A Specific, UV-Induced RNA–Protein Cross-Link Using 5-Bromouridine-Substituted RNA†

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Received January 24, 1991; Revised Manuscript Received March 26, 1991

ABSTRACT: The well-characterized RNA binding site of the bacteriophage R17 coat protein has been used to investigate the cross-linking of protein to 5-bromouridine (BrU)-substituted RNA using medium-wavelength UV light. We have demonstrated a specific RNA–protein cross-link and identified the site on the RNA of protein attachment. Formation of the covalent complex is dependent upon the presence of BrU at position −5 of the RNA and specific binding of the RNA by coat protein. The amount of cross-linking increases with time and depends on the light source and conditions used. Irradiations using a broad-spectrum UV transilluminator (peak at 312 nm) or monochromatic XeCl excimer laser (308 nm) gave levels of cross-linking exceeding 20 and 50%, respectively. The quantum yield of photo-cross-linking, determined with 308-nm excitation, was 0.003. While little strand breakage or debromination of the RNA occurred, significant protein photodamage was observed.

One means of probing protein–nucleic acid interactions entails the production of UV-induced cross-links [reviewed by Shetlar (1980) and Hanna (1989)]. 5-Bromodeoxyuridine (BrdU) has been incorporated into DNA to enhance its photosensitivity both in vivo (Weintraub, 1973) and in vitro (Lin & Riggs, 1974; Ogata & Gilbert, 1977). More recently, 5-bromouridine (BrU) has been shown to be readily incorporated into RNA transcripts by T7 RNA polymerase and used to study the interaction between yeast tRNA ligase and precursor tRNA (Tanner et al., 1988).

The photochemistry of BrU is significantly wavelength-dependent (Dietz et al., 1987). The UV spectrum of BrU shows a π–π^* transition at 276 nm and a poorly resolved n–π^* transition as a shoulder at 300 ± 10 nm. The first excited triplet state is the state proposed as reactive in protein cross-linking; model studies implicate an electron-transfer mechanism with the intermediacy of ion radicals (Ito et al., 1980; Swanson et al., 1981; Dietz et al., 1987; Dietz & Koch, 1989). While either the ^{1}n,π^* or the $^1\pi,\pi^*$ singlet state can undergo intersystem crossing to the triplet manifold, only the $^1\pi,\pi^*$ state undergoes competitive C–Br bond homolysis to yield the highly reactive 5-uracilyl radical. Formation of the uracilyl radical can result in localized photodamage such as strand breaks (Hutchinson & Köhnlein, 1980). Thus, using longer wavelength light to selectively populate the ^{1}n,π^* singlet state, which yields the triplet state but no uracilyl radical, may provide an advantage when the goal is cross-linking to an associated protein.

A number of previous studies with BrU and model compounds utilized a monochromatic XeCl excimer laser operating at 308 nm to excite selectively the n–π^* transition (Dietz et al., 1987; Dietz & Koch, 1987, 1989). Here we extend these experiments to an RNA–protein interaction. The bacteriophage R17 coat protein interacts specifically with a small RNA hairpin within the phage genome (Bernardi & Spahr, 1972; Krug et al., 1982). This interaction plays two roles during phage infection: the coat protein acts as a translational repressor of replicase synthesis (Eggen & Nathans, 1969), and the complex serves as a nucleation site for encapsidation (Ling et al., 1970; Beckett et al., 1988). In vitro, the coat protein binds to isolated hairpins with high affinity, and the system has been extensively characterized [see Witherell et al. (1991) for a recent review]. We demonstrate here the formation of a specific, covalent photoadduct between the R17 coat protein and a BrU-containing RNA binding site and show that the site of cross-linking is at a particular BrU within the loop of the RNA hairpin.

Materials and Methods

RNAs. RNA fragments were prepared by in vitro transcription from synthetic DNA templates by T7 RNA polymerase (Milligan et al., 1987). Transcription reactions contained 40 mM Tris-HCl (pH 8.1 at 37 °C), 1 mM spermidine, 5 mM dithiothreitol, 50 μg/mL bovine serum albumin (BSA), 0.1% (v/v) Triton X-100, and 80 mg/mL poly(ethylene glycol) (M_r 8000). Labeled RNAs were prepared in a 40-μL transcription reaction with 1 mM each of three NTPs (using either UTP or BrUTP), 0.25 mM [α-^{32}P] NTP (5 μCi), 6 mM $MgCl_2$, 100–400 nM template, and 0.05–0.1 mg/mL T7 RNA polymerase. Nucleotides, including 5-BrUTP, were purchased from Sigma. RNA fragments were gel-purified by electrophoresis on 20% denaturing polyacrylamide gels run in 90 mM Tris–borate/2 mM EDTA (TBE), cut out, eluted overnight in 0.1 M Tris-HCl (pH 8)/1 mM EDTA, and ethanol-precipitated in the presence of 0.4 M ammonium acetate.

Coat Protein Expression System. The overexpression system of Studier and Moffat (1986) was used to isolate large amounts of phage coat protein. A derivative of plasmid pT7-2 (U.S. Biochemical Corp.) was used which contains a 832 bp *Nrul–Nael* fragment encoding the 3′ end of the phage maturation protein and the entire coat protein gene under the control of a phage T7 promotor. The upstream sequences (*Nrul–Xbal*) were derived from pSIU510 (Parker & Precup, 1986), while the coat protein gene (*Xbal–Nael*) was subcloned from pCOAT184 (Berkhout, 1986). This plasmid, pTCT5, was used to transform BL21 (DE3) which contains a single copy of the bacteriophage T7 RNA polymerase gene integrated into the bacterial genome (Studier & Moffat, 1986) under control of the *lac* promotor. Induction with isopropyl β-D-thiogalactopyranoside (IPTG) resulted in high levels of

† Supported by National Institutes of Health Postdoctoral Fellowship Grant GM-12452 to J.M.G., by National Science Foundation Grant CHE-8903637 to T.H.K., and by National Institutes of Health Grant GM-36944 to O.C.U.
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expression of the R17 coat protein (approximaately 20 mg/L).

Coat Protein Purification. BL21 (DE3) cells containing plasmid pTCT5 were grown to mid-log phase in YTG medium (per liter: 10 g of Bactotryptone, 5 g of yeast extract, 5 g of NaCl, and 4 g of glucose) supplemented with 150 μg/mL ampicillin. IPTG was added to a final concentration of 0.4 mM, and cells were incubated an additional 3 h at 37 °C with shaking to allow accumulation of coat protein. Cells were harvested, resuspended in 50 mM NH_4Cl/20 mM Tris (pH 8 at 4 °C)/3 mM DTT/1 mM EDTA/5% glycerol, and lysed at 4 °C for 15 min by the addition of lysozyme to a final concentration of 0.45 mg/mL. The cell lysate was then brought to 14 mM $MgCl_2$, 15 units/mL RNase-free DNase (Boehringer Mannheim) was added, and the mixture was incubated another 30 min on ice. The lysate was centrifuged at 4 °C at 27000*g* for 50 min, and the supernatant was subjected to further centrifugation for 90 min, 4 °C, at 186000*g*. Pellets were resuspended overnight with gentle shaking in 200 mM NaCl/50 mM MOPS (pH 7)/1 mM EDTA/5 mM β-mercaptoethanol at 4 °C. Samples were passed over a DEAE column (TSK-gel DEAE 650S, Toyopearl) equilibrated in the same buffer and then extracted with 2 volumes of chilled glacial acetic acid, vortexing 10 s every 10 min for 1 h. After centrifugation at 4 °C, 16000*g* for 10 min, samples were dialyzed vs. three changes of 1 mM acetic acid, and protein concentration was determined by optical density.

Filter Binding Assays. The association constant between coat protein and each RNA fragment was determined with a nitrocellulose filter retention assay described in detail by Carey et al. (1983a). A constant, low concentration of ^{32}P-labeled RNA was mixed with a series of coat protein concentrations between 0.1 nM and 1 μM in 10 mM magnesium acetate, 80 mM KCl, 80 μg/mL serum albumin, and 100 mM Tris-HCl (pH 8.5 at 4 °C) (TMK). After incubation at 4 °C for 20 min, the mixture was filtered through a nitrocellulose filter and the amount of complex retained on the filter determined by liquid scintillation counting. For each experiment, the data points were fit to a retention efficiency and a K_d value, assuming a bimolecular equilibrium (Carey et al., 1983a).

Irradiations. RNA (1–10 nM) and coat protein (80–120 nM) were incubated on ice in 100 mM Tris-HCl (pH 8.5 at 4 °C)/80 mM KCl/10 mM magnesium acetate/80 μg/mL BSA for 15–60 min before irradiation. RNAs (in water) were heated to 90 °C for 3 min and quick-cooled on ice before use to ensure that the RNAs would be in a conformation bound by the coat protein (Groebe & Uhlenbeck, 1988).

A hand-held Spectroline (Spectronics Corp.) medium-wavelength UV transilluminator (peak at 312 nm) was used for some irradiation experiments. A volume of 10 μL of fthe RNA–coat protein solution was distributed into each well of a microtiter plate and irradiated at a distance of 1 cm for the indicated time. The samples were kept on ice at all times.

A Lambda Physik EMG-101 excimer laser was used for laser irradiation and was operated at 308 nm by charging with 60 mbar of xenon and 80 mbar of 5% hydrogen chloride in helium and filled to 2500 mbar with helium. The laser output was passed unfocused through a 7-mm-diameter circular beam mask and into a 1-cm-square cuvette in a thermostated cell holder. The cell was bubbled with a small stream of argon to remove the oxygen and stir the sample. The laser power output was measured with a Scientech 36-001 disk calorimeter power meter. The monochromatic light passing through the beam mask was measured to be in the range of 5 mJ/pulse at 10 Hz for most of the experiments. For all laser irradiations, the temperature was regulated at 4 ± 2 °C with an MGW Laude RC3 circulating bath. The cuvette windows were kept dry with a constant flow of nitrogen into the cell holder.

Irradiated samples were heated to 95 °C for 3 min in 2 M urea/1% SDS/0.01% bromophenol blue and electrophoresed at 100 V through 15% polyacrylamide/SDS gels made according to Maniatis et al. (1982). Gels were dried and either subjected to autoradiography or quantitated using a Phosphor Imager (Molecular Dynamics).

Nuclease Digestions of Irradiated Species. RNA variant **1** was synthesized by using BrUTP and [α-^{32}P]ATP and irradiated as above. RNA and RNA–coat protein complexes were separated on 20% acrylamide/7 M urea/TBE gels, eluted, and dialyzed against water. Approximately 4000 cpm of each sample was digested with either 150 units/mL RNase T1 or 330 μg/mL RNase A in 20 mM Tris-HCl (pH 7.5)/1 mM EDTA. After 1 h at 60 °C, samples were heated gradually (over 15 min) to 80 °C before addition of 0.5 volume of 7 M urea/TBE/0.4% bromophenol blue/0.4% xylene cyanol and being loaded onto a 20% acrylamide/7 M urea gel in TBE. Samples were electrophoresed for 75 min at 1000 V/20 mA, and the gel was subjected to autoradiography.

RNA variant **1** was synthesized by using BrUTP and [α-^{32}P]ATP and irradiated as described. Approximately 2000 cpm of isolated RNA or RNA–coat protein complex was digested with 25 units/mL RNase T1, 20 units/mL RNase T2, 5 μg/mL RNase A, and 1 μg/mL proteinase K in 30 mM Tris-HCl (pH 7.5)/5 mM ammonium acetate for 45 min at 37 °C. Samples were spotted onto 10 cm × 10 cm Kodak Chromagram cellulose sheets (no. 13254 with fluorescent indicator 6065) along with cold standards and subjected to chromatography in two dimensions. Solvent systems used were as follows: first dimension, isobutyric acid/0.5 M NH_4OH/0.1 M EDTA, 75:25:0.75 (v/v); second dimension, 2-propanol/HCl/water/0.1 M EDTA, 70:15:20:1 (v/v).

Results

Formation of an RNA–Protein Cross-Link. Initial cross-linking studies utilized an RNA fragment (**1** in Figure 1A) specifically designed for this work. This RNA hairpin contains all the elements essential for tight binding to R17 coat protein determined by previous mutagenesis studies (Romaniuk et al., 1987), including an unpaired purine within the stem and a four-nucleotide loop with adenosines at positions −4 and −7 and a pyrimidine at −5. The sequence of **1** was designed to include a single U at position −5 so that 5-BrU could be specifically incorporated by in vitro transcription. Position −5 was chosen because it is very sensitive to nucleotide substitutions (Carey et al., 1983b) and is a potential site of RNA–coat protein contact (Romaniuk & Uhlenbeck, 1985).

In Figure 1B, the binding of BrU-containing **1** (BrU–RNA **1**) to R17 coat protein is compared to the U-containing **1** (U–RNA **1**) using a nitrocellulose filter binding assay. As has been previously noted (Talbot et al., 1990), the introduction of 5-BrU increases the affinity of binding about 6-fold (from K_d = 21 nM to K_d = 3.5 nM), suggesting that the substitution does not greatly alter the nature of the RNA–protein interaction. In both cases, about 70% of the RNA was retained on the filter at saturating protein concentrations. A significant amount of the remainder was bound to the protein, but not trapped by the nitrocellulose filter (Carey et al., 1983a). Thus, most RNA molecules were bound to coat protein when cross-linking was carried out at high protein concentrations.

Irradiation of 120 nM coat protein and a low concentration of ^{32}P-labeled BrU–RNA **1** at 308 nm resulted in the formation of a new band on a denaturing SDS/polyacrylamide

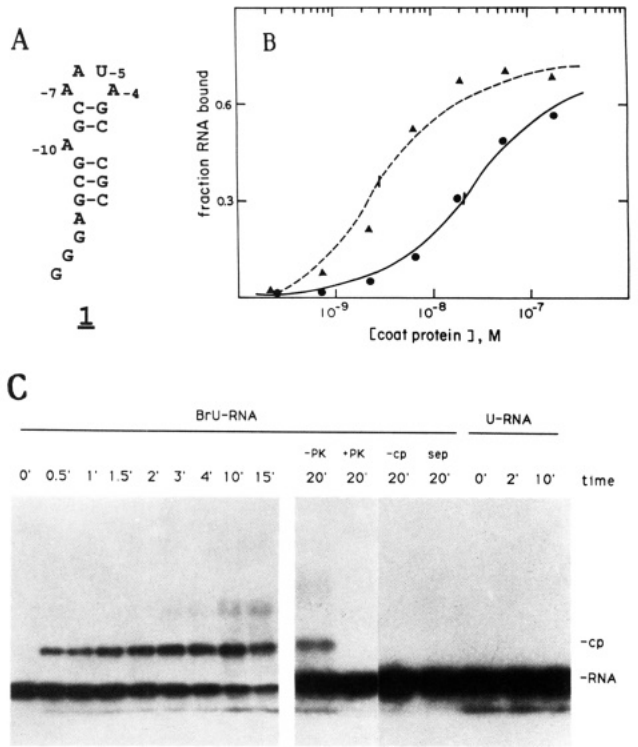

FIGURE 1: (a) Sequence and proposed secondary structure of RNA variant **1**. For ease of comparison to previous work, the nucleotides are numbered according to their position relative to the genomic binding site for coat protein. In this context, the first nucleotide of the replicase initiation codon is designated as +1. (b) Coat protein excess binding curves for ^{32}P-labeled U–RNA **1** or BrU–RNA **1** in TMK buffer at 4 °C. Theoretical binding curves were calculated by using a retention efficiency of 63% and a half-saturation of 21 nM for U–RNA **1** (solid line) and 72% and 3 nM for BrU–RNA **1** (dashed line). (c) SDS/acrylamide gel of irradiation products. Lanes 1–9: 9 nM BrU–RNA **1** and 400 nM coat protein irradiated at 308 nm for 0, 0.5, 1, 1.5, 2, 3, 4, 10, or 15 min, respectively; lanes 10 and 11, 3.5 nM BrU–RNA **1** and 120 nM coat protein irradiated at 308 nm for 20 min and incubated in the absence (lane 10) or presence (lane 11) of proteinase K; lane 12, 3.5 nM BrU–RNA **1** irradiated in the absence of coat protein; lane 13, 3.5 nM BrU–RNA **1** and 120 nM coat protein irradiated separately and then mixed; lanes 14–16, 3.5 nM U–RNA **1** and 120 nM coat protein irradiated at 308 nm for 0, 2, or 10 min, respectively.

gel that migrated more slowly than the free RNA (Figure 1C). The species comigrated with stained R17 coat protein monomer. The amount of product increased with irradiation time (lanes 1–9), and at longer times, a small amount of a second, more slowly migrating product was observed. Both photoproducts were composed of RNA and protein since treatment with proteinase K yielded a product that migrated with free RNA (lanes 10 and 11). The presence of the photoproducts required the simultaneous presence of the RNA and the protein during the irradiation. No new band was observed when the RNA was irradiated in the absence of the protein (lane 12) or when the RNA and protein were irradiated separately and then mixed (lane 13). Formation of the photoproduct appears to require the formation of complex since the photoproduct was not observed at protein concentrations below the K_d where most of the RNA was not bound. Finally, the appearance of the photoproduct required the presence of BrU; U–RNA **1** did not form the slower moving band (lanes 14–16).

The products of irradiation were also analyzed on 7 M urea/polyacrylamide gels which are expected to denature both RNA and protein (data not shown). Again, a slower moving photoproduct was observed which was dependent on the

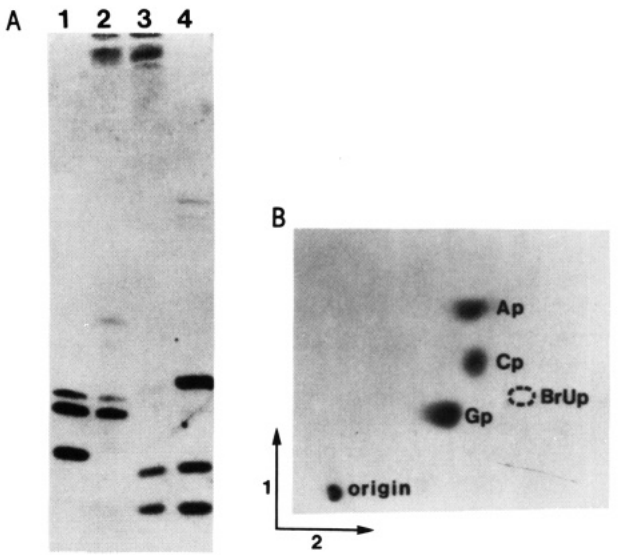

FIGURE 2: (a) RNase digestion of BrU–RNA **1** and the BrU–RNA/coat protein adduct. BrU–RNA **1** (lanes 1 and 4) or isolated RNA/coat protein adduct (lanes 2 and 3) digested with either RNase A (lanes 1 and 2) or RNase T1 (lanes 3 and 4). (b) Two-dimensional thin-layer chromatography. Isolated RNA–coat protein adduct was digested with RNases A, T1, and T2 and proteinase K and chromatographed in two dimensions. Standards were visualized via UV shadowing before autoradiography.

presence of BrU in the RNA. Relatively little photodamage of the BrU–RNA **1** was observed. At long irradiation times, an additional RNA species became apparent which migrated slightly slower than the original RNA. However, this species appeared whether or not protein was present and never exceeded 10% of the total radioactivity, even after 20 min of irradiation.

In summary, the available data indicate that the major photoproduct between BrU–RNA **1** and R17 coat protein is a covalent cross-link that forms when a preformed RNA–protein complex is irradiated. The minor photoproduct, which is also composed of RNA and protein and appears to contain one or more photo-cross-links, was not studied further.

Identification of the RNA Cross-Link Site. While the BrU residue in **1** is the most likely site of cross-link formation with the protein, the possibility that cross-linking occurred at another site had to be eliminated. Incorporation of BrU into RNA may alter the interaction subtly, allowing photochemistry that is not effective with the U–RNA. Alternatively, while BrU is the only nucleotide that absorbs at 308 nm, the energy absorbed by BrU could have been transferred to another base or amino acid residue before cross-linking occurred.

The site of cross-linking was localized to one of the loop nucleotides through a series of experiments utilizing BrU–RNA **1** synthesized with [α-^{32}P]ATP. Digestion of this RNA with RNase T1 should give three labeled products, Gp, CpGp, and CpApApBrUpApGp, while digestion with RNase A should give ApApBrUp, GpApGpCp, and pppGpGpGpApGpCp. As shown in Figure 2A, bands of the expected mobility were observed on a denaturing polyacrylamide gel. However, when the isolated RNA–protein adduct was digested with these enzymes, the longest RNase T1 fragment (CpApApBrUpApGp) and the shortest RNase A fragment (ApApBrUp) were missing, and radioactivity appeared high on the gel where the cross-linked material migrated. This experiment suggested that BrU is the site of the cross-link but did not eliminate A_{-6} or A_{-7}.

Confirmation of BrU as the site of cross-linking was accomplished by digestion of the cross-linked product with

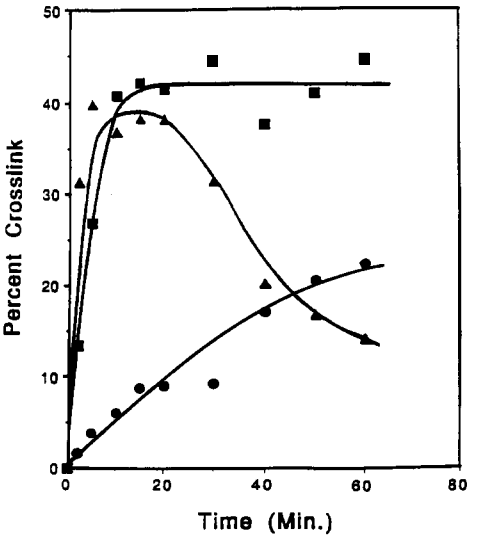

FIGURE 3: Time course of cross-link formation. 5 nM ^{32}P-labeled BrU–RNA **1** and 120 nM coat protein irradiated at 308 nm, 2.7 mJ/pulse at 10 Hz (■); 308 nm, 5.0 mJ/pulse at 10 Hz (▲); or with a medium-wavelength UV transilluminator, 312-nm peak (●).

proteinase K and ribonucleases A, T1, and T2. The resulting nucleotides were separated by two-dimensional thin-layer chromatography. Radiolabeled Gp, Cp, Ap, and BrUp were expected based on the sequence of **1**. The cross-linked product gave Gp, Cp, and Ap, but the spot for BrUp was missing and a new spot appeared at the origin (Figure 2B). Since the shift in mobility was quantitative compared to the results with un-cross-linked RNA, the counts which remained at the origin are likely to be the result of attachment of one or a few amino acids to the BrUp rather than the result of incomplete digestion of the RNA. Digestion of irradiated but uncomplexed RNA gave only the expected four products, indicating that no photoreduction of BrU to U (Campbell et al., 1974) was occurring under these conditions.

Properties of the Cross-Linking Reaction. The quantum yield of cross-linking was calculated from the data shown in Figure 3 from 308-nm excitation with 2.7 mJ/pulse at 10 Hz. The molar extinction coefficient at 308 nm for the BrU chromophore in BrU–RNA **1** complexed to the coat protein was assumed to be 385 M^{-1} s^{-1}, the same as for 5-bromouridine in pH 7 water. We calculated a quantum yield of cross-linking equal to 0.003 from the measurement which showed 13% cross-linking after 200 s of irradiation of a 1.0-mL sample. In the calculation, we assumed that the probability of a molecule being excited more than once in a single laser pulse was negligible since the probability for the first excitation in a single pulse was only 0.05. We also assumed that cross-linking did not occur via an upper excited state from two-photon laser excitaation since significant photo-cross-linking was observed with excitation with transillumination.

Qualitatively similar results were seen when irradiations were carried out using a broad-spectrum UV transilluminator (maximum at 312 nm) rather than the XeCl excimer laser. The properties of the cross-linking reaction were the same except that an hour of irradiation was required to achieve maximum levels of cross-linking, which were still significantly less than laser yields (Figure 3). Since use of 1-mm-thick polystyrene or 3-mm Pyrex glass reduced the yield of photo-cross-link, most transilluminator experiments were carried out without a filter. Even so, neither debromination nor strand cleavage of the RNA was observed with the broad-spectrum light.

Since the chromophore of the cross-linked product is expected to absorb in the region of 308 nm (Dietz & Koch, 1987), some destruction of the cross-linked species might be expected. This was only seen at high laser power (Figure 3) and was related to the energy input per pulse rather than the frequency of pulsation. Little or no photocleavage of the cross-linked product occurred during 308-nm irradiation at 2.7 mJ/pulse and 20 Hz (not shown). Possibly, at high energy per pulse, the photoproduct undergoes a two-photon cleavage of the cross-link. Since the extinction coefficient of the product chromophore at 308 nm is probably significantly higher than that of the BrU chromophore, the product chromophore has a higher probability for sequential absorption of two photons during a single laser pulse than the BrU chromophore of the starting RNA fragment.

Table I: Binding and Cross-Linking of BrU-Substituted RNA Variants to R17 Coat Protein

variant	K_d (nM)	cross-link
1	1.8	+
2	$>10^4$	–
3	3	+
4	450	±
5	1.5	–
6	300	–

With either light source, the amount of cross-linked product leveled off when significantly less than 100% of the RNA was cross-linked (Figure 3). This was unexpected since the uncomplexed RNA can be isolated and shown to bind coat protein in a nitrocellulose filter binding assay and little evidence of either strand breakage or debromination of the RNA was apparent. To account for the incomplete cross-linking, the possibility of accumulation of protein photodamage was examined. Unexpectedly, irradiation of coat protein in the absence of RNA resulted in the inactivation of coat protein in binding assays. Consistent with this, allowing the RNA and coat protein to dissociate and reassociate under conditions of protein excess between periods of irradiation did not lead to more cross-link formation unless fresh coat protein was added. Thus, the extent of cross-linking appears to be limited by the amount of protein photodamage.

RNA Sequence Requirements for Cross-Linking. Five additional RNA fragments were tested for cross-linking to R17 coat protein (Figure 4). Fragment **2** is very similar to **1**, but cannot bind R17 coat protein because it lacks the two loop-closing base pairs. As would be expected, BrU–RNA **2** did not show detectable binding to coat protein and did not form a cross-link (Table I). This experiment shows that cross-linking requires prior specific binding and is not the result of random collision between RNA and protein.

Fragment **3** has a sequence quite different than **1**, but binds coat protein with a similar K_d (Wu & Uhlenbeck, 1987). When the six U residues in **3** were replaced by BrU, the oligonucleotide bound coat protein tightly and cross-linked to it as well as **1**. The unsubstituted **3** did not cross-link. Fragment **4** is a sequence isomer of **3** where the position of the bulged A residue is changed with respect to the remainder of the molecule. As a result of this change, BrU–RNA **4** has a 150-fold weaker affinity to coat protein. When cross-linking was attempted at 80 nM coat protein, a much lower yield of cross-linked product was obtained. This result is consistent with the lower fraction of complex that would be formed at that protein concentration.

Fragments **5** and **6** were used to test whether photo-cross-linking could occur at any position in the binding site other than the one tested by **1**. Both **5** and **6** lack a U residue at position −5 but are able to bind coat protein when substituted

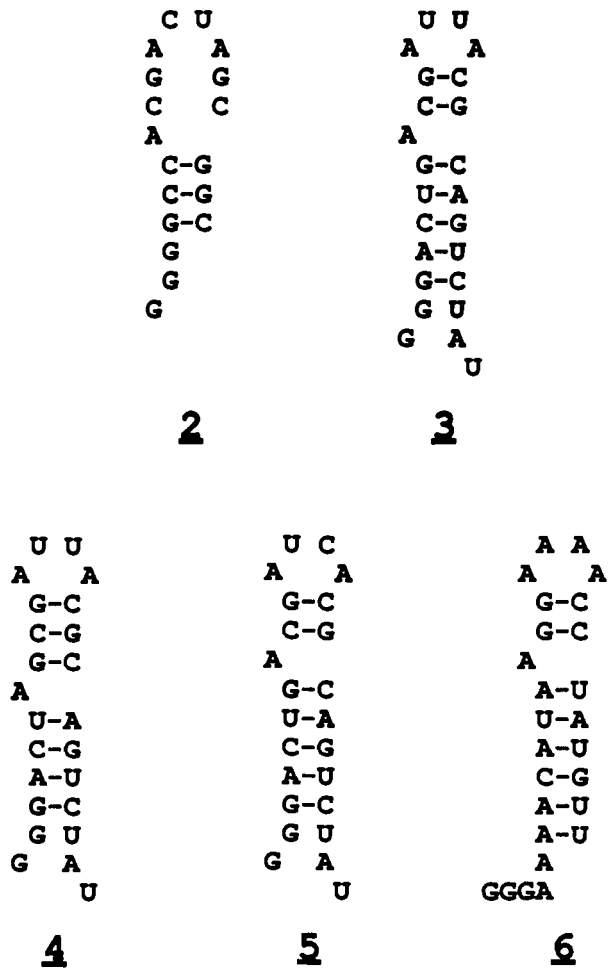

FIGURE 4: Sequence and proposed secondary structures for RNA variants **2**–**6**. All RNAs have 5′-terminal triphosphates.

with BrU (Table I). Quite strikingly, neither fragment showed detectable cross-linking. This indicates that the BrU residues in **5** and **6** are not able to form photo-cross-links. Although this is not as surprising for the BrU residues in helices (positions −12, −1, 2, 4, and 5 in **6** and −12 and +3 in **5**), it is striking that the three single-stranded BrU residues in **5** (positions −6, +5, and +7) do not form cross-links. Of nine positions tested, only RNAs containing a BrU at −5 formed the cross-linked product. Clearly, the position of the BrU in the RNA is critical for cross-link formation.

Discussion

The RNA–protein cross-link demonstrated here is dependent upon prior specific binding of the RNA to coat protein. This is illustrated by the variant RNAs, which have different affinities for the coat protein, and by experiments done at a variety of coat protein concentrations. Similar specificity has been reported for UV cross-linking experiments involving the *lac* repressor and BrdU-substituted *lac* operator (Lin & Riggs, 1974).

The requirement for BrU at position −5 of the RNA hairpin suggests that a precise juxtaposition of the BrU and amino acid side chain is required for cross-linking. The high extent of cross-linking in this system is not unexpected, since it is likely that the residue involved in the cross-link is held in a particular orientation with respect to the BrU in the RNA–protein interaction. Although this has worked to advantage in the R17 system, this will not always be true. Thus, the applicability of the technique to other systems will depend on the molecular details of an individual interaction and must be tested independently.

Studies with model compounds suggest that only a limited number of amino acid side chains are likely to form BrU-related cross-links. BrU has been shown to couple to derivatives of tryptophan when irradiated in organic solvents (Ito et al., 1980). In aqueous solution, cross-linking has been demonstrated to peptide linkages (Dietz et al., 1987), cysteine and glutathione (Varghese, 1974), and peptide-like derivatives of tryptophan, tyrosine, and histidine (Dietz & Koch, 1987). Cross-linking to a peptide-like derivative of cysteine has recently been shown to require prior oxidation of the cysteine to cystine (Dietz & Koch, 1989). Since the coat protein contains no histidines, the most likely sites of cross-linking are its two tryptophans and four tyrosines. The crystal structure of the MS2 phage has recently been determined, and most of the tryptophan and tyrosine residues lie within the protein interior (Valegard et al., 1990). However, Tyr-85 is located near electron density attributed to viral RNA, and Tyr-129 is in a region which may be flexible in the protein dimer, making these residues likely candidates for cross-linking with the BrU at position −5.

The quantum yield of photo-cross-linking, 0.003, is the product of the efficiency of three steps: intersystem crossing to the triplet state, electron transfer from the reactive amino acid side chain to triplet BrU, and cross-link formation from the resulting triplet ion radical pair. Studies of BrU photoreduction to U in 2-propanol solvent estimate the intersystem crossing efficiency of the $^1n,\pi^*$ singlet state to be 0.06. This places the product of the efficiencies of the second and third steps in the range of 0.07. At present, we do not have sufficient information to assign the individual efficiencies for the second and third steps.

While little is known about the photochemistry of BrU in RNA, BrdU-containing DNA is highly susceptible to UV-induced strand breakage (Hutchinson, 1973; Ogata & Gilbert, 1977). The most likely mechanism involves homolysis of the C–Br bond, resulting in the formation of a vinyl radical. This radical can abstract a proton from the 5′ neighboring sugar, leading to strand scission (Hutchinson, 1973). Studies with model compounds have shown that while the $^1\pi,\pi^*$ singlet state of BrU can undergo significant C–Br bond homolysis, the $^1n,\pi^*$ singlet state does not (Dietz et al., 1987). Since the π–π^* and n–π^* transitions have been assigned to the band at 276 nm and the shoulder at 300 ± 10 nm, respectively, we chose to irradiate at longer wavelengths to populate selectively the $^1n,\pi^*$ state. However, Sugiyama et al. (1990) have recently shown that even 308-nm irradiation of specific BrdU-containing duplex DNA oligonucleotides results in debromination and the formation of alkali-labile bonds. Consequently, we have looked for evidence of various types of photodamage. Neither irradiation for 20 min with a monochromatic laser (308 nm) nor irradiation for 3 h with the UV transilluminator (peak at 312 nm) resulted in debromination of the RNA. Strand scission was also negligible, with less than 3% of the RNA degraded at the longest time points. Thus, RNA photodamage does not accumulate to appreciable levels under these conditions.

Given the lack of RNA photodamaage, nearly all of the RNA would be expected to cross-link to the R17 coat protein. Instead, with irradiation at 308 nm, the extent of cross-linking leveled off between 40 and 60% of the input RNA. This was shown to be due to photodamage to the coat protein, a somewhat surprising result since the protein absorbs very weakly at this wavelength. Protein photodamage was also

found to be a problem by Tanner et al. (1988), particularly at shorter wavelengths. This suggests that the lower extent of reaction seen with the transilluminator (20–25%) is the result of faster inactivation of the coat protein by broad-spectrum light. The most photolabile amino acid residue with irradiation in the region of 300 nm is tryptophan. Degradation of tryptophan residues occurs in both aerobic and anaerobic environments, and the efficiency depends on the hydrophobicity of the medium (Pigault & Gerard, 1988), the conformation (Ras et al., 1990), the presence of oxygen (Hibbard et al., 1985), and the neighboring amino acid residues (Tallmadge & Borkman, 1990). The reduced cross-linking seen with irradiated protein may be a consequence of either reduced binding of RNA or a direct effect on the amino acid involved in the cross-link. Higher extents of cross-linking have now been achieved by occasional addition of fresh protein and reequilibration of the sample during irradiation.

ACKNOWLEDGMENTS

We thank Tim Dietz and John Milligan for their preliminary experiment, Stewart Stricker for assistance with the calculation of the quantum yield, and June Gustafson for preparation of the manuscript. We also thank the Keck Foundation for their generous support of RNA science on the Boulder campus.

Registry No. BrU, 957-75-5.

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