Effects of (Hydroxymethyl)trimethylpsoralen on Structure and Function of Bacteriophage MS2 Ribonucleic Acid†

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ABSTRACT: Treatment of bacteriophage MS2 with 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen and 360-nm light caused a dose-dependent decline in the infectivity of the virus. Covalent photobinding of a single psoralen molecule on the phage genome was a lethal event. Ribonucleic acid (RNA) extracted from psoralen and light-treated virus had a dose-dependent 385-nm fluorescence emission but was unaltered in its physical properties compared to control RNA samples. Phage adsorption and penetration in *Escherichia coli* host cells were unaffected, but in vivo replication of the treated virus was affected to the same extent as infectivity. The cell-free translational activity of the MS2 RNA was also severely reduced after psoralen and light treatment of the phage. Examination of the in vitro translation products revealed that the synthesis of the viral replicase protein was most substantially affected. Psoralen treatment of purified, protein-free MS2 RNA promoted an even greater reduction in cell-free synthesis of all viral proteins. This difference in translational function was consistent with the observation that virion-free RNA bound ~4 times as much psoralen as did RNA treated within the phage capsid. It was concluded that the replicase gene is the most sensitive region of the viral RNA molecule for psoralen binding.

The genomes of the ribonucleic acid (RNA)[1] bacterial viruses represent an extreme example of the sequestration and control of expression of genetic information. Only four proteins are coded for by the single-stranded RNA chromosome, with one expressed from an overlapping sequence (Capecchi & Webster, 1975; Atkins et al., 1979). Yet both gene expression and replication of the RNA in infected cells are controlled by complex regulatory constraints from the protein products of the RNA and by the RNA structure itself (Lodish, 1975; Robertson, 1975; Steitz, 1979). In addition, several host proteins are subverted for use in the specific replication of the viral RNA (Kamen, 1975).

Recently, the complete nucleotide sequence of the MS2 phage RNA has been determined (Min Jou et al., 1972; Fiers, 1975; Fiers et al., 1976). In spite of this impressive achievement, relatively little is known about the overall secondary and tertiary structure of the RNA molecule (Fiers et al., 1976; Steitz, 1979). This information is important in order to have a complete understanding of the structure and conformation of the RNA as it exists in the virion and during translation and replication. Furthermore, the nature of changes in the RNA structure under these different conditions is not well established (Paranchych, 1975; Lodish, 1975; Robertson, 1975).

A new approach for examining RNA structures involves the use of photoreactive, intercalating agents like psoralen and its derivatives (Issacs et al., 1977; Hearst & Thiry, 1977). This methodology has been used by a number of investigators to examine the specific structures of messenger RNA (Calvert & Pederson, 1979), transfer RNA (Ou & Song, 1978), and ribosomal RNA (Karathanasis & Champney, 1979; Thammana et al., 1979; Wollenzien et al., 1979). In addition, the RNA genomes of several different RNA viruses have been studied for alterations in structure and function after psoralen treatment (Hearst & Thiry, 1977; Nakashima & Shatkin, 1978; Nakashima et al., 1979).

We have initiated a study on the effects of (hydroxymethyl)trioxsalen on both the biological function and RNA structure of the bacteriophage MS2. Replication of viral RNA in infected cells was severely affected by moderate doses of the drug, concomitant with a decline in translational ability of the sequences for the phage-coded replicase protein. A psoralen-sensitive site within the secondary structure of this gene is proposed to account for the observed affects on phage infectivity.

Materials and Methods

Materials. 4,5′,8-Trimethylpsoralen (trioxsalen) was purchased from Paul B. Elder Co., recrystallized twice from chloroform and hexane, and dissolved in ethanol at 1 mg/mL. 4′-(Hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT)[1] was purchased from Calbiochem and was dissolved in 35% ethanol at a final concentration of 0.4 mg/mL. [^{3}H]HMT was a gift of Dr. John Hearst. It had a specific activity of 1.49×10^5 cpm/μg and was dissolved in 35% ethanol at a final concentration of 0.4 mg/mL. Carrier-free $^{32}PO_4$ was purchased from Amersham. [^{14}C]Uracil (54.4 mCi/mmol) and [^{3}H]leucine (60 Ci/mmol) were purchased from New England Nuclear. Rifampicin (Sigma Chemical Co.) was dissolved in 97% ethanol at a final concentration of 2.5 mg/mL. Sodium lauryl sulfate was purchased from Gallard-Schlesinger Chemicals. ATP, GTP, $PEPNa_3$, and pyruvate kinase were purchased from P-L Biochemicals. All other chemicals were purchased from the Sigma Chemical Co.

MS2 Phage Purification and Labeling. Escherichia coli strain D10 (Gesteland, 1966) was used as the host for the propagation and titer of phage MS2. The phage was purified from infected cultures after the method of Nathans (1968),

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[1] Abbreviations used: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; HMT, 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; moi, multiplicity of infection; $NaDodSO_4$, sodium dodecyl sulfate.

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as described previously (Rahi et al., 1979). Labeled phages were prepared by infecting cells in the presence of $^{32}PO_4$ (55 μCi/mL). The labeled phages were purified by the procedure of Gesteland & Spahr (1970) and had a specific activity of 2.6×10^5 cpm/μg. Viral RNA was extracted from the phage with phenol and $CHCl_3$ in the presence of 10 mM EDTA (pH 7) exactly as described for the purification of ribosomal RNA (Karathanasis & Champney, 1979). This procedure is sufficient to remove all unbound psoralen molecules from the RNA (Issacs et al., 1977).

Psoralen Treatments. MS2 phage at 80 μg/mL in TM/2 buffer (10 mM Tris-HCl and 10 mM $MgCl_2$, pH 7.6) or MS2 RNA at the same concentration in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.6) was irradiated at 4 °C with a 360-nm light source giving 3 J/(m^2 s) exactly as previously detailed (Karathanasis & Champney, 1979). The light dose received was controlled by the exposure time. HMT was present in solution at 20 μg/mL, and trioxsalen, when used, was at 1 μg/mL for a phage suspension at 4.8 μg/mL. After irradiation, unreacted psoralen and other photochemical by-products were removed by the RNA extraction procedure.

Characterization of HMT-Treated MS2 RNA. The ultraviolet absorbance and fluorescence spectra and the thermal denaturation analysis of treated RNA were determined as previously described (Karathanasis & Champney, 1979). The homogeneity of the RNA samples was examined by denaturing gel electrophoresis as described (Duesberg & Vogt, 1973; Karathanasis & Champney, 1979).

Assay for Phage Adsorption and RNA Penetration. Cellular adsorption of MS2 phage was determined by infecting log phase cells in the presence of 1 mM $CaCl_2$ at 37 °C with ^{32}P-labeled phage, at a multiplicity of infection of ~0.1 (Silverman & Valentine, 1969). After 15–20 min of incubation, EDTA and pancreatic RNase were added to give final concentrations of 2 mM and 5 μg/mL, respectively. Five minutes later the cells were collected by centrifugation (10000 rpm, 15 min), and the cell-free supernatant and pellet were measured for radioactivity. For examination of viral RNA penetration, after adsorption of the labeled phage, the samples were blended in a Sorvall Omnimixer to remove phage bound to the F-pilus. The cells were then centrifuged and washed with TE buffer, and the radioactivity was counted. Measurements of radioactivity on filters and in liquid were performed as previously detailed (Karathanasis & Champney, 1979).

Phage RNA Replication. $CaCl_2$ and uracil at final concentrations of 2 mM and 4 μg/mL were added to growing *E. coli* cells at a density of 5×10^8/mL. The cells were incubated for 10 min at 37 °C and then infected with MS2 phage (moi = 1), followed by rifampicin (50 μg/mL) and [^{14}C]uracil (2 μCi/mL) at 10-min intervals (Fromageot & Zinder, 1968). The infected cells were incubated for an additional 30 min and were sampled during this period for the incorporation of [^{14}C]uracil into RNA (Champney, 1979). The incorporation into uninfected host cells was used to measure the background RNA synthesis.

In Vitro Protein Synthesis Assays. A translationally active S30 extract was made from *E. coli* strain Q13 cells grown in rich media following the procedures of Miller (1972). The cell-free protein synthesis system was adapted from Miller (1972) and optimized such that maximal incorporation of [^{3}H]leucine into viral proteins occurred after a 20-min incubation at 37 °C in a mixture containing 10 μg of MS2 RNA. The methods for quantitation of in vitro protein synthesis have been described (Rahi et al., 1979).

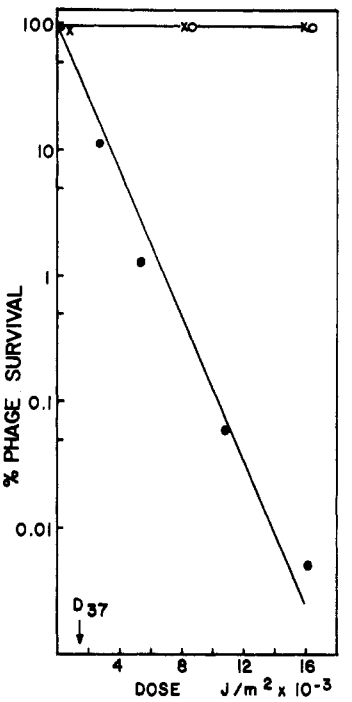

FIGURE 1: Loss of plaque-forming ability by HMT- and light-treated MS2 phage. Phage samples treated with HMT and various doses of 360-mn light were assayed for infectivity as described under Materials and Methods. D_{37} indicates the dose for 37% survival (=1/*e*). HMT- and light-treated samples (●); HMT-treated samples (○); light-treated samples (X). The data are the average of three experiments.

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. Separation and detection of the specific viral proteins synthesized in the in vitro translational system were carried out by electrophoresis in 15% polyacrylamide gels as previously described (Alton & Vapnek, 1979). For quantitative determinations, the appropriate areas of the gel were cut out and measured for radioactivity. Alternatively, the intensity of the bands on the fluorographic negative (Bonner & Laskey, 1974) was measured by scanning the negative at 600 nm in a densitometer as previously described (Karathanasis & Champney, 1979). The method of Dottavio-Martin & Revel (1978) was used to prepare the ^{14}C-labeled protein standards by reductive methylation.

Results

Treatment of bacteriophage MS2 with HMT and increasing doses of 360-nm light resulted in a dramatic decline in the infectivity of the phage preparation. As Figure 1 indicates, survival was reduced by a factor of 10^4 by using a light dose of 16×10^3 J/m^2. The D_{37} for the loss of infectivity was 2×10^3 J/m^2. Phage treated with HMT in the dark or irradiated in the absence of HMT showed no loss of infectivity (Figure 1). Identical killing curves were observed for phage treated at several different multiplicities of infection (moi = 0.1–10) and after treatment with another derivative, 4,5′,8-trimethylpsoralen (trioxsalen). These observations suggested that structural alterations of the phage RNA by HMT treatment were interfering with the productive infectivity by the virus.

The interaction of HMT with MS2 RNA was quantitated by measuring the binding of ^{3}H-labeled drug to the phage and to purified viral RNA. The binding of drug as a function of light dose for each sample is indicated in Figure 2. RNA within the virion structure bound ~16 molecules of HMT/phage particle at the highest dose used. Under these same

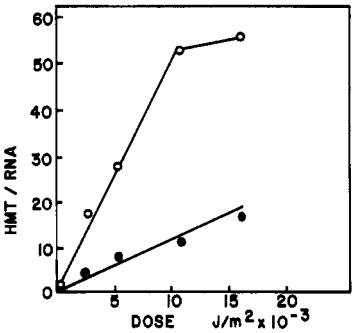

FIGURE 2: Binding of [^{3}H]HMT to treated MS2 phage and to free RNA. (●) Saturation curve for [^{3}H]HMT binding to MS2 RNA. Phages were irradiated for various lengths of time, and the binding of [^{3}H]HMT to the RNA was determined as described under Materials and Methods. From the specific activity of [^{3}H]HMT, the number of molecules bound per molecule of phage RNA was calculated. (○) Saturation curve for the binding of [^{3}H]HMT to free MS2 RNA. The average results from two assays are shown.

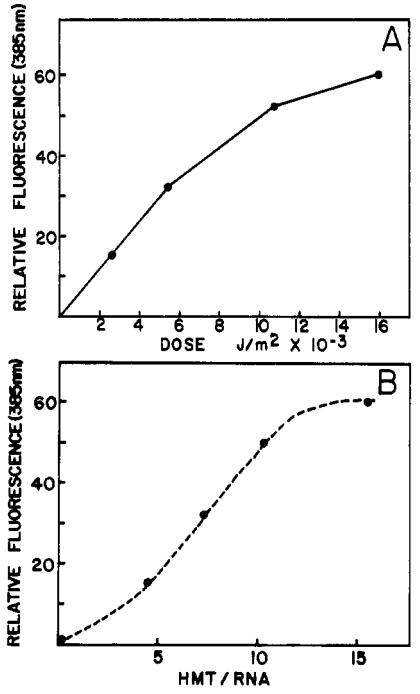

FIGURE 3: Relative fluorescence of viral RNA extracted from HMT- and light-treated MS2 phage as a function of dose received and number of covalently bound HMT molecules. The relative fluorescence was measured as the difference (in millimeters of pen excursion) between the 385-nm fluorescence emission (λ_{ex} = 340 nm) of HMT-treated samples and the fluorescence of controls as described previously (Karathanasis & Champney, 1979). In (A) the fluorescence is given as a function of light dose received, and in (B) the dose has been converted into molecules of HMT bound, from the information in Figure 2.

conditions, purified MS2 RNA was capable of binding ~60 molecules of HMT. In both cases, the half-saturation dose was 5.5 × 10^3 J/m^2.

Examination of the treated RNA by sucrose gradient sedimentation and by electrophoresis in denaturing, formamide gels indicated that the HMT-containing RNA was homogeneous and not fragmented by exposure to the drug and light (Karathanasis & Champney, 1979). In contrast to our earlier observations on trioxsalen-treated 16S ribosomal RNA, there were no significant differences after thermal denaturation in either hyperchromicity or T_m for HMT-treated MS2 RNA compared to control samples. As observed with the 16S RNA, there was a dose-dependent increase in the 385-nm fluorescence of HMT-treated RNA, indicative of the formation of

Table I: Adsorption and RNA Penetration by HMT and Light-Treated MS2 Phage[a]

sample	time of irradiation (min)	light dose received [(J/m^2) × 10^4]	input phage adsorbed (%)	RNA penetration (%)
control (−HMT)	0	0	43	100
control (−HMT)	90	1.6	31	57
control (+HMT)	0	0	50	nd[b]
control (+HMT)	90 (dark)	0	44	68
+HMT	0	0	46	75
+HMT	90	1.6	35	44

[a] Phage adsorption to growing *E. coli* host cells was measured as described under Materials and Methods. Adsorbed phage is defined as ^{32}P radioactivity in the cell pellet/input ^{32}P radioactivity in MS2 phage. Phage penetration was determined as cell-associated ^{32}P radioactivity after infection and removal of the pili by blending as described under Materials and Methods. The average results of duplicate experiments are presented. [b] nd = not determined.

monoaddition products (Figure 3A). The half-saturation value for this effect, as with the drug binding, was 5 × 10^3 J/m^2, equal to the binding of 8 molecules of HMT (Figure 3B).

The similarity of the excitation and emission maxima of HMT-containing MS2 RNA with the maxima observed for 8-(methoxy)psoralen-containing DNA (Ou & Song, 1978) indicates that the 385-nm fluorescence observed for the RNA molecules was probably due to 4′,5′-HMT monoadduct formation. For low doses of 360-nm light there was a linear increase of the relative fluorescence at 385 nm indicating the initial formation of 4′,5′-HMT monoadducts. At higher dose points, however, there was a decline of the fluorescence indicating that the 4′,5′ monoadducts were converted to diadducts which do not fluoresce in the 385-nm region. There was also a linear increase of fluorescence with increasing numbers of bound HMT molecules followed by a plateau at higher doses. At this plateau the rate of 4′,5′ monoadduct formation and the rate of conversion of 4′,5′ monoadduct to diadduct were equal. Consequently, ~4 of the 16 HMT adducts, present in the viral RNA treated with HMT and light at a dose of 1.62 × 10^4 J/m^2, must represent cross-links.

One possible consequence of the alteration of the phage RNA structure by HMT could be an inability of the phage to adsorb to the cells or to inject RNA. Adsorption was investigated by examining the ability of ^{32}P-labeled phage to bind to growing cells. Between 45 and 50% of the input phage was able to bind to the cells, and this adsorption was only reduced ~25% by prior irradiation of the phage in the presence or absence of HMT (Table I). Furthermore, penetration of the labeled RNA into the cells was only affected to about the same extent (Table I). RNA injection was measured as cell-associated radioactivity present after blending of the cells to remove phage adsorbed to the F-pilus. Thus, neither phage adsorption nor RNA penetration was significantly affected by an HMT dose which gave ~16 adducts to the RNA.

HMT modification of the RNA structure could substantially alter its ability to serve as a template for in vivo replication of the phage genome. MS2 RNA replication is carried out by a replicase complex consisting of a phage-coded replicase protein and three host proteins (Fedoroff, 1975; Kamen, 1975).

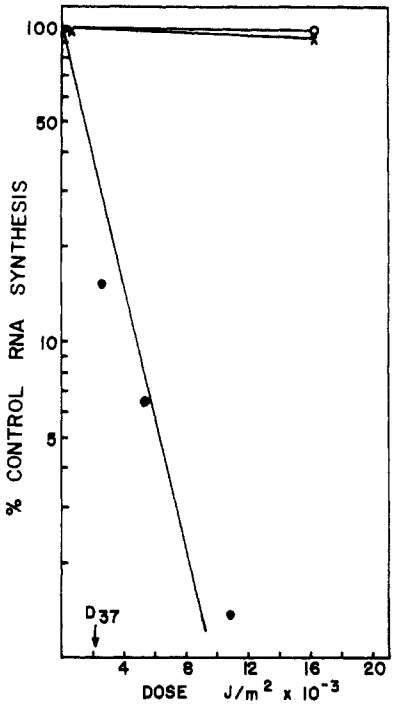

FIGURE 4: Effect of HMT treatment of MS2 phage on in vivo replication of RNA. The incorporation of [^{14}C]uracil into infected, rifampicin-treated cells was measured for control and HMT- and light-treated MS2 phage. The decline in RNA synthesis is given as a function of light dose for HMT- and light-treated samples (●), for HMT-treated samples (○), and for light-treated samples (X). The D_{37} is indicated by an arrow. The data are averaged from two experiments.

A characteristic of this activity is its insensitivity to inhibition by rifampicin, a transcriptional inhibitor of the host RNA polymerase (Fromageot & Zinder, 1968). We therefore examined RNA synthesis in infected cells treated with rifampicin by using both control and HMT-treated phage. The in vivo replication of MS2 RNA declined substantially as a function of light dose for HMT-treated phage (Figure 4). The D_{37} value for the decline in replicating ability was 2×10^3 J/m^2, equivalent to 2 molecules of HMT bound/RNA. The replication of control RNA proceeded normally in rifampicin-treated cells (Figure 4).

The similarities observed between the HMT effects on phage viability and RNA replication suggested that an inhibition of RNA synthesis could account for the loss of infectivity. However, the inability to copy new phage RNA molecules from HMT-treated RNA could result from either an alternation in template ability or an alteration in message function leading to reduced synthesis of the phage replicase protein. The translational activity of HMT-treated MS2 RNA was tested in an in vitro translational system. As Figure 5 indicates, the total translation capacity of HMT-treated virion RNA was reduced after exposure to light. About 50% of the message activity was lost by RNA treated with the highest dose of HMT and light.

To examine more specifically the effects of the altered RNA structure on translation, we fractionated the protein products of the in vitro incorporation system by polyacrylamide gel electrophoresis. Figure 6A shows a fluorogram of the protein formed in this system, using control and treated MS2 RNA as message. It is very clear that formation of the replicase protein was most drastically affected by moderate doses of HMT and light, using RNA extracted from treated phage. The synthesis of maturation protein declined less rapidly, and coat protein formation was least severely affected by the

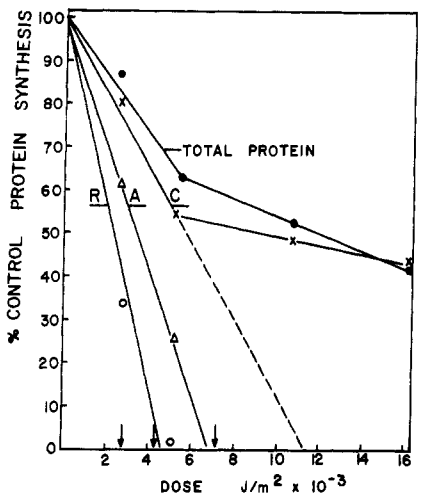

FIGURE 5: Decline in total and phage-specific protein synthesis as a function of dose of HMT and light. The amounts of total and phage-specific proteins synthesized in vitro were measured from the net incorporation and from the gel bands by the procedures described under Materials and Methods. The decline in the relative amount of each protein is presented as a function of the dose of 360-nm light for HMT-treated RNA, used as the messenger in the cell-free assays. The D_{37} valves for each protein are indicated by arrows. The results from four assays have been combined.

treatment. A quantitative comparison of the decline in the synthesis of each protein species is presented in Figure 5. The D_{37} for the loss of replicase protein was 2.5×10^3 J/m^2, very similar to the value found for the loss of infectivity and in vivo replicating activity. This strongly suggests that binding of 2 HMT molecules to the RNA interfered with replicase gene translation and accounts for the loss of infectivity observed for treated phage. For the maturation and coat proteins the D_{37} values were 4.2×10^3 and 7.2×10^3 J/m^2. The background polypeptides observed in the gels may be due to either premature termination of protein synthesis in the in vitro translational system or to some heterogeneity of the viral RNA used as template, as observed by others (Atkins et al., 1979).

The dramatically increased susceptibility of purified viral RNA to HMT binding was also reflected in its in vitro translational efficiency. Figure 6B compares the loss of MS2 proteins translated from purified viral RNA treated with HMT and light. The relative amounts of total and coat proteins synthesized in vitro were reduced even more rapidly when virion-free RNA was used as messenger (Figure 7). The D_{37} values were 4.4×10^3 and 3.0×10^3 J/m^2 for total and coat proteins, respectively. Due to the rapid reduction in in vitro synthesis of replicase and A proteins, it was not possible to obtain an accurate estimate of the decline rates of these proteins. Replicase formation was effectively abolished at a dose of 5.4×10^3 J/m^2.

The presence of HMT adducts on the MS2 RNA molecule inhibited its ability to serve as template for in vitro protein synthesis. The number and the location of the covalent HMT adducts on the viral genome are dependent upon the specific secondary and tertiary structural arrangements of the molecule. Thus, the loss in total protein synthesis function of the RNA with increased numbers of HMT adducts is directly related to the specific structural arrangement of the molecule. In Figure 8, the decline rates for total protein synthesis are presented, for MS2 RNA which had been treated with HMT and light, either within the virion or as free RNA. As can be seen in this figure, protein synthesis with encapsulated RNA required 14 HMT adducts to give 63% inhibition (=D_{37}), while 28 HMT adducts were required to cause the same extent of inhibition in the case of free RNA. This observation suggests

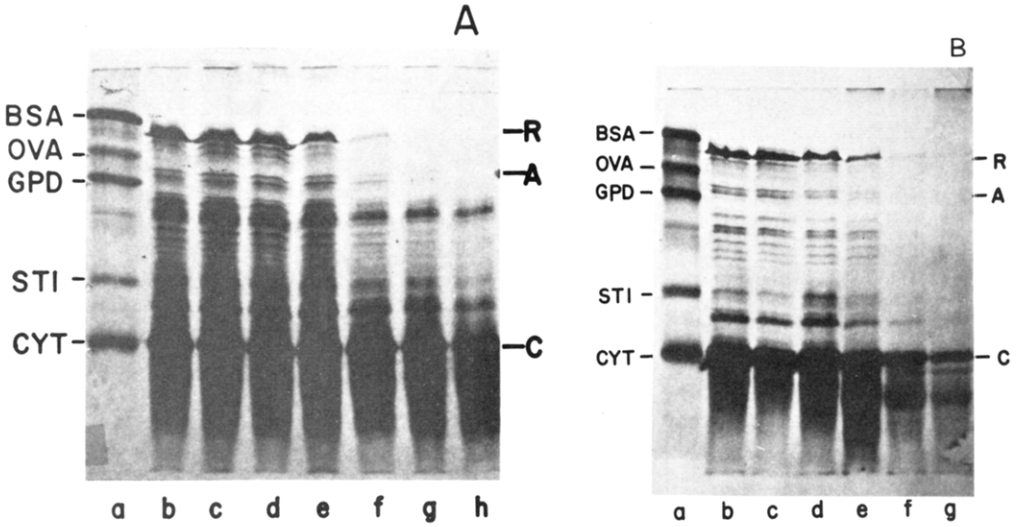

FIGURE 6: Polyacrylamide gel electrophoresis patterns of proteins synthesized with control and HMT-treated MS2 RNA. The reaction products were separated on 15% polyacrylamide gels as described under Materials and Methods. A fluorogram of a 12-day exposure of the dried gels is presented. (A) Translation products of virion-treated RNA. (B) Translation products of purified RNA. The positions of the three phage gene products are indicated (R = replicase; A = maturation protein; C = coat protein), along with several radioactive molecular weight standards. Gel lanes are as follows: (a) molecular weight markers; (b) translation from MS2 phage RNA, HMT treated in the dark; (c) RNA irradiated at 1.62×10^4 J/m^2 in the absence of HMT; (d) RNA irradiated in the presence of HMT at 0 J/m^2, (e) 0.27×10^4 J/m^2, (f) 0.54×10^4 J/m^2, (g) 1.08×10^4 J/m^2, and (h) 1.62×10^4 J/m^2.

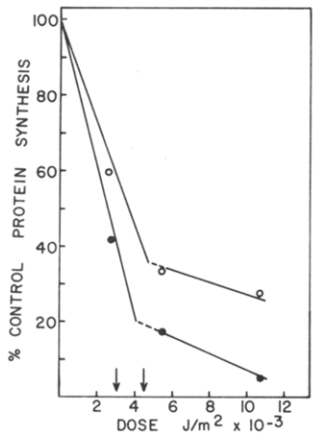

FIGURE 7: Decline in total and coat protein synthesis from HMT- and light-treated MS2 RNA. The amounts of total and coat protein were determined as described in the legend of Figure 5. Total Cl_3CCOOH-precipitable protein (○); coat protein (●). The D_{37} values are indicated by arrows. Three assays were run and the results averaged.

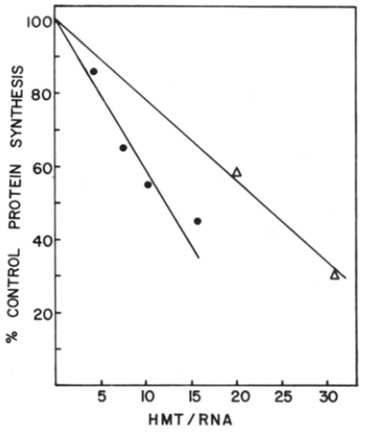

FIGURE 8: Decline in total protein synthetic capacity of HMT- and light-treated MS2 RNA as a function of the number of covalently bound HMT molecules. The number of bound HMT molecules per RNA (Figure 2) for virion and free viral RNA was related to the percent of total in vitro protein synthesis at each dose point (Figures 5 and 7). Virion-treated RNA (●); free MS2 RNA (△).

that the structural arrangements of the RNA are not the same under the two conditions examined.

Discussion

It is becoming increasingly apparent that specific secondary and tertiary structural arrangements play a critical role in determining the biological function of RNA molecules. Hairpin structures in RNA molecules have been hypothesized to provide the control mechanisms for specific RNA splicing (Khoury et al., 1979), attenuation (Oxender et al., 1979), termination (Hirashima et al., 1979), and pausing of Qβ replicase (Mills et al., 1978) and ribosomes (Chaney & Morris, 1979). It is also apparent that the secondary structure (and tertiary interactions) of messenger RNA molecules is a critical determinant in the degree to which a particular sequence may be translated (Steitz, 1979). Many studies have suggested that the differential translation of the three genes of the RNA bacteriophage genome is due to differences in secondary structure in various parts of the molecule (Lodish, 1975; Atkins et al., 1979). These constraints on the three-dimensional conformation of the RNA may be important both for packaging the RNA within the virion and for regulation of the translation of the four gene sequences.

Several workers have recently reported that psoralen derivatives can be used to stabilize the structure of RNA molecules (Isaacs et al., 1977; Wollenzien et al., 1978, 1979; Karathanasis & Champney, 1979). Psoralen-treated RNA molecules showed complete loss of their biological function (Ou & Song, 1978; Karathanasis & Champney, 1979). These findings have suggested that psoralens could be used to examine the structure–function correlations in RNA molecules and ribonucleoprotein complexes.

A dramatic effect of HMT and light on MS2 phage was observed when the plaque-forming ability of treated particles was examined. Less than two HMT molecules, and possibly a single HMT molecule, covalently bound on the MS2 viral genome were sufficient to cause a lethal event. It has been observed that reovirus infectivity and the template activity of the viral RNA were specifically reduced by treatment with trioxsalen derivatives (Nakashima & Shatkin, 1978; Naka-

shima et al., 1979). Psoralen treatment has also been shown to inactivate the infective ability of the single-stranded vesicular stomatitis virus (Hearst & Thiry, 1977).

Our results indicate that HMT binding on the MS2 virus genome did not substantially interfere with the attachment and penetration processes. Thus, the lethal event resulting from HMT covalent binding must occur at either the translational or replicational steps. Furthermore, this finding suggests that if structural rearrangements of the genomic RNA do take place during ejection, they must be limited to a region less susceptible to HMT binding. This rules out extensive unwinding of the viral RNA molecule during ejection as has been previously suggested (Paranchych, 1975).

Studies on the base specificity of psoralen binding to DNA (Cole, 1970) and RNA (Bachellerie & Hearst, 1980) have suggested that the most probable reaction sequence in an RNA duplex region would be

An inspection of the known sequences of the three MS2 RNA genes for such sites within the proposed secondary structure models is informative (Min Jou et al., 1972; Fiers, 1975; Fiers et al., 1976). Comparison of the sequences of the first one-third of the replicase, maturation, and coat genes shows a frequency of occurrence of this site of 11, 7, and 4 times, respectively. Clearly, the replicase gene is enriched in potential psoralen reactive sites relative to the other two genes of the phage. Interestingly, the ratios of the site frequencies in the initial one-third of the three genes (11/7 = 1.57 and 7/4 = 1.75) are very similar to the ratios of the respective D_{37} values for translational inhibition from these same sequences (4.2/2.5 = 1.68 and 7.2/4.2 = 1.71; Figure 5). This observation gives support to the idea that a preferential binding of HMT to the initial part of the replicase gene sequence may be responsible for the observed effects on gene translation and infectivity. A total of 73 sites can be found within the secondary structure of MS2 RNA. Our observation that 60 molecules of HMT were bound to each RNA suggests that a majority of these proposed duplex structures actually exist and are available for HMT photobinding.

With the assumption of a Poisson distribution of covalent HMT binding on the viral genome, it would be expected that if complementation occurred, then the survival of treated phage at high multiplicities of infection should have increased. An increase in phage survival due to complementation should have resulted in greater values of D_{37}, but this result was not observed. This suggests either that the replicase complex is unable to replicate past an HMT adduct located anywhere on the RNA or that HMT binding leads to a preferential inhibition of replicase protein synthesis. Experimentally, the first alternative predicts that the inactivation curve of the replicating capacity of treated phage should have a greater D_{37} value that that obtained when infectivity was examined. The second alternative would predict that these D_{37} values must be equal. This is what was found.

Disassembly of the tertiary interaction between the initiation site of the replicase gene and the proximal segment of the coat protein gene by ribosomes transversing this region is a prerequisite for synthesis of replicase protein (Lodish, 1975). Thus, the presence of HMT adducts in the early region of the coat protein gene would be expected to decrease the synthesis of coat and replicase proteins proportionally. However, the amount of replicase formed in the translational assay was substantially less than the amounts of coat and maturation proteins. This result is in agreement with the speculation that the replicase gene preferentially binds HMT and that the adducts inhibit replicase protein formation. Since photochemical binding of HMT on the phage genome is dependent upon the secondary interactions present in the molecule, we can conclude that the extent of such interactions within the replicase gene may be much greater than those within either coat or maturation protein RNA segments. A mathematical relationship between the size of the secondary structural target and the protein synthesis inactivation rate for an RNA gene has been developed (Karathanasis, 1980). From such an analysis, it appears that the replicase and maturation protein genes have 2.6- and 1.6-fold more total secondary structure that the coat protein gene. These predictions are in accord with the relative differences in total size of the three genes in MS2. The higher susceptibility of the MS2 replicase gene to binding by polycyclic aromatic hydrocarbons compared to the other viral genes has been reported (Sagher et al., 1979). These authors have also suggested that the greater reactivity of the replicase gene was mainly due to the larger target size of this sequence compared to the other viral genes.

A preliminary comparison of the effects of HMT and light treatments on purified MS2 RNA was carried out. The RNA was 3.6-fold more sensitive to HMT and light than RNA within the viral capsid. The difference in photoreactivity of free and protein-complexed nucleic acids has been observed in a number of different systems (Wiesehahn et al., 1977; Karathanasis & Champney, 1979). It is not clear, however, if this phenomenon occurs as a consequence of a less organized secondary structure of the RNA within the virion or as a consequence of limited psoralen permeability through the capsid structure. The D_{37} values for either total or coat protein synthesis were drastically reduced when using treated RNA compared to the corresponding values for translation of RNA treated inside the viral capsid. This reduction in D_{37} values corresponds to the increased HMT photobinding observed and indicates the enhanced sensitivity of purified RNA compared to RNA within the capsid to HMT and light treatments.

Our results not only point to the potential of using psoralens to directly examine the secondary structure of single-stranded nucleic acid molecules but also indicate that the individual secondary structures present in each gene of a polycistronic mRNA may be identified by using this method, in conjunction with other techniques such as electron microscopy (Jacobson, 1976; Thammana et al., 1979; Wollenzien et al., 1979). It may also be possible to directly determine the secondary and/or tertiary interactions of a nucleic acid molecule within an RNA–protein complex.

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