Unusual properties of two branched RNA's with circular and linear components

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Received 15 February 1985; Accepted 31 May 1985

ABSTRACT

Irradiation with ultraviolet light was used to create two nonlinear RNA molecules. Circular potato spindle tuber viroid (PSTV) RNA was crosslinked at a single site to generate a figure eight-shaped molecule; 5S rRNA from HeLa cells was transformed into an alpha-shaped molecule with a small circular element and two arms (1). Crosslinked RNA's could be separated from their untreated counterparts by electrophoresis in polyacrylamide gels containing urea. The gel mobility of crosslinked PSTV was not altered by boiling, treatment with *E. coli* RNase III or glyoxalation. However, mild nuclease digestion ("nicking") produced derivatives which migrated more slowly than the starting material in gels of certain polyacrylamide concentrations, but not in others. Limited nuclease digestion of crosslinked 5S rRNA did not generate any detectable products with reduced mobility in the gels tested. Thus, the ability of the "nicking assay" to reveal circular elements within nonlinear RNA's can vary depending upon the composition of the gel chosen for analysis and on the size of the circular element relative to the rest of the molecule.

INTRODUCTION

Anomalous electrophoretic mobility in polyacrylamide gels is a key property distinguishing nonlinear RNA molecules. Viroid RNA's--the first circular RNA's to be discovered (2, 3)--were found to have an apparent molecular weight which varied depending upon the composition of the polyacrylamide gel used for separation (4, 5, 6). Recent studies of eukaryotic mRNA splicing intermediates indicate that some members of this class of RNA molecules also have a nonlinear geometry--i.e., a circular and a linear component attached together in a "lariat-shaped" molecule--and a distinctive set of electrophoretic properties (7, 8, 9). Given the increasing prominence of circular and branched circular RNA's, it is necessary to explore their properties in more detail. A few well-defined and readily available examples of branched RNA's composed of both circular and linear components are needed in order to establish a set of procedures for their detection and purification.

We have used two highly specific ultraviolet light-induced crosslinking reactions [recently discovered in this laboratory (1)] to construct two model

RNA's. By introducing a crosslink into potato spindle tuber viroid (PSTV) RNA at a single site, we produced a molecule shaped like a figure eight. With the same approach, we transformed HeLa cell 5S rRNA into an alpha-shaped molecule. The exact positions of these two UV crosslinking sites are known (1), giving the size and location of each circular and linear portion in these RNA's. In this paper we provide data on the electrophoretic properties of both the PSTV and 5S rRNA crosslinked derivatives either as intact molecules or following various treatments such as heat denaturation or limited nuclease digestion.

MATERIALS AND METHODS

Sources of Nucleic Acids.

PSTV was propagated in tomato plants (Lycopersicon esculentum cv. Rutgers). Plant nucleic acids were extracted by homogenization in phenol/chloroform (10), precipitated in ethanol and viroid RNA purified by gel electrophoresis as described before (10, 11). To prepare large quantities of viroid RNA, aliquots containing 250 μ g (per lane) of the LiCl supernatant fraction of nucleic acids extracted from PSTV-infected plants were separated in 5% non-denaturing polyacrylamide gels containing a Tris-acetate buffer (11), stained with ethidium bromide, exposed to ultraviolet light and the prominent fluorescent band containing viroid RNA cut from the gel. The RNA in the gel slice was then fractionated in a second 5% polyacrylamide gel containing a Tris-borate buffer and 7 M urea to separate circular, crosslinked, and linear forms (12). Sufficient UV-crosslinked RNA was produced by this procedure so that yields as high as 10-20% of circular RNA could be obtained as the crosslinked species. RNA was recovered from ethidium-stained gel bands and purified by chromatography on cellulose CF11 (13) or by chromatography on hydroxylapatite followed by chromatography on cellulose CF11 (14). 32 P-labeled 5S rRNA was prepared from HeLa cells (labeled at a density of 5×10^6 cells/ml) which had been incubated in the presence of 250 μ Ci/ml of 32 P-labeled inorganic phosphate for 2½ hours under conditions described before (15) except that actinomycin D was omitted. Nucleic acids from the cytoplasmic fraction were extracted with chloroform/phenol heated to 65° (16), purified by chromatography on cellulose CF11, precipitated with ethanol, resuspended in water and the 5S rRNA separated from other species by electrophoresis as described in the legend to Figure 8. 32 P-labeled influenza viral RNA's (WSN strain) were synthesized in vitro by incubation of detergent-treated virions in the presence of 32 P-labeled GTP (17) and double-stranded forms selected by

two rounds of chromatography on cellulose CF11 (13).

Two-Dimensional Gel Electrophoresis.

Nucleic acid samples [in 0.0004 M Tris-HCl (pH 7.6), 10% sucrose, and 0.04% bromphenol blue dye] were applied to 5% non-denaturing gels composed of 5% polyacrylamide/0.25% bis acrylamide and a Tris-acetate buffer [0.040 M Tris-acetate (pH 7.2), 0.020 M sodium acetate, 0.001 M EDTA (11)] for separation in the first dimension. Preparative scale gels (3.0 mm thick) were run at 4°; analytical gels (1.5 mm thick), at room temperature. After the bromphenol blue dye marker migrated 6-10 cm, the gel was sliced and individual lanes cast into a second gel containing 0.045 M Trizma base (pH 8.2), 0.044 M boric acid, 0.0014 M EDTA, 7 M urea, 0.04% (w/v) ammonium persulfate and 0.1% (v/v) TEMED and acrylamide/bis acrylamide in the ratio of 19:1. This same gel recipe was used for various one-dimensional analyses, including ultrathin gels 0.3 mm thick. All gels containing urea were run with the accelerating voltage necessary to maintain the temperature at about 55°.

Other Gel Procedures.

For chemical denaturation, nucleic acid samples (containing a minimum of 1.0 µg RNA) were incubated in a 12 µl volume containing 1 M deionized glyoxal, 0.010 M sodium phosphate buffer (pH 7.1), 50% dimethylsulfoxide for 1 hour at 50° (18) and then fractionated by electrophoresis in 5% polyacrylamide gels containing 0.010 M sodium phosphate buffer. Electrophoresis was carried out at 4°, with buffer recirculation.

UV Irradiation.

Nucleic acids (either contained in a non-denaturing gel; dissolved in a drop of Tris-acetate buffer; or in a quartz cuvette) were placed on a protective sheet of Saran Wrap and were usually irradiated using a UV light box (Ultra-Violet Products, Inc. Model TS-15) equipped with 4 shortwave tube bulbs (254 nm peak); however, other UV sources were also effective. When irradiating nucleic acids in a gel slice, the gel strip was covered with a second sheet of Saran Wrap and then a plastic bag filled with ice water was placed on top to prevent heating. Typically, samples contained in a gel slice were irradiated for one minute intervals, alternating with 30 second cooling periods. Nucleic acid samples dissolved in a Tris-acetate buffer were irradiated for 2 minute intervals on a pre-cooled UV light box, alternating with one minute cooling periods during which time the samples were placed on ice.

In Vitro Iodination.

The Commerford reaction for in vitro iodination of RNA (19) was carried out with the modifications described before (20), using carrier-free Na¹²⁵I

(New England Nuclear, Product NEZ 033L).

RESULTS

Preparation of Crosslinked PSTV.

The gel procedure used to identify crosslinked RNA's [similar to the two-dimensional procedure of Schumacher et al. (21)] is illustrated in Figure 1. Nucleic acids of PSTV-infected tomato plants were fractionated in a 5% non-denaturing gel, which was then sliced into individual lanes. Samples to be irradiated were exposed to a standard UV source of the type commonly used to stimulate fluorescence of ethidium-stained nucleic acids and then they were fractionated in a second gel containing 7 M urea. Following UV treatment, a number of new RNA species can be seen, including a cross-linked form of PSTV (identified by an arrow in Fig. 1, panel B) migrating slightly faster in the urea gel than its circular counterpart (marked "PSTV")

Figure 1. Two-Dimensional Gel Electrophoresis of UV Crosslinked RNA's.

Aliquots containing 25 μ g of nucleic acids from the LiCl supernatant fraction purified from PSTV-infected tomato plants were separated in the first dimension by electrophoresis in a 5% polyacrylamide gel run under non-denaturing conditions. After the bromphenol blue dye marker migrated 7-8 cm, the gel was sliced into individual lanes. Gel strips either received no UV exposure or UV irradiation for a total of 4 min under conditions described in Materials and Methods. Samples were then fractionated in the second dimension by electrophoresis in a 5% polyacrylamide gel containing 7 M urea, stained with ethidium bromide and photographed. A control sample is shown in panel A; the positions of circular PSTV and tomato 5S rRNA are identified. UV treated nucleic acids are shown in panel B; the crosslinked forms of PSTV and 5S rRNA are designated by arrows. The pair of horizontal and vertical arrows indicates the directions of first and second dimension separations, respectively.

in Fig. 1, panel A). Qualitatively identical results were obtained when the UV treatment was carried out on RNA's free in solution [see ref. (1), Fig. 1]. To obtain material for subsequent analysis, the crosslinked viroid RNA was recovered from urea gels similar to the one shown here and aliquots were iodinated in vitro (19, 20).

In order to determine the over-all shape of the modified viroid RNA, the position of the crosslink was mapped by a combination of procedures. One-dimensional analysis of RNase T1-resistant oligonucleotides revealed that the crosslinked RNA contains a distinct, new oligonucleotide. Two-dimensional fingerprinting, secondary analysis and primer extension [see ref. (1) for details] indicated that the crosslink joins bases G-98 and U-260 [using the numbering scheme for PSTV established by Gross and co-workers (22)]. Crosslinking of these two bases transforms the PSTV circle [359 bases in circumference (22)] into a figure eight with two circular components of approximately equal size (see Fig. 2).

Stability of the Crosslinked RNA to Heating and Chemical Denaturation.

Before proceeding with gel electrophoretic studies, two kinds of experiments were performed to assess the stability of the crosslinking site and, thus, to determine whether the crosslinked RNA could be used as a test molecule for a wide variety of gel conditions. First, RNA samples were heated in a boiling water bath under conditions leading to the complete denaturation of double-stranded influenza virus RNA. The mobility of the crosslinked RNA was not altered by this treatment (see Fig. 3). Next, the viroid RNA's were chemically denatured by treatment with glyoxal and examined in the electron microscope. The unusual, potato-like morphology of the crosslinked RNA demonstrated its durability (see Fig. 4, panel C).

Figure 2. Geometric Shapes of PSTV Molecules.

Circular PSTV is converted into a molecule with a figure eight shape by UV induction of a crosslink in the central conserved region of the RNA (27, 28, 29). Nuclease cleavage at any one site transforms the crosslinked RNA into a molecule shaped like the Greek letter alpha. Further cleavage produces X-shaped molecules and linear fragments.

Figure 3. Gel Mobility of Crosslinked PSTV RNA Is Not Altered by Heating.

Various RNA's [dissolved in 0.01 M Tris-HCl (pH 7.6), 0.001 M EDTA] were treated as described below and then fractionated in a 0.3 mm thick 5% polyacrylamide gel containing 7 M urea (30). Lanes (a-d) show ^{32}P -labeled influenza virus RNA (initially present as double-stranded RNA; see Materials and Methods); lanes (e-h) represent a mixture of ^{125}I -labeled PSTV circular and linear RNA; lanes (i-l) depict ^{125}I -labeled crosslinked PSTV. Samples (a, e, and i) were kept on ice. Samples (b, f, and j) were denatured by heating in a boiling water bath for 30 sec and then quenched on ice. Samples (c, g, and k) were incubated with RNase III [an enzyme which cleaves double-stranded, but not single-stranded RNA (31)] for 30 min at 37°. Samples (d, h, and l) were denatured by heating, quenched and then incubated with RNase III. "O" marks the origin of electrophoresis; an arrow indicates the position of a xylene cyanol blue dye marker.

Gel Mobility of Crosslinked PSTV.

Gel electrophoresis of the crosslinked PSTV RNA revealed a number of distinctive properties. Although the crosslinked RNA separated from circular and linear forms during electrophoresis in 5% polyacrylamide gels containing 7 M urea (as shown in Figs. 1 and 3), all three forms co-migrated during electrophoresis in 5% non-denaturing polyacrylamide gels (see Fig. 5, panel A). This co-migration presumably results because the three forms all have both the same molecular weight and the same conformation. When the

Figure 4. Electron Micrographs of PSTV RNA's.

Seven and one-half nanograms of PSTV circular (panel A), linear (panel B), or crosslinked (panel C) RNA were denatured in 70% formamide/1 M glyoxal for 1 hour at 54°. The RNA was then mixed with cytochrome c and spread using a formamide hypophase, picked up on Parlodion-coated 200 mesh copper grids, stained with ethanolic uranyl acetate, rotary shadow cast and examined, essentially as described by Kaback and colleagues (32).

native conformation was disrupted, as by treatment with glyoxal, the three forms of PSTV each formed a separate band during electrophoresis in 5% polyacrylamide gels (see Fig. 5, panel B). This result, showing that glyoxalated RNA's do not necessarily migrate according to their molecular weights, illustrates the hazard of using gel electrophoresis of glyoxalated RNA's as the sole means for establishing the size of an unknown RNA.

Response of the Crosslinked RNA to Mild Nuclease Digestion ("Nicking").

Electrophoresis of nuclease- or magnesium-treated RNA's has been used to distinguish circular and linear forms of PSTV (12, 23) and more recently to analyze intermediates of mRNA splicing (7, 8, 9). This simple test for non-linear structure was applied to the crosslinked viroid RNA. ¹²⁵I-labeled circular, linear, and crosslinked PSTV were cleaved at one or more sites by incubation with a small amount of RNase T1 and then fractionated by electrophoresis in a 5% polyacrylamide gel containing 7 M urea. As expected, mild nuclease digestion produced a continuous array of bands (diminishing in size with increasing digestion time) from the sample of linear PSTV. Nicking of circles generated linear molecules and smaller fragments. However, upon nuclease digestion, the crosslinked RNA acquired products which migrated both more slowly and more quickly than the starting material (see Fig. 6). As diagrammed in Figure 2, cleavage of the crosslinked viroid RNA at a single site transforms the figure eight-shaped species into an alpha-shaped mole-

Figure 5. Electrophoresis of Native and Fully Denatured PSTV RNA's.

Panel A. PSTV circular (lane a), crosslinked (lane b) and linear (lane c) RNA's were fractionated by electrophoresis in a 5% non-denaturing gel containing a Tris-acetate buffer (see Materials and Methods), stained with ethidium bromide and photographed. "O" indicates the origin of electrophoresis of the gels in panels A and B. The arrowhead in panel A marks the position of a bromphenol blue dye marker.

Panel B. ^{32}P -labeled viroid RNA's (prepared as described below) were fully denatured by treatment with glyoxal (18) and then separated by electrophoresis in a 5% polyacrylamide gel containing 0.01 M sodium phosphate buffer (pH 7.1). Gel lanes depict the following RNA's: linear citrus exocortis viroid [371 bases in length; ref. (33)] (lane a); linear PSTV (lane b); circular PSTV (lane c); linear PSTV (lane d); crosslinked PSTV (lane e); circular and crosslinked PSTV (lane f); crosslinked and linear PSTV (lane g); crosslinked PSTV (lane h).

The crosslinked PSTV shown in panel B was prepared from 0.50 μg of linear PSTV pre-treated with calf alkaline phosphatase. The RNA was labeled by incubation with 100 μCi of gamma [^{32}P]-ATP and polynucleotide kinase. The 5' end-labeled RNA was circularized by incubation with bacteriophage T4 RNA ligase (P-L Biochemicals). After phenol extraction and CF11 cellulose chromatography (13), the circular RNA was exposed to UV light and the resulting crosslinked derivative purified by gel electrophoresis.

cule. Additional cleavage creates a complex mixture of geometric forms, including X's and linear species of various lengths. The reduced mobility of the alpha and X-shaped RNA's reflects the presence of a branch point and two or more free ends, as also observed using branched DNA molecules (24, 25).

Partial digestion products were examined in a second gel system to determine whether the same results were obtained. As shown in Figure 6 (compare lanes d and e) some of the digestion products of the crosslinked RNA migrate at about the rate of circular PSTV during electrophoresis in 5% polyacrylamide gels. In subsequent experiments, aliquots of the crosslinked RNA

Figure 6. Partial Digestion Products: Circular, Crosslinked or Linear PSTV.

^{125}I -labeled PSTV RNA's were either dissolved in 0.01 M Tris-HCl (pH 7.6), 0.001 M EDTA containing 1 $\mu\text{g}/\mu\text{l}$ tRNA and then kept on ice (lanes a-c) or dissolved in the same buffer containing 5 $\mu\text{g}/\text{ml}$ RNase T1 and 1 $\mu\text{g}/\mu\text{l}$ tRNA and then incubated for 1 min (lanes d-f), 10 min (lanes g-i), or 40 min (lanes j-l) prior to electrophoresis in an 0.3 mm thick polyacrylamide gel containing 7 M urea. Lanes (a, d, g, and j) show a mixed preparation of circular and linear PSTV; lanes (b, e, h, and k) depict crosslinked PSTV, lanes (c, f, i, and l) represent linear PSTV (500 dpm/time point). "0" marks the origin of electrophoresis. An arrow and an arrowhead indicate the positions of xylene cyanol and bromphenol blue, respectively.

were digested with either RNase T1 or nuclease S1 for variable periods of time. Reactions were stopped by the addition of a formamide dye mix, split, and matched samples fractionated by electrophoresis in either a 5% or a 3% polyacrylamide gel (both containing urea). While samples analyzed in the 5% gel had prominent bands migrating in the region of the circular PSTV size marker, those analyzed in the 3% gel did not (see Fig. 7). All digestion products migrated at about the same rate as the starting material (or more quickly) in the 3% gel--results identical to those expected of a linear mole-

Figure 7. 5% or 3% Polyacrylamide Gel Analysis of the Partial Digestion Products of Crosslinked PSTV.

^{125}I -labeled crosslinked PSTV was incubated in the presence of either 5 $\mu\text{g}/\text{ml}$ RNase T1 [in a reaction mixture containing 1 $\mu\text{g}/\text{ul}$ tRNA in 0.010 M Tris-HCl (pH 7.6), 0.001 M EDTA] or 1.00 unit/ μl nuclease S1 [in a reaction mixture containing 1 $\mu\text{g}/\text{ul}$ tRNA in 0.30 M NaCl, 0.030 M sodium acetate, 0.0045 M ZnSO_4 (pH 4.5)] for various times at 37°. Reactions were stopped by the addition of 3 volumes of a dye mix made up of 99% formamide and 0.01% each of bromphenol blue and xylene cyanol. The samples were then divided into equal fractions so that matched aliquots could be analyzed in a 5% (panel A) and a 3% (panel B) polyacrylamide gel containing 7 M urea.

Gel lanes depict the following RNA's: ^{125}I -labeled circular (lane a), linear (lane b), and crosslinked (lane c) PSTV; crosslinked PSTV incubated with RNase T1 for 1 min (lane d), 5 min (lane e), 10 min (lane f), or 40 min (lane g); crosslinked PSTV incubated with nuclease S1 for 1 min (lane h), 5 min (lane i), 10 min (lane j), or 40 min (lane k). Arrows mark the origins of electrophoresis, while arrowheads indicate the positions of the xylene cyanol blue dye markers.

cule. Thus, when seeking evidence of unusual structure by gel electrophoresis of partial digestion products, it may be necessary to assay the products in a number of different systems.

Purification of HeLa 5S rRNA Containing a UV-Induced Crosslink.

Preliminary studies using nucleic acids of tomato plants indicated that UV irradiation produces a crosslinked derivative of tomato 5S rRNA (see

Figure 8. Purification of Control and UV Crosslinked 5S rRNA from HeLa Cells.

³²P-labeled nucleic acids were extracted from the cytoplasmic fraction of HeLa cells after a 2½ hour incubation of the cells in the presence of 250 µCi of [³²P]-inorganic phosphate per ml as described before (15, 16). Aliquots (about 100 µg containing 6.6 x 10⁷ dpm) were then fractionated by electrophoresis in a 3.0 mm thick 5% non-denaturing gel. Gel bands containing 5S rRNA (identified in panel A) were excised and either stored on ice (to provide a control sample) or exposed to ultraviolet light for a total of 8 minutes prior to electrophoresis in a second gel composed of 10% polyacrylamide, 7 M urea and a Tris-borate buffer. Control 5S rRNA (marked by the arrow in panel B, lane a) was eluted in parallel with UV crosslinked 5S rRNA (marked by the arrow in panel B, lane b), using procedures described before (14). Eluted RNA's were purified by cellulose CF11 chromatography (13), precipitated with ethanol and 10 µg tRNA and then fractionated in an 0.3 mm thick 12% polyacrylamide gel containing 7 M urea (panel C). Highly purified 5S rRNA (panel C, lane a) and its crosslinked derivative (panel C, lane b) were recovered from the positions indicated by the arrows. For panels A-C, the notations at the side of the gels were used to align autoradiograms for accurate excision of gel bands.

Fig. 1), suggesting that 5S rRNA from other eukaryotes might also be susceptible to UV crosslinking. ³²P-labeled 5S rRNA from HeLa cells was used to test this possibility because it is relatively easy to purify and has been studied extensively (26). To purify crosslinked 5S rRNA, ³²P-labeled nucleic acids, extracted from HeLa cells using phenol/chloroform (15, 16), were fractionated in 5% non-denaturing gels. The position of 5S rRNA was determined by autoradiography (Fig. 8, panel A). To generate crosslinked 5S rRNA, several gel bands containing 5S rRNA were exposed to UV light. Then they were cast into a second gel (composed of 10% polyacrylamide and 7 M urea) to separate the crosslinked and uncrosslinked forms of 5S rRNA. Control 5S rRNA, which was not exposed to UV light at any point, was prepared in parallel.

Figure 9. Partial Digestion Products of Crosslinked 5S rRNA Fractionated in Either a 12% or an 8% Polyacrylamide Gel.

³²P-labeled 5S rRNA and its crosslinked derivative were incubated in the presence of either 0.020 or 2.00 µg/ml RNase T1 [in reaction mixtures containing 2 µg/µl tRNA in 0.010 M Tris-HCl (pH 7.6), 0.001 M EDTA] for various times at 37°. Reactions were stopped by the addition of 3 volumes of a pre-chilled dye mix. Samples were divided and matched aliquots analyzed in either a 12% (panel A) or an 8% (panel B) polyacrylamide gel containing 7 M urea. Cross-linked 5S rRNA is shown in lanes (a-g) following incubation in buffer alone (lanes a and g) or after digestion by 0.020 µg/ml RNase T1 [for 1 min (lane b), 5 min (lane c), or 20 min (lane d)] or after digestion by 2.00 µg/ml RNase T1 [for 30 sec (lane e) or 8 min (lane f)]. Control 5S rRNA is depicted in lanes (h-m) following incubation in buffer alone (lane h) or after digestion by 0.020 µg/ml RNase T1 [for 1 min (lane i), 5 min (lane j), or 20 min (lane k)] or after digestion by 2.00 µg/ml RNase T1 [for 30 sec (lane l) or 8 min (lane m)]. "O" indicates the origin of electrophoresis; an arrow the position of xylene cyanol blue dye; an arrowhead the position of bromphenol blue dye.

Irradiated samples contained an additional major band not present in control samples (Fig. 8, panel B). However, preliminary fingerprint analysis showed that the crosslinked form of 5S rRNA co-migrates in the 10% gel with a minor RNA present in both control and irradiated samples. This contaminant has a distinctive fingerprint which is not related to that of 5S rRNA. It separated from crosslinked 5S rRNA during electrophoresis in ultrathin 12% polyacrylamide gels containing 7 M urea (Fig. 8, panel C). Highly purified crosslinked and control 5S rRNA's were eluted from 12% gels and the position of the crosslink was mapped by RNA fingerprinting and secondary analysis. The crosslink in HeLa 5S rRNA was found to join bases U-76 and G-99 [see ref. (1)]

for details]. Thus, the linear 5S rRNA is transformed by UV-induced cross-linking into an alpha-shaped molecule with a 24-base circular element flanked by linear regions of 75 bases (on the 5' side) and 21 bases (on the 3' side). Electrophoresis of Crosslinked 5S rRNA following Mild Nuclease Digestion.

Mild nuclease digestion of the crosslinked 5S rRNA did not generate any detectable bands migrating more slowly than the starting material when assayed in either a 12% or an 8% gel (see Fig. 9). The absence of any prominent digestion products migrating more slowly than the starting material probably reflects the small target size presented by the circular element, which constitutes only about 15% of the total length of crosslinked 5S rRNA. However, the pattern of bands produced by partial digestion of 5S rRNA and its crosslinked derivative differed in both distribution and clarity. Products of the crosslinked RNA formed fuzzy bands in several regions of both gels.

DISCUSSION

UV irradiation was used to generate two novel RNA's of known geometry. One crosslinked RNA, derived from PSTV, had the shape of a figure eight. The other, derived from HeLa 5S rRNA, was shaped like an alpha. Thus, both molecules contain at least one circular element and a branch point. They were analyzed in a number of gel systems in order to determine the effect of their unusual geometry on a number of assays commonly used to characterize RNA molecules.

The crosslinked viroid RNA separated from circular and linear forms during electrophoresis in 3% and 5% polyacrylamide gels containing 7 M urea and during electrophoresis as a glyoxalated RNA; however, it co-migrated with the other forms of PSTV during electrophoresis in non-denaturing gels. Mild nuclease treatment of crosslinked PSTV produced partial digestion products which migrated more slowly than the starting material when analyzed in a 5% polyacrylamide gel containing urea, but such unconventional products were not evident when these same products were analyzed in a 3% gel of the same type. Furthermore, no species migrating more slowly than the starting material were detected in digestion products of the crosslinked 5S rRNA, although two different gel systems were used for this analysis. Thus, results concerning partial digestion products are highly variable and may depend on the composition of the gel chosen for analysis or on the size and nuclease sensitivity of the circular element relative to the rest of the molecule.

While the detection of partial digestion products migrating more slowly

than the starting material provides strong evidence of unusual structure, the failure to detect slow moving digestion products does not rule out the possibility of unusual geometry. However, if left undetected, an exotic structural element could cause misinterpretation of data since, as noted above, it may alter gel mobility even following treatment with glyoxal. To ensure detection of branch points, gel electrophoresis can be coupled with other procedures, such as electron microscopy, one- or two-dimensional oligonucleotide analysis, or primer extension (1, 7, 8, 9).

The crosslinks studied here were introduced into the RNA's with great ease. In fact, crosslinked forms of both PSTV and 5S rRNA were unavoidably produced at low levels by use of our standard equipment for detecting ethidium bromide-stained gel bands. Thus, RNA exposed to UV irradiation at any point during its preparation could contain unusual structures created as artifacts of the preparative procedure. To avoid artifactual branch points, it may be useful to prepare some samples of any RNA under investigation for nonlinear geometry using detection methods [e.g., autoradiography; toluidine blue staining (11)] not involving exposure to ultraviolet light.

In a parallel study to this one (1), we demonstrated that the sequence and secondary structure surrounding the ultraviolet light-induced crosslinks are nearly identical in PSTV and HeLa cell 5S rRNA's, suggesting that the crosslinks form in a characteristic area of previously undetected local tertiary structure. Other RNA molecules with similar structural elements have been identified and their physical properties and functional significance are under investigation.

ACKNOWLEDGEMENTS

We thank Kerry K. Willis and J. F. Shaw for outstanding technical assistance and Drs. Sidney Altman, Charles C. Cantor, David B. Kaback, Richard J. Roberts, and Olke C. Uhlenbeck for helpful discussions and suggestions; and Dr. David Kaback for generous help with, and providing facilities for, electron microscopy. This work was supported in part by NSF grant PCM 83-03943 to HDR and ADB; and by a McKnight Foundation Individual Award in Plant Biology, NSF grant PCM 82-08406, and NIH grant GM-28294 to HDR.

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