photoreactive crosslinking agents for nucleic acids. Although relatively unreactive with single-stranded polynucleotides, psoralens readily intercalate into double-stranded regions in DNA or RNA. Upon long wavelength ultraviolet (UV) irradiation, the intercalated psoralen first forms a covalent monoadduct with a pyrimidine base and then a covalent diadduct by reacting with an adjacent pyrimidine base located on the opposite strand of the base paired region. Short wavelength UV irradiation reverses this reaction. Psoralens react mostly with uracil bases in RNA and thymine bases in DNA although there is some reaction with cytidines in both polynucleotides. The properties of the various psoralen compounds, their reactions with nucleic acids and their uses for investigating DNA and RNA structure have recently been summarized (1).

Compared to other psoralens, 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT) and 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT) photoreact more rapidly, have higher dark binding affinities, higher yields of photoreaction products, and higher rates of photodegradation (2). HMT has been widely used to characterize psoralen photoreaction with DNA and RNA and to map crosslinkable sites in tRNA (3) and rRNA (4,5). AMT has an aqueous solubility that is 150 times greater and a binding affinity for RNA that is 100 times greater than HMT (2). These properties, combined with the ability of

psoralens to benignly invade biological structures (including the cell) and react with their component nucleic acids, make AMT a potentially powerful reagent for investigating the role of RNA structure in post-transcriptional regulation of gene expression.

In the experiments below, we describe both the reaction of AMT with hen oviduct mRNA and its use as a reagent for the analysis of ovalbumin mRNA secondary structure by a DNA primer extension assay. A similar approach has previously been used by others to determine addition of HMT to bases in a local region of secondary structure in 16s ribosomal RNA (6). Ovalbumin mRNA contains 1872 nucleotides (excluding the 3' poly A tail) 41% of which are intramolecularly base-paired (7). Ovalbumin mRNA secondary structure is relatively AU rich (46% AU base pairs) and is sensitive to the ionic environment (7,8). In order to understand the role of ovalbumin mRNA secondary structure in the initiation of translation, it was first necessary to unambiguously determine the secondary structure of the 5' portion of the ovalbumin mRNA molecule.

EXPERIMENTAL METHODS

Materials.

4'-Aminomethyl-4,5',8-trimethylpsoralen - HCl (HRI Associates, Berkeley, CA) was dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, 1mM EDTA) at a final concentration of 2 mg/ml and stored at -20°C. in the dark. AMT remained stable under prolonged storage as indicated by periodically measured, reproducible UV absorbance spectra (data not shown). 4'-[³H(G)]-Aminomethyl-4,5',8-trimethylpsoralen (1.6 Ci/mmol) was obtained from New England Nuclear, Boston, MA., as a 16 ug/ml solution in water:ethanol (v:v = 9:1). Deionized formamide, rabbit reticulocyte poly A(+) RNA (containing >80% 10s globin mRNA) and poly A(+) RNA molecular weight markers were obtained from Bethesda Research Laboratories, Gaithersburg, MD. Dideoxy- and deoxynucleoside triphosphates were obtained from Pharmacia Inc., Piscataway, NJ. Autoradiography was accomplished using Kodak XAR-5 X-ray film.

Poly A(+) RNA was prepared from White Leghorn Hen oviducts essentially as described (9). Briefly, total RNA was alkaline (pH 8.3) phenol - sodium dodecyl sulfate (SDS) extracted from frozen oviducts and chromatographed on successive oligo dT cellulose columns (Types 2 and 3 respectively, Collaborative Research, Inc., Lexington, MA) with continuous absorbance monitoring at 260 nm until unbound material could no longer be detected. Poly A(+) RNA preparations were evaluated for the absence of rRNA and tRNA and for mRNA integrity by electrophoresis on denaturing 1.5% agarose gels and were stored at -80°C. as 1 mg/ml stock solutions in HE buffer (10 mM HEPES-KOH, pH 7.55, 1 mM EDTA).

AMT Photoreaction with RNA.

An appropriate volume of hen oviduct or rabbit reticulocyte poly A(+) RNA stock solution was heated at 90°C. for 15 seconds and diluted into cold (4°C.) TE or HE buffer. RNA concentration was determined prior to the addition of AMT by measuring the absorbance at 260 nm of the buffer solution of mRNA and calculating the concentration from the expression: 1 A_{260} unit = 40 ug/ml. Exact concentrations of AMT and mRNA are given in the text and figure legends. Since the molar concentration of ovalbumin mRNA in the oviduct poly A(+) RNA was not determined, molar ratios are expressed as AMT:ribonucleotide (average molecular weight = 340). Broad band UV irradiation [wavelength maximum = 350 nanometers (nm)] of the reaction mixture was performed at 4°C. in an RMR-500 Photochemical Mini-Reactor with four RPR-3500 A lamps and a rotating reaction vessel carrousel (Southern New England Ultraviolet Company, Hamden, CT). RNA was recovered by ethanol precipitation from reaction mixture aliquots, collected by centrifugation, washed with cold 95% ethanol, vacuum dried, and dissolved in the same volume of HE buffer as the original aliquot for fluorescence emission spectra (FES) and specific activity analysis or in TE buffer for gel and primer extension analysis (see below). A second ethanol precipitation and wash showed less than a 10% decrease in ^3H -AMT-RNA specific activity indicating that one ethanol precipitation and wash was sufficient to remove most of the noncovalently bound AMT from oviduct mRNA. Photoreversal of AMT-reacted mRNA was accomplished by irradiating in HE buffer at 250 nm in a quartz cuvette placed at the surface of a UVSL-58 hand lamp (Ultra-Violet Products, Inc., San Gabriel, CA)

Fluorescence Emission Spectroscopy and Specific Activity Measurements

Fluorescence emission spectra were determined for the AMT-mRNA reaction mixture and for mRNA that was recovered from the reaction mixture and dissolved in HE buffer as described above. Fluorescence measurements were made with an SLM Instruments Model 8000 photon counting, scanning spectrofluorometer equipped with a 450 watt xenon arc lamp, dual holographic-grating excitation monochromator, single holographic-grating emission monochromator, EMI 9635A photomultiplier tubes and thermostated sample compartment. The spectrofluorometer was interfaced to a Hewlett Packard 9815 programmable calculator for data acquisition and manipulation. Emission spectra were obtained between 340 and 580 nm with a resolution of 4 nm and an excitation wavelength of 330 nm. Spectra were not corrected for photomultiplier or instrumental response. Spectral integrals were calculated from the sum of all emission photons detected when scanning over the stated wavelength range less the sum detected for a buffer blank scanned in an identical manner. Samples were maintained at 4°C. during all fluorescence measurements.

Following fluorescence emission spectroscopy, absorbance measurements were made

at 260 nm to determine mRNA recovery; 50 μ l of each RNA solution was then added to 5 ml ACS liquid scintillation cocktail (Amersham Corp., Arlington Heights, IL) and counted for 5 minutes in a Beckman LS100-C liquid scintillation counter using the standard tritium window (total cpm ^3H -AMT-mRNA recovered). The total amount of ^3H -AMT radioactivity (measured as cpm) in each reaction mixture was determined by diluting 5 μ l aliquots of the reaction mixture, taken at the beginning and end of the reaction, to 50 μ l with HE buffer and counting in an identical manner (total cpm ^3H -AMT in the reaction mixture). Radioactivity background was determined by counting 50 μ l of HE buffer and all data points were background subtracted. Specific activity was expressed either as cpm or as pmoles ^3H -AMT bound per μ g of mRNA recovered. In the latter case, pmoles of ^3H -AMT bound was calculated by multiplying the total pmoles of ^3H -AMT in the reaction mixture by the fraction: (total cpm ^3H -AMT-mRNA recovered) / (total cpm ^3H -AMT in the reaction mixture).

Gel Electrophoresis.

RNA relative electrophoretic mobilities (REM) were determined by electrophoresis on both nondenaturing and denaturing 1.5% agarose gels. Nondenaturing gels were prepared in TBE buffer (90 mM Tris - 90mM boric acid, pH 8.3, 2.5 mM EDTA) or in TAE buffer (40 mM Tris-HOAc, pH 8.0, 2mM EDTA). Each RNA sample was dissolved in 10 μ l TE buffer, heated at 90°C. for 10 seconds and rapidly chilled to 4°C. in an ice bath. Denaturing gels were prepared in MOPS buffer (20 mM 3-(N-Morpholino)propanesulphonic acid, 5 mM Na acetate, pH 7.0, 1 mM EDTA) containing 2.2M formaldehyde. Each RNA sample was incubated at 65°C. for 5 minutes in 10 μ l MOPS buffer containing 2.2M formaldehyde and 25% (v/v) deionized formamide. In both systems, 3 μ l of 0.25% xylene cyanol ff - 0.25% bromophenol blue in 30% glycerol was added to each sample before loading on the gel. REM values were determined from a standard semi-log plot of the RNA molecular weight markers as a function of electrophoretic mobility (300 nucleotide RNA marker REM = 1).

Northern Blot Hybridization.

RNA was transferred by diffusion from 1.5% agarose gels to activated aminophenylthioether paper (Schleicher and Schuell, Inc., Keene, NH) or to Hybond-N nylon membrane (Amersham Corp., Arlington Heights, IL) using the protocols supplied by the manufacturers. The resulting RNA blot was preincubated for 4 hours at 42°C. in prehybridization solution (5xSSC, 0.1% SDS, 1% Glycine, 5x Denhardt's solution (10), 150 μ g/ml sonicated, denatured herring sperm DNA in 50% deionized formamide) and a heat-denatured 5'-end labelled ^{32}P -DNA probe was added. Hybridization was for 18 hours at 42°C. in the same solution except that 2x Denhardt's solution was used. Following hybridization, the RNA blot was successively washed two times with 2xSSC-0.1% SDS and 2 times with 0.1xSSC-0.1% SDS at room temperature and

autoradiographed at -80°C . with intensifying screens. ($1\times\text{SSC} = 0.15\text{M NaCl}-0.015\text{M Na citrate}$.) When necessary, the first DNA probe was completely removed by washing the blot at 50°C . in 99% deionized formamide and verified by autoradiography before hybridization of a second DNA probe was performed.

Two DNA probes were prepared by restriction digestion of ovalbumin expression plasmid pUC1001 (11). The 3'-end probe was an 820 bp Xba I - Eco R1 fragment containing 527 nucleotides from the 3' end of ovalbumin mRNA (nucleotides 1345 - 1872 starting from the Xba I site). The 5'-end probe was a 440 bp Eco R1 - Pst I fragment containing 434 nucleotides from the 5' end of ovalbumin mRNA (nucleotides 43 - 477) and an Eco R1 cloning linker.

Primer Extension Analysis of Secondary Structure

Hen oviduct poly A(+) RNA was reacted with dimethylsulfate using the stabilizing "high Mg^{++} conditions" or destabilizing "low Mg^{++} conditions" described by Inoue and Cech (12) and recovered by ethanol precipitation. All RNAs for primer extension analysis (i.e. unreacted controls, methylated and AMT photoreacted) were dissolved in TE buffer at a concentration of 1 $\mu\text{g}/\text{ul}$. Reverse transcription was accomplished in 50 μl of 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 9 mM MgCl_2 , 10 mM 2-mercaptoethanol containing 0.25 mM each of dATP, dGTP, dCTP and dTTP, 1 μg RNA template and 30-40 ng oligodeoxynucleotide primer. The reaction mixture was heated to 90°C . and allowed to equilibrate to 37°C .. Primer extension (30 minutes at 37°C .) was initiated by the addition of 10 units of avian myeloblastosis virus reverse transcriptase (Life Sciences Inc., St. Petersburg, FL). The reaction was stopped by the addition of EDTA (20 mM final concentration), 1.5 μg carrier tRNA and two volumes of cold ethanol. Nucleic acids recovered by centrifugation were dissolved in 20 μl of 50 mM Tris-borate, pH 8.3, 1 mM EDTA, 0.1% xylene cyanol ff, 0.1% bromophenol blue in 80% (v/v) deionized formamide. After heating at 90°C . for 2 minutes and rapidly chilling on ice, 20,000 cpm of each reaction (determined by measuring the Cerenkov radiation of each nucleic acid pellet in the tritium channel of a liquid scintillation counter) was applied to parallel wells of a 0.4 mm thick, 8% polyacrylamide sequencing gel (13). Following electrophoresis, the gel was dried and autoradiographed at -80°C . with intensifying screens.

The oligodeoxynucleotide primer used in these experiments was a 21mer that is complementary to nucleotides 36-56 of ovalbumin mRNA. This 21mer was synthesized on a Biosearch Sam One DNA Synthesizer and was generously provided by Biosearch, San Rafael, CA. The 21mer was purified from shorter oligodeoxynucleotides by electrophoresis on a 20% polyacrylamide sequencing gel after 5'-end radiolabelling with T4 polynucleotide kinase (Boehringer-Mannheim, Indianapolis, IN) and gamma ^{32}P -ATP ($>4000\text{ Ci}/\text{mmole}$, ICN Pharmaceuticals Inc., Irvine, CA). The 21mer was recovered from the gel by the "crush and soak" technique (13) and its purity verified by analytical

Figure 1. Fluorescence emission spectra of the photoreaction of AMT with hen oviduct poly A(+) RNA. The photoreaction was interrupted at successive irradiation times and four minute fluorescence emission spectra were recorded. Relative fluorescence emission intensity is shown as a function of emission wavelength. Each spectrum is labelled with its irradiation time in minutes. Insert: Photographic negative of ethidium bromide stained 1.5% agarose-TBE gel containing mRNA samples recovered from 50 μ l aliquots taken at successive irradiation times (indicated above each gel lane). The prominently stained band in each gel lane is ovalbumin mRNA.

sequencing gel electrophoresis.

Dideoxynucleotide sequence analysis was a modification of the method of Zimmer and Kaesburg (14). Reactions were performed in 20 μ l of RT buffer containing 2 μ g poly A(+) RNA, 0.2 μ g 5'-end, 32 P-labelled oligodeoxynucleotide primer (see above), 5 units reverse transcriptase and the following concentrations of dideoxy- and deoxynucleoside triphosphates: A-reaction: 75 μ M ddATP and 450 μ M of each dNTP (see above); T-reaction: 50 μ M ddTTP and 200 μ M of each dNTP; C-reaction: 25 μ M ddCTP and 125 μ M of each dNTP; G-reaction: 25 μ M ddGTP and 125 μ M of each dNTP. Primer extension reaction and gel electrophoresis were as described above.

RESULTS

Reaction of AMT with Hen Oviduct mRNA.

The photoaddition of AMT to hen oviduct mRNA was initially followed by fluorescence emission spectroscopy as a general indicator of crosslinking. A reaction

Figure 2. Photoreaction of $3.1 \mu\text{M}$ ^3H -AMT with $9.7 \mu\text{g/ml}$ hen oviduct poly A(+) RNA (AMT:ribonucleotide molar ratio = 1:9.2) in HE buffer at 4°C . RNA was recovered from 400 μl aliquots taken at successive irradiation times and analyzed as described in Methods. a. Fluorescence emission spectra of recovered RNA samples. Each spectrum is labelled with the irradiation time in minutes. Spectra generated between 6 and 15 minutes could not be resolved on this scale and are therefore represented by a single curve. b. Specific activity (expressed as pmoles ^3H -AMT per μg RNA) of recovered RNA (●) is plotted as a function of irradiation time. The integral value for each fluorescence emission spectrum in (a) was expressed per μg of recovered RNA and plotted as a function of irradiation time (Δ).

mixture containing $20 \mu\text{g/ml}$ hen oviduct poly A(+) RNA and $5.7 \mu\text{M}$ AMT in TE buffer (AMT:ribonucleotide molar ratio = 1:10) was irradiated for 70 minutes as described in Methods. Figure 1 shows fluorescence emission spectra taken at successive times throughout the reaction. During the first 10 minutes the emission wavelength of maximum fluorescence shifted from 450 nm (free AMT, 0-time) to 385 nm and the amplitude of the fluorescence signal at 385 nm increased. From 10 to 70 minutes, the amplitude of the fluorescence signal at 385 nm decreased. Similar fluorescence emission spectra have been reported for the photoreaction of AMT with the alternating copolymer poly (dA-dT) (15) where the amplitude increase at 385 nm was interpreted as

Figure 3. Increase in ovalbumin mRNA REM as a function of photoreaction time. 10 μ g/ml hen oviduct poly A(+) RNA was combined with 3.4 μ M AMT (AMT:ribonucleotide molar ratio = 1:8.7) in TE buffer at 4°C, and irradiated as described in Methods. RNA recovered from 400 μ l aliquots taken at successive irradiation times was applied to a 1.5% agarose. Each gel lane is labelled with the irradiation time or with an "M" to indicate poly A(+) RNA molecular weight markers whose sizes in nucleotides are shown on either side of the gel. The left panel shows the photographic negative of an ethidium bromide stained nondenaturing gel in TAE buffer. The right panel shows the autoradiogram of a Northern blot hybridization; mRNA was transferred from a denaturing gel (see Methods) to a nylon membrane and hybridized with the 5'-end 32 P-labelled oligodeoxynucleotide used in the primer extension experiments (see Methods). Lane "C" on this gel is a control containing unreacted oviduct poly A(+) RNA with "OV" indicating the position of ovalbumin mRNA. The table below each gel indicates the REM and apparent size in nucleotides of the prominent band of ovalbumin mRNA in the gel at each irradiation time.

monoaddition and the subsequent amplitude decrease was interpreted as the conversion of monoadducts to diadducts (i.e. crosslinking). Thus the population of mRNAs prepared from the hen oviduct appear to undergo a biphasic reaction with AMT similar to the model compound poly (dA-dT); that is, rapid monoaddition followed by slower crosslinking. The decline in the fluorescence signal at 450 nm in Figure 1 resulted both from AMT addition to mRNA and from AMT photodegradation (data not shown). The insert in Figure 1 shows that the electrophoretic mobility of ovalbumin mRNA (which constitutes 60% - 70% of the oviduct mRNA) was increased by photoreaction with AMT. This electrophoretic mobility increase was fully developed by 5 minutes and remained constant for the rest of the reaction. Neither UV irradiation alone nor dark binding of AMT affected the stability or electrophoretic mobility of ovalbumin mRNA (data not shown).

The kinetics of this biphasic reaction were further investigated as described in Figure 2. In these experiments, ^3H -AMT-mRNA was recovered by ethanol precipitation and washed with ethanol in order to remove unreacted and photodegraded ^3H -AMT before fluorescence emission spectrum and specific activity measurements were made. Recovery usually exceeded 90% of the starting mass of mRNA and the recovered mRNA exhibited little or no degradation on agarose gels. The fluorescence emission spectra of ^3H -AMT-mRNA recovered at successive photoreaction times are shown in Figure 2a. A time dependent increase and subsequent decrease in the amplitude of a single fluorescence peak with a maximum at 385 nm is apparent for AMT modified mRNA. Figure 2b shows the addition of ^3H -AMT to mRNA as a function of photoreaction time. Greater than 80% of the total addition measured at one hour had occurred during the first 15 minutes of UV irradiation. The integral value for each fluorescence emission spectrum in Figure 2a was expressed relative to the amount of mRNA recovered (i.e. as FES integral per μg mRNA) and also expressed as a function of photoreaction time. These integral values increased rapidly to a maximum value between 6 and 15 minutes and correlated well with the time dependent increase in ^3H -AMT addition to mRNA which starts to level off between 12 and 15 minutes of reaction. The pattern of spectral changes for isolated AMT-mRNA indicates that the similar changes in reaction spectra at 385 nm observed in Figure 1 reflect the photoaddition of AMT to RNA. The similar kinetics for the addition of AMT to mRNA and the increase in FES amplitude and integral values indicates that the initial reaction of AMT with mRNA produces largely monoadducts. The decline in FES integral values between 15 and 60 minutes reflects the decline in the amplitude of the fluorescence peak at 385 nm which is indicative of crosslinking. During this crosslinking phase, there is a slow but steady increase in ^3H -AMT addition to mRNA suggesting that there may be two classes of reaction sites.

The reaction in Figure 2 was performed at an approximately half-fold lower concentration of both AMT and mRNA (while maintaining a similar molar ratio of AMT to ribonucleotides) than the reaction in Figure 1. This lower concentration of reactants produced a more gradual increase in the electrophoretic mobility of ovalbumin mRNA as shown in Figure 3. The increase in relative electrophoretic mobility (REM) and apparent decrease in size of ovalbumin mRNA as a function of photoreaction time is apparent on both nondenaturing (Figure 3, left panel) and denaturing (Figure 3, right panel) agarose gels. Furthermore, the time-dependent increase in REM was similar in both gels and occurred largely during the monoaddition phase (0-15 minutes) of the photoreaction as defined by the FES analysis in Figure 2.

The inability of nonreversible denaturation to abolish the photoreaction-dependent increase in ovalbumin mRNA electrophoretic mobility (Figure 3) strongly suggests a more compact molecule whose structure is stabilized by intramolecular crosslinking. The

Figure 4. Northern blot hybridization of 1 μ g each of unreacted (lane 1), photoreacted (lane 2), and 10 minute photoreversed (lane 3) ovalbumin mRNA. 10 μ g/ml hen oviduct poly A(+) RNA was irradiated for 5 minutes at 4°C. with 6.8 μ M AMT in HE buffer (AMT:ribonucleotide molar ratio = 1:4.3). Panel A is the photographic negative of an ethidium bromide stained 1.5% agarose-TAE gel from which the RNA was transferred for Northern blot hybridization (see Methods). Native ovalbumin mRNA (Ov) is indicated by the arrow. Panels B and C are autoradiograms of blot hybridizations using successive 3' end and 5' end ovalbumin cDNA probes, respectively, as described in Methods.

Increase in ovalbumin mRNA REM also could result from the loss of approximately 650 nucleotides (Figure 3, right panel) from one or both ends of the mRNA molecule. In order to evaluate this possibility, cDNA probes containing 527 nucleotides of ovalbumin mRNA 3'-end sequence and 434 nucleotides of ovalbumin mRNA 5'-end sequence were successively hybridized to a Northern blot of unreacted, photoreacted and photoreversed ovalbumin mRNA. Figure 4 (panel A) shows the increase in electrophoretic mobility of photoreacted ovalbumin mRNA relative to unreacted ovalbumin mRNA and the photoreversal induced decrease to an REM similar to that of unreacted ovalbumin mRNA. Apparently equal hybridization (Figure 4, panels B and C) of both ovalbumin cDNA probes to unreacted, photoreacted and photoreversed ovalbumin mRNAs indicates that no large truncation has occurred exclusively at either end of the ovalbumin mRNA molecule. The loss of 650 nucleotides by truncation of both ends of the mRNA molecule would not be detected by this analysis since the resulting mRNA would still contain sufficient sequence at each end to hybridize with both cDNA probes. However, the ability to reverse the REM of photoreacted ovalbumin mRNA to that of the unreacted molecule strongly indicates that the mRNA molecule is intact. Since primer extension experiments (reported below) also demonstrate that the 5'-end of ovalbumin mRNA is intact, the more compact ovalbumin mRNA molecule produced by photoaddition of AMT appears to result from intramolecular crosslinking.

Comparison of Ovalbumin and Globin mRNAs.

The secondary structure of ovalbumin mRNA has been reported to consist largely of AU rich hairpin structures (averaging 5 base pairs each) distributed along the length of

Figure 5. Panel A. Photoreaction of 3.1 μM ^3H -AMT with 10 $\mu\text{g}/\text{ml}$ hen oviduct poly A(+) RNA (solid line) or with 9.3 $\mu\text{g}/\text{ml}$ rabbit reticulocyte poly A(+) RNA (dashed line) in HE buffer at 4°C. The specific activity (cpm/ μg) of ^3H -AMT-RNA recovered from 400 μl reaction aliquots is plotted as a function of irradiation time. Panel B. Photographic negative of an ethidium bromide stained 1.5% agarose-TBE gel containing unreacted (lane 1), 30-minute photoreacted (lane 2) and 12-minute photoreversed (lane 3) reticulocyte poly A(+) RNA. Globin mRNA (GI) is indicated by the arrow.

the molecule (7). Since psoralens react primarily with uracil bases in RNA (16,17), modifying ovalbumin mRNA with AMT may stabilize its AU rich secondary structure resulting in the more compact molecule observed by gel electrophoresis. This interpretation suggests that the REM of a mRNA with a GC rich secondary structure such as globin mRNA (18) may not be affected by reaction with AMT because of its inherently greater stability under the same conditions. Figure 5 (panel A) shows the results of an experiment in which similar concentrations of hen oviduct mRNA (> 60% ovalbumin mRNA) and rabbit reticulocyte mRNA (>80% 10s globin mRNA) were photoreacted with ^3H -AMT under identical reaction conditions. As expected, both the initial rate and final extent of ^3H -AMT addition to reticulocyte mRNA are less than those for oviduct mRNA. (Since two ethanol precipitations and washes were needed to achieve a constant specific activity for reticulocyte mRNA, the GC rich secondary structure of globin mRNA may also result in a higher dark binding constant for AMT intercalation.) Both photoreacted (30 minutes) and photoreversed globin mRNA exhibited no change in electrophoretic mobility relative to unreacted globin mRNA (Figure 5, panel

Figure 6. Hairpin structure at the 5' end of ovalbumin mRNA.

B). At similar levels of AMT addition (i.e. 3 minutes for ovalbumin mRNA and 30 minutes for globin mRNA), ovalbumin mRNA had already shown an increase in electrophoretic mobility as indicated in Figure 3. It would therefore appear that AMT does not have the same "compacting" effect on globin mRNA that it has on ovalbumin mRNA.

Secondary Structure Analysis of Ovalbumin mRNA.

The hairpin structure at the 5' end of ovalbumin mRNA (Figure 6) was first described in the course of its sequence analysis (19) and was selected as a convenient structure on which to determine whether photoaddition of AMT resulted in secondary structure specific base modification. The stem of this hairpin contains three AU base pairs at its base with uridines in a crosslinkable array; the remainder of the stem is more GC rich. Since trimethylpsoralens react almost exclusively with uridine residues in RNA and prefer a "loose" helix for intercalation (17,20), we would expect to see preferential addition of AMT to the uridines at the base of this hairpin stem. We therefore employed a primer extension method to map this 5' end hairpin structure and to determine its pattern of base modification by AMT.

In order to unambiguously establish the presence of this hairpin structure at the 5' end of ovalbumin mRNA, native and denatured versions of oviduct poly A(+) RNA were each subjected to partial methylation with dimethylsulfate and analyzed by ovalbumin-specific DNA primer extension using reverse transcriptase as described in Methods. Dideoxynucleotide sequence analysis of ovalbumin mRNA using the same DNA primer was run on the same gel in order to unambiguously establish the position and identity of the methylated nucleotides in the secondary structure analysis. Inoue and Cech (12) have shown that dimethylsulfate primarily reacts with adenine and cytosine bases in RNA and that bases engaged in secondary structure are relatively resistant to methylation. Nucleotides engaged in secondary structure can be revealed by comparing

Figure 7. Analysis of secondary structure at the 5' end of ovalbumin mRNA by ovalbumin specific DNA primer extension. Experimental details are described in Methods. Right: Autoradiogram of 8% polyacrylamide sequencing gel of primer extensions using native (lane 1), methylated native (lane 2) and methylated denatured (lane 3) hen oviduct poly A(+) RNA. Lanes A,T,C and G are dideoxy sequencing reactions using an ovalbumin mRNA template and the same ovalbumin DNA primer. Left: The sequences of the primer extended cDNA and ovalbumin mRNA template (nucleotides 1 - 35) are shown. Reverse transcriptase pause sites (○—); methylation-specific reverse transcriptase stop sites (—); nucleotides that are methylated in both native and denatured mRNA (*); nucleotides that are methylated only in denatured mRNA (+); and the base-paired nucleotides in the stem of the hairpin structure (dashed arrows) are also indicated on these sequences. The dashed box encloses the region of the autoradiogram corresponding to this hairpin structure.

the methylation patterns of native and denatured mRNA. This is accomplished by using reverse transcriptase to extend a complementary DNA primer through a local region of the methylated mRNA. The reverse transcriptase stops at each RNA template nucleotide on the 3' side of a methylated base thereby producing a DNA fragment whose length corresponds to the distance from the 5' end of the DNA primer to the methylated nucleotide in the template. Since the DNA primer used in the reverse transcription is 5'

Figure 8. Primer extension analysis of the 5' end of photoreacted ovalbumin mRNA. 10 ug/ml hen oviduct poly A(+) RNA was combined with 0.68 uM AMT in TE buffer, irradiated at 4°C. for 10 minutes and recovered for primer extension analysis. Right: Autoradiogram of an 8% sequencing gel of primer extensions of an ovalbumin mRNA template which is unreacted (lane 1), photoreacted (lane 2), photoreacted in the presence of 75% DMSO (v/v) (lane 3), and photoreacted in the presence of 80% formamide (v/v) (lane 4). Lanes A, T, C and G are dideoxy sequencing reactions as described in Figure 7. Left: The sequences of the primer-extended cDNA and ovalbumin mRNA template (nucleotides 1 - 35) are shown. Reverse transcriptase pause sites (o--); AMT-specific reverse transcriptase stop sites (x); and the stem of the hairpin structure (dashed arrows) are also indicated on these sequences. The dashed box encloses the region of the autoradiogram corresponding to this hairpin structure.

end labelled with ³²P, these fragments appear as distinct bands on the autoradiogram of a sequencing gel.

Figure 7 shows the results of this type of secondary structure analysis of the ovalbumin mRNA 5' end. The cDNA sequence (shown in the figure) can be read from the dideoxysequencing lanes (A,T,C,G) and is the exact complement of nucleotides 2-33 of

Figure 9. Autoradiogram of an 8% sequencing gel of primer extensions of ovalbumin mRNA. 10 ug/ml Hen oviduct poly A(+) RNA was irradiated for 10 minutes at 4°C. in 3.4 μ M AMT (lanes 1, 3 and 5) or 6.8 μ M AMT (lanes 2, 4 and 6) in TE buffer (lanes 1 and 2), or in 1.2 mM $MgCl_2$ -2.4 mM Hepes, pH 7.55, containing either 110 mM KCl (lanes 3 and 4) or 110 mM KOAc (lanes 5 and 6). The dideoxy sequencing lanes (A,T,C,G) are as described for Figure 7. The dashed lines enclose the region of the autoradiogram corresponding to the ovalbumin mRNA 5' end hairpin.

ovalbumin mRNA template (also shown). Each of the methylation specific bands in lanes 2 and 3 of Figure 7 corresponds to a DNA molecule which terminates at a nucleotide that is 3' to an A or C residue in the ovalbumin mRNA template. Only the three A residues predicted to lie in the hairpin loop and the A residue in the base pair closing this loop are methylated in the native mRNA. Methylation of these residues is apparently enhanced by denaturation while additional A and C residues predicted to lie in the hairpin stem are methylated only when the mRNA is denatured. Thus, the pattern of base methylation is entirely consistent with the predicted hairpin structure at the 5' end of ovalbumin mRNA as shown in Figure 6. It should be noted that a control reaction (lane 1) is routinely performed in order to differentiate "natural" reverse transcriptase pause sites from true methylation sites in the mRNA template.

Preliminary experiments using different photoreaction times and AMT:ribonucleotide molar ratios indicated that a ratio of 1:45 and an irradiation time of 10 minutes was minimally sufficient to detect AMT modified bases in ovalbumin mRNA by primer extension (data not shown). This reaction produce approximately 50% of the

maximum increase in ovalbumin mRNA electrophoretic mobility observed in Figure 3. Figure 8 (lane 2) shows the pattern of DNA molecules produced by reverse transcription of AMT modified ovalbumin mRNA using the same DNA primer as in the methylation experiments. Each of the bands corresponds to a DNA molecule that ends at a nucleotide which is 3' to an AMT modified U residue (labeled with an "x") in the ovalbumin mRNA template. The major sites of AMT addition appear to be the three adjacent uracil bases at positions 27- 29 and the four uracil bases (positions 4, 20, 22 and 23) at the base of the hairpin stem. (Uracil 29 is not obviously modified in this figure but is indicated as such because it often appeared so in other experiments; see for example Figures 9 and 11.) There is apparently no addition to cytidine and uracil bases in the GC rich portion of the hairpin stem. The absence of strong bands at the top of lane 2 - corresponding to DNA primers that have been extended all the way to the 5' end of the mRNA template - indicates that essentially all of the ovalbumin mRNA molecules have been modified by AMT in their 5' region. AMT addition to uracil 22 in the AU rich base of the hairpin stem and uracil 23 immediately adjacent to the hairpin stem was especially pronounced. The weaker signals at uracils 4 and 20 probably reflect the blockage of reverse transcriptase by the AMT addition to uracils 22 and 23. The relative distribution of AMT addition sites suggests partial crosslinking predominantly between uracil 4 and 22 and less so between uracil 4 and 20. The detection of AMT addition to uracil 4 (which is on the 5' side of the hairpin) could not occur were all of the hairpins crosslinked at their base. Figure 8 also shows the primer extension analysis of ovalbumin mRNA photoreacted under the same conditions as in lane 2 but in the presence of 75% DMSO (lane 3) or 80% deionized formamide (lane 4). These denaturants inhibited the structure specific addition of AMT to ovalbumin mRNA observed in lane 2 and restored the synthesis of fully extended primers.

Figure 9 shows the effect of higher AMT:ribonucleotide ratios as well as the effect of mono- and divalent cations on the pattern of AMT base addition at the 5' end of ovalbumin mRNA. Higher ratios (lanes 1 and 2) progressively eliminate the detection of AMT modifications at uracils 4 and 20. This result could reflect the higher level of monoaddition to downstream uracils that is expected to occur at higher psoralen to base pair ratios or more crosslinking involving uracil 22. Mono- and divalent cations should produce a "tighter" helix in the hairpin stem which would be more refractory to AMT intercalation resulting in a lower level of AMT addition. Figure 9 (lanes 3-6) shows that inclusion of K^+ and Mg^{++} cations in the photoreaction reduces and limits the primer extension to the same uracil bases on the 3' side of the hairpin and results in more fully extended primers (as evidenced by the dark bands at the top of lanes 3-6). The sites of addition again indicate AMT intercalation into the "looser" region of the helix stem.

If DMSO inhibition of AMT modification of the ovalbumin mRNA 5' end was due to its

Figure 10. Effect of DMSO on ovalbumin mRNA photoreaction with AMT. 10 $\mu\text{g}/\text{ml}$ hen oviduct poly A(+) RNA was irradiated for 10 minutes at 40°C . in $0.68 \mu\text{M}$ AMT in TE buffer containing 0, 10, 25, 50 or 75% concentrations (v/v) of DMSO. RNA recovered from each 100 μl reaction was applied to a 1.5% agarose-TAE gel. Each gel lane is designated by the percent DMSO in the photoreaction. Gel lane "N" contains native (unreacted) ovalbumin mRNA (Ov) whose position is indicated by the arrow. Outer lanes (M) contain poly A(+) RNA molecular weight markers whose sizes in nucleotides are indicated at the right of the gel. This is a photographic negative of an ethidium bromide stained gel.

effect on ovalbumin mRNA secondary structure, it should also effect the overall AMT induced change in ovalbumin mRNA electrophoretic mobility. Figure 10 shows that 50% DMSO was sufficient to completely inhibit the electrophoretic mobility shift apparent in the absence of DMSO (lane 0), while 10% and 25% DMSO produced correspondingly less inhibition. (In this particular experiment, the mRNA recovery varied but was sufficient to demonstrate the effect.) Absorbance measurements at 280 nm of oviduct mRNA in the presence of increasing concentrations of DMSO indicated a pronounced hyperchromic effect at $>25\%$ DMSO (data not shown). An experiment identical to that in Figure 10 but using deionized formamide indicated that 20% formamide (the lowest concentration tested) was sufficient to completely inhibit the electrophoretic mobility shift (data not shown). The ability of formamide to completely inhibit the electrophoretic mobility shift at a lower concentration than DMSO is consistent with its report as a more potent denaturant of AU secondary structure (21).

The effect of increasing concentrations of DMSO on the structure specific addition of AMT to bases at the ovalbumin mRNA 5' end was also evaluated using the primer extension assay. Again, the lower concentrations of DMSO (Figure 11, lanes 3 and 4) appeared to inhibit but not abolish the structurally specific addition of AMT to the 5' end hairpin (Figure 11, lane 2). The band intensities in lanes 3 and 4 probably overestimate the level of inhibition by 10% and 25% DMSO; bands of similar intensity at

Figure 11. Autoradiogram of an 8% sequencing gel of primer extensions of an ovalbumin mRNA template which is unreacted (lane 1), photoreacted with AMT (lane 2), and photoreacted with AMT in 10% DMSO (lane 3), 25% DMSO (lane 4), 50% DMSO (lane 5) or 75% DMSO (lane 6). Ovalbumin mRNA photoreactions were as described in Figure 10. Lanes A, T, C and G are dideoxy sequencing reactions as described in Figure 7. The dashed lines enclose the region of the gel corresponding to the ovalbumin mRNA 5' end hairpin shown in Figure 6. The arrows indicate the AMT-specific modification sites associated with this hairpin.

the top of these gel lanes suggests a poor recovery of RNA template for this primer extension assay. At 50% DMSO (Figure 11, lane 5), there is a considerable amount of fully extended primer (as indicated by the dark bands at the top of the gel) and the specific additions apparent in lane 2 are nearly absent. At 75% DMSO, the specific modifications are completely inhibited. The similar pattern of inhibition by DMSO on the AMT induced change in ovalbumin mRNA electrophoretic mobility and on base specific modification suggest that the inhibitory effect of DMSO is the result of its denaturing effect on mRNA secondary structure. Direct inhibition of the photochemical reaction by DMSO is unlikely since DMSO does not absorb ultraviolet light or chemically react with AMT under these photoreaction conditions (data not shown).

DISCUSSION

As noted in the introduction, the photocycloaddition of various psoralen compounds including AMT to DNA and RNA has recently been reviewed in detail (1) and will not be

discussed here except to summarize some general features of the reaction.

4,5',8-Trimethylpsoralens form primarily 4'5' furan side monoadducts by cycloaddition to pyrimidine bases in DNA (98%) and RNA (80%). The rarer 3,4 pyrone side monoadduct is more prevalent in RNA than in DNA because the uracil bases in RNA do not have the sterically blocking 5-methyl group that is present on thymine bases in DNA. Because of its ability to absorb light above 320 nm, the 4'5' monoadduct is eventually converted to a diadduct by 3,4 pyrone side cycloaddition to an adjacent pyrimidine on the opposite strand.

The final diadduct to monoadduct ratio in DNA and RNA can be effected by the intensity and timing of light delivery, by the wavelength of light, and by the molar ratio of psoralen to base pairs. The high intensity, short duration (nanosecond) irradiation produced by laser favors monoaddition because the helix does not have sufficient time to change its shape to accomodate diadduct formation from 4'5' monoadducts (22,23). Performing the photoreaction at wavelengths greater than 380 nm also favors monoaddition since the resulting 4'5' monoadducts absorb light poorly above 380 nm and therefore are not converted to diadducts (24). In both of these cases, re-irradiation will efficiently convert 4'5' monoadducts to diadducts. However, since the initial yield of 4'5' monoadducts is relatively low, these techniques were not considered readily adaptable for primer extension analysis of secondary structure.

Conditions of low ionic strength and low temperature have been shown to promote maximal incorporation of HMT into model RNAs (20). Therefore, photoreactions performed at 4°C. in HE or TE buffer (1-2 mM Na⁺) would be expected to maximize AMT reaction. Likewise, sub-saturating psoralen to RNA base pair ratios should favor crosslinking since the relatively low proportion of occupied monoaddition sites will leave more unmodified bases available for diadduct formation. The fluorescence emission spectra and kinetic data described in Figures 1 and 2 demonstrate an apparently rapid and extensive (early) photoreaction consistent predominantly with monoaddition. By 15 minutes, 34% of the starting AMT (measured in pmoles) has been covalently added to RNA; this represents 82% of the total addition at 60 minutes. This would correspond to approximately 250 AMT molecules per ovalbumin molecule if all the mRNAs were ovalbumin. The slower addition of AMT during the crosslinking phase of the photoreaction may result from reaction with a weaker class of intercalation sites (e.g. GC rich hairpin stems) or from the creation of additional intercalation sites as the secondary structure is stabilized by crosslinking.

In contrast to the fluorescence data, the photoreaction-dependent electrophoretic mobility increase (Figure 3) of ovalbumin mRNA in the absence of any major mRNA truncation (Figure 4) indicates that crosslinking is occurring during the putative monoaddition phase of the reaction. This conclusion is strongly supported by the

inability of nonreversible denaturation to prevent the photoreaction-dependent increase in ovalbumin mRNA electrophoretic mobility. Assuming (for the sake of discussion) that oviduct poly A(+) RNA is 100% ovalbumin mRNA, an AMT:ribonucleotide ratio of 1:10 would result in approximately one AMT molecule for every two base pairs (which constitute a crosslinkable site) in ovalbumin mRNA. Since only 46% of these base pairs are AU, the actual ratio of AMT to reactable base pairs would be more like 1:1. However, the presence of other mRNA species and the simultaneous photodegradation of AMT during the reaction reduces this ratio to a value that is probably too low to result in saturation of reactable sites by monoaddition. (Other relatively abundant oviduct mRNAs - often visible on agarose gels - show no AMT photoreaction dependent shift in electrophoretic mobility. Perhaps AMT monoaddition to these mRNAs "masks" observation by FES of ovalbumin mRNA crosslinking.) Thus, although no attempt was made to measure the extent of ovalbumin mRNA crosslinking, the data strongly supports its wide-spread occurrence even during the putative monoaddition phase of the reaction.

The ability of DMSO and formamide to inhibit the increase in ovalbumin mRNA electrophoretic mobility at concentrations which correspond to their relative potencies as denaturants of AU base pairs (Figure 10) indicate that AMT modification of ovalbumin mRNA is secondary structure dependent and suggests that AMT is stabilizing the AU rich secondary structure of ovalbumin mRNA. The absence of a similar effect on globin mRNA electrophoretic mobility (Figure 5) is consistent with its more stable GC rich secondary structure. Since both ovalbumin and globin mRNAs have been shown to have stable secondary structures at low ionic strength (1 mM K^+) at 4°C. (7), the psoralen induced decrease in ovalbumin mRNA size is unlikely to be the result of the induction of new secondary structure. Furthermore, the observed 31% decrease in ovalbumin mRNA size (Figure 3, right panel) is well within the expected 19-39% decrease in size (depending on the arrangement of secondary structure) that should occur by stabilizing the existing secondary structure in ovalbumin mRNA. The most likely structural basis for this stabilizing effect is rapid crosslinking at a set of highly reactive sites such as the crosslinking between uracil 4 and 20 or 22. The progressive nature of the decrease in ovalbumin mRNA size suggests a hierarchy of such sites. Stabilization of secondary structure by increased stacking interactions resulting from AMT monoaddition seems less likely given the conformational change in the base paired helix accompanying monoaddition. Simple intercalation cannot be the mechanism since dark binding of AMT did not effect the electrophoretic mobility of ovalbumin mRNA.

Figure 8 shows that the selective addition of AMT to uracils 4, 20 and 22 at the 5' end of ovalbumin mRNA occurs in a secondary structure specific manner. These additions are consistent with the reported preference of AMT for monoaddition to uracil bases contained in "loose" helical structures (17,20) as indicated by the absence of AMT

addition to uracils 10 and 18 in the GC rich portion of the hairpin stem. AMT modification of all ovalbumin mRNA molecules at the low AMT:ribonucleotide ratio of 1:50 suggests that the 5' end hairpin is a preferred reaction site. Inclusion of mono- and divalent cations (Figure 9) or denaturants (Figure 11) in the photoreaction inhibit the structure-specific photoaddition observed in Figure 8. K^+ and Mg^+ cations reduce the extent of addition because the resulting "tighter" helix inhibits the AMT intercalation necessary for photoaddition while the disruption of secondary structure by DMSO and formamide removes the necessary intercalation sites.

Two additional base modifications at the 5' end of ovalbumin mRNA require structural explanation; namely the strong AMT addition to uracil 23, which lies outside, but adjacent to, the 3' side of the hairpin stem, and to uracils 27-29 which also appear to lie in a single-stranded region of the mRNA. In the first case, a number of studies have demonstrated that the 7-methyl guanosine of the cap structure can form a stacked array with the first two nucleotidyl residues at the mRNA 5' end (25,26). In ovalbumin mRNA these three nucleotides could form a continuous base stacking array with the base pairs of the immediately adjacent hairpin stem. This arrangement could create the site necessary for AMT intercalation and subsequent photoaddition to the most available reactive base, namely uracil 23 which would be immediately adjacent to the intercalation site. This reaction would probably be restricted to monoaddition although the formation of a diadduct between cytidine 2 and uracil 23 might be possible. In the second case, control experiments indicate that reverse transcriptase sometimes "pauses" at uridine 27 perhaps because the three successive uridine residues slow the progress of the enzyme. Uridines 27-29 may also be part of the stem of a previously undetected hairpin. This conclusion is suggested by data from Figure 7 which indicates that cytidines 25 and 26 and adenine 30 are methylated only in denatured ovalbumin mRNA. Inspection of the downstream ovalbumin mRNA sequence reveals a possible base pairing arrangement between nucleotides 26-32 and nucleotides 39-47 which are masked from analysis by the DNA primer. Since this hairpin does not contain a crosslinkable array of pyrimidines, AMT reaction may be confined to simple monoaddition to uracils 27-29. A two nucleotide "bulge" at bases 42 and 43 in this second hairpin would render it less stable than the 5' end hairpin. This relative instability may limit reaction with AMT sufficiently to account for the ability of the primer extension to detect base modification in the more distal 5' end hairpin. Experiments using a more downstream primer to detect this second hairpin structure are in progress.

To summarize our conclusions regarding the photoreaction of AMT with ovalbumin mRNA under conditions of low ionic strength and low temperature:

1. Fluorescence emission spectroscopy of AMT modified hen oviduct poly A(+) RNA does not appear to be an accurate indicator of intramolecular crosslinking of its major

constituent ovalbumin mRNA.

2. The AMT photoreaction-dependent increase in ovalbumin mRNA electrophoretic mobility is secondary structure dependent and reflects a more compact molecular structure that is most likely the result of the stabilization of AU base pairs by AMT crosslinking.

3. A specific DNA primer extension analysis of the 5' end of ovalbumin mRNA was able to identify all the uridine residues lying upstream from the primer (a distance of 35 nucleotides) that were modified by AMT addition. Furthermore, AMT photoaddition to uracil bases in this region (whether as monoadduct or diadduct) was both qualitatively and quantitatively selective and was entirely consistent with the 5'-end hairpin structure determined by other means. This result indicates the utility of this analysis in identifying base paired uridine residues. A manuscript describing the use this analysis to evaluate alternative secondary structures predicted for the initiation region of ovalbumin mRNA from its nucleotide sequence (8,27) is in preparation.

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