Extensive Regions of Single-Stranded DNA in Aphidicolin-Treated Melanoma Cells†

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ABSTRACT: We have looked for the presence of single-stranded DNA in human melanoma cells. Single-stranded DNA was observed by lysis of cells in dilute alkali (to partly denature the DNA) followed by CsCl gradient centrifugations. In normally growing cells we did not observe single-stranded DNA whereas large amounts were present in cells treated with aphidicolin (an inhibitor of DNA polymerase α). The single-stranded DNA is much larger (>20 kb) than Okazaki fragments. When the cells were washed free of aphidicolin, the single-stranded DNA was converted to high molecular weight DNA. Furthermore, when DNA synthesis is recovering after drug treatment, the single-stranded DNA disappears. The single-stranded DNA represents a transient step during the maturation of newly synthesized DNA.

DNA replication is very complex and is usually believed to involve the initiation of synthesis, the formation of replication forks which proceed to elongate, and finally the joining of adjacent replicons (Campbell, 1986; Taylor, 1984). There exist different results regarding the increasing size of nascent DNA, with a discontinous increase in primary cultures of mouse embryo cells (Kowalski & Cheevers, 1976) whereas there is a linear increase in C3H 10T1/2 cells (Cordeiro et al., 1985). Also in some systems one can detect long-lived intermediates (Funderud et al., 1978; Lönn & Lönn, 1985a) that do not appear in other cells (Cordeiro et al., 1985).

So far most interest has focused on the DNA replication fork and the possible presence of specific initiation points (Campbell, 1986; Mechali & Kearsy, 1984; Cusick et al., 1984). However, recently also another mechanism to replicate chromosomal DNA has been suggested in which replication forks are not formed (the strand-separation hypothesis) (Gaudette & Benbow, 1986). This hypothesis suggests that replication may begin with DNA duplex destabilization and strand separation. The resulting single-stranded DNA may then serve as substrate for the DNA polymerase-primase complex.

We wished to examine the presence in replicating mammalian cells of single-stranded DNA not appearing as traditional DNA replication intermediates. To examine this question one can manipulate the system using the drug aphidicolin—an inhibitor of DNA polymerase α (Huberman, 1981). We have earlier shown that in cells treated with aphidicolin no new DNA replication intermediates are detected but joining of already formed large intermediates can occur (Lönn & Lönn, 1983). Aphidicolin therefore reduces the number of replication forks but may still allow the presence of single-stranded DNA as required by the strand-separation hypothesis.

In this paper we have looked for the presence of single-stranded DNA in aphidicolin-treated cells. To reveal the single-stranded DNA, we lyse cells in dilute alkali and then separate the DNA in CsCl gradients. The results show that a large fraction of DNA is single-stranded and represents a transient step during the maturation of new DNA.

Materials and Methods

Cells and Labeling Conditions. Human melanoma cells were grown at 37 °C in 5% CO_2 in air as described earlier (Lönn, 1982). The culture medium was Eagle's MEM with Earle's salts, containing 2 mM L-glutamine, 10% fetal calf serum, and antibiotics. Cells to be used in experiments were seeded in small Petri dishes (35 × 10 mm) with 3 mL of medium.

The cells were treated with aphidicolin (10 μg/mL) for 60 min. Labeling of the DNA was performed by addition of 50 μCi of [^{3}H]thymidine (25 Ci/mmol; Amersham Inc.) to the culture medium during the last 30 min of drug treatment. As a density label we have added bromodeoxyuridine (BrdU) (3 μg/mL) with the [^{3}H]thymidine.

Cell Lysis. To lyse cells in dilute alkali, the medium was drained from the Petri dish, and 2.25 mL of 0.03 M NaOH was added (Ahnström & Erixon, 1981; Lönn & Lönn, 1986). After 30 min at 0 °C in the dark, the solution was neutralized by the addition of 0.9 mL of 0.067 M HCl/0.02 M NaH_2PO_4; 1-mL samples were then sonicated for 3 × 15 s in a Branson sonifier equipped with a microtip set at maximum power. Finally, sarkosyl was added to 1% final concentration, and density gradient analysis was performed. Alternatively, the sample was treated with nuclease S_1 (see below) before analysis. The samples to be analyzed by gel electrophoresis were lysed according to the same procedure with the omission of the sonication step.

For cell lysis at neutral pH the cells were scraped off from the Petri dish with a rubber policeman and suspended in 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8, containing 0.001 M ethylenediaminetetraacetic acid (EDTA). The sample was then sonicated and the detergent finally added.

Treatment with Nuclease S_1. Treatment with nuclease S_1 was performed immediately after sonication of the sample (1 mL); 100 μL of 300 mM NaOAc, pH 4.6, 0.5 mM ZnOAc, and 750 mM NaCl was then added together with 200 IU/mL nuclease S_1 (Sigma Chemical Co.) and the mixture incubated for 30 min at 37 °C. The digestion was stopped by the addition of detergent and EDTA (Lönn & Lönn, 1986).

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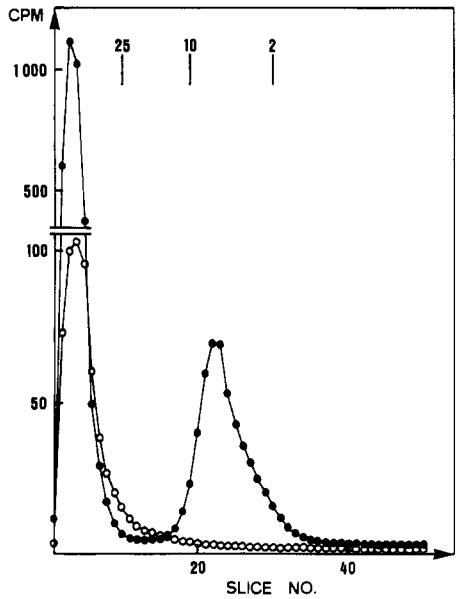

FIGURE 1: Gel electrophoretic separations of DNA from cells labeled with [^{3}H]thymidine for 30 min (●) or cells treated with aphidicolin for 60 min and labeled with thymidine during the last 30 min (○). The cells were lysed in dilute alkali, and the DNA was separated in 0.75% agarose gels. Double-stranded high molecular weight DNA is located at slices 3–6 and single-stranded 10-kb DNA at slices 20–24. 25, 10, and 2 denote the size (in kilobases) and location of single-stranded DNA markers.

Gradient Centrifugations and Gel Electrophoresis. The CsCl gradient centrifugations were performed in a Beckman L2-65B ultracentrifuge run at 38 000 rpm at 23 °C for 40 h with a Ti 50 rotor. CsCl was added to the solution containing DNA (0.01 M Tris, pH 8, 0.001 M EDTA) to give a refractive index of 1.3990. After centrifugation the gradients were divided into 30–40 fractions, trichloroacetic acid (TCA) was added to each fraction, and the labeled DNA was collected on Whatman GF/C filters. The radioactivity was measured in a toluene-based scintillation fluid containing 3% Soluene 100, on a Packard scintillation counter.

The 0.75% agarose gel electrophoresis were performed as described (Lönn & Lönn, 1986) in an LKB Multiphor electrophoretic unit. The gels were sliced into 1 mm thick slices, and the radioactivity was measured in a Packard scintillation counter.

RESULTS

Gel Electrophoretic Analysis. To examine DNA synthesis, we have developed a procedure to lyse cells in dilute alkali (0.03 M NaOH). The treatment in dilute alkali results in a partial denaturation of DNA: The denaturation is initiated at gaps and/or alkali-sensitive regions present in the DNA, e.g., at replication forks. After neutralization of the alkaline solution there is a renaturation of the large DNA (>20 kb), which then appears as double-stranded DNA in neutral CsCl gradients. The DNA fragments (<20 kb) remain, however, as single-stranded DNA fragments (Erixon, 1980; Ahnström & Erixon, 1981; Lönn, 1982; Lönn & Lönn, 1986). The approach can be visualized as a selective melting of replicons to facilitate the analysis of DNA replication.

Figure 1 shows the gel electrophoretic analysis of DNA from cells treated with aphidicolin for 60 min and pulsed with [^{3}H]thymidine during the last 30 min. The separation shows a low level of high molecular weight DNA. In comparison, cells incubated with thymidine alone show, apart from high molecular weight DNA, also a peak of 10-kb DNA replication intermediates. Furthermore when the cells are washed free of aphidicolin and incubated in fresh medium for 24 h, one can detect only high molecular weight DNA.

The data confirm our earlier data that aphidicolin largely, but not completely, prevents the incorporation of label into DNA. Although there are no detectable DNA replication intermediates, a small residual incorporation amounting to 5–10% of untreated cells can be detected (Lönn & Lönn, 1983). This high molecular weight DNA can be used as an analytical tool to examine whether there exist also in mammalian cells extensive regions of single-stranded DNA during DNA synthesis. The labeling of the DNA occurs during a pulse for 30 min. However since the incorporation of label occurs in a high molecular weight DNA, the size characteristics of this labeled DNA mimic those of parental DNA.

Control Experiments: Density Gradient Analysis of DNA from Cells Lysed at Neutral pH. Melanoma cells were incubated with [^{3}H]thymidine for 30 min or with aphidicolin for 60 min with [^{3}H]thymidine present during the last 30 min. In another set of experiments, BrdU was also present with the [^{3}H]thymidine. When the incubations had been terminated, the cells were scraped off from the Petri dish and sonicated, and the detergent was added. The DNA was then banded in neutral CsCl gradients.

The results obtained with cells incubated in the absence of BrdU are shown in Figure 2A. The labeled material bands as unsubstituted double-stranded DNA irrespective of whether the incubations were performed in the presence or absence of aphidicolin.

In cells incubated in the presence of BrdU there is a density shift in DNA as compared with cells incubated with [^{3}H]-thymidine only (Figure 2B). In cells preincubated with aphidicolin there is, however, no density shift. The labeled DNA of these cells appears as unsubstituted DNA. The low level of incorporation of BrdU in these cells does not result in a density shift.

As pointed out in the section about gel electrophoresis, the DNA labeled in the presence of aphidicolin has the size characteristics of parental DNA. The results of Figure 2B furthermore agree with the interpretation that the incorporation of label is due to a DNA repair mechanism. If the DNA is damaged by, e.g., X-irradiation, the incorporation of label in this peak increases due to DNA repair synthesis (Lönn & Lönn, 1985b). The DNA of the cells is, however, not experimentally damaged. Therefore, one has to look for other explanations, e.g., the possibility that the incorporation of label is due to a mechanism as that described for the strand-separation hypothesis.

Density Gradient Analysis of DNA from Cells Lysed in Dilute Alkali. We next wished to examine whether a partial denaturation of the DNA may reveal a single-stranded DNA with characteristics other than those of traditional DNA replication intermediates. Therefore, the cells were lysed in dilute alkali (0.03 M NaOH; 0 °C; 30 min) prior to sonication and CsCl gradient centrifugation.

The cells are incubated with [^{3}H]thymidine for 30 min or treated with aphidicolin for 60 min with thymidine present during the last 30 min. One set of cells is incubated with BrdU present together with the [^{3}H]thymidine. When cells are incubated in the absence of BrdU, the results show banding in CsCl gradients as double-stranded DNA irrespective whether the incubation is performed in the presence or absence of aphidicolin (not shown).

In cells incubated in the presence of BrdU there is some material showing a small density shift and also some material banding at the bottom of the gradient (Figure 3A).

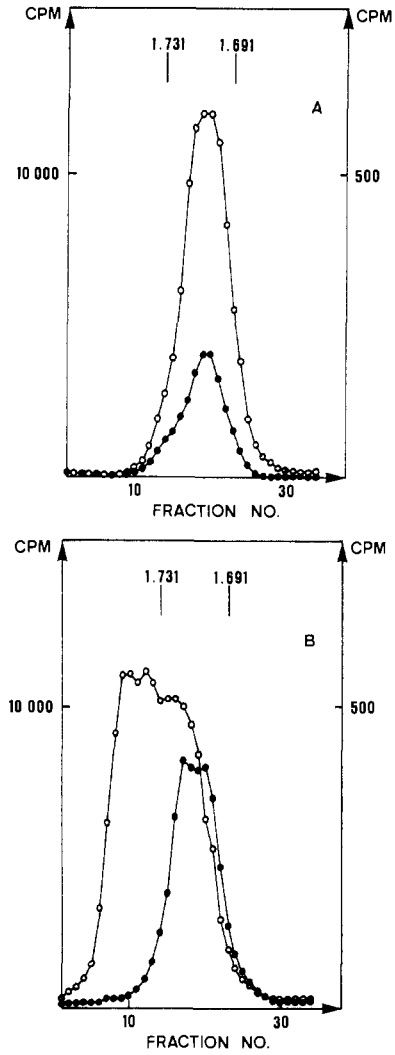

FIGURE 2: (A) Density gradient analysis of DNA from cells lysed at neutral pH. The cells were incubated with [^{3}H]thymidine (○) for 30 min or aphidicolin (10 μg/mL) for 60 min with [^{3}H]thymidine present during the last 30 min (●). After cell lysis and sonication the DNA was banded in neutral CsCl gradients. 1.731 and 1.691 denote the location and density (g/mL) of double-stranded DNA markers. The scale to the left refers to (○) whereas the scale to the right refers to (●). (B) The same protocol as in (A) with the difference that BrdU was added together with the [^{3}H]thymidine.

Next, we examined samples that had been treated with nuclease S_1 after the sonication (Figure 3B). This enzyme digests single-stranded DNA but not double-stranded DNA (Ando, 1966). In cells not treated with aphidicolin the enzyme digestion does not induce any change in the banding pattern. In contrast, in cells incubated with aphidicolin the material banding at the bottom of the gradient is removed by the digestion (Figure 3B). Hence, this material is single-stranded DNA. Remaining in the gradient is double-stranded DNA banding with a small density shift.

Figure 3C shows a control experiment with cells labeled with [^{3}H]thymidine and BrdU and where aphidicolin is added to the cell lysate prior to centrifugation. The results show no additional appearance of single-stranded DNA, ruling out the possibility of a nonenzymatic interaction of aphidicolin and nascent DNA.

Next we examined cells labeled in the presence of aphidicolin and then incubated in fresh medium for 60 min or 24 h, before the cells were lysed in dilute alkali, and the DNA was then separated in the gradient. Figure 4 shows that when the cells have been incubated in fresh medium for 60 min, the

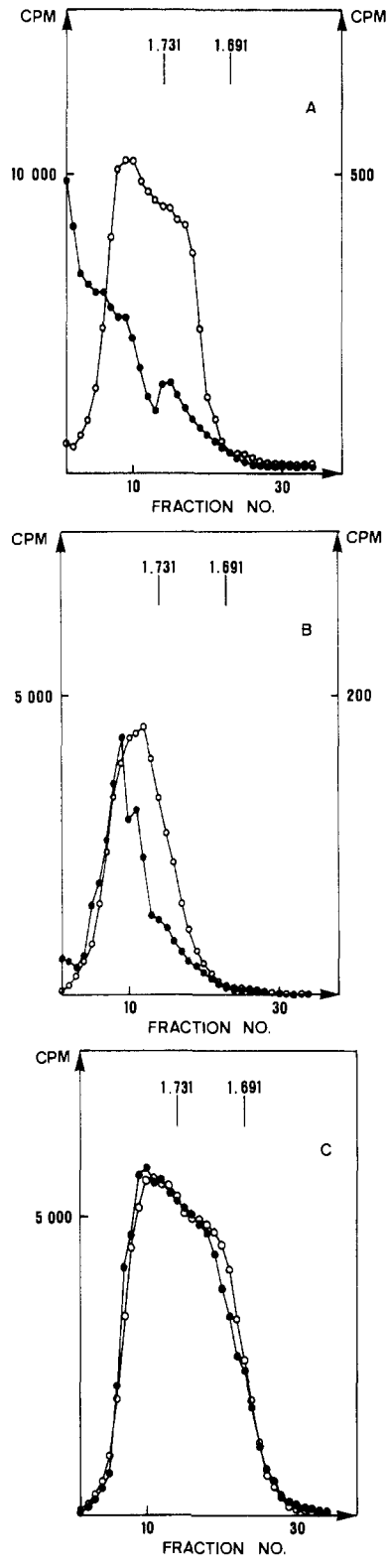

FIGURE 3: (A) Density gradient analysis of DNA from cells lysed in dilute alkali. The cells were incubated with [^{3}H]thymidine and BrdU for 30 min (○) or with aphidicolin (10 μg/mL) for 60 min and [^{3}H]thymidine and BrdU present during the last 30 min (●). After cell lysis and sonication the DNA was banded in neutral CsCl gradients. 1.731 and 1.691 denote the location and density (g/mL) of double-stranded DNA markers. The scale to the left refers to (○) whereas the scale to the right refers to (●). (B) The same protocol as in (A) but the sample was treated with nucleases S_1. The scale to the left refers to (○) whereas the scale to the right refers to (●). (C) Cells incubated with [^{3}H]thymidine and BrdU for 30 min. The cell lysate was divided into two parts. To one part was added aphidicolin (10 μg/mL) (●) but not to the other part (○). The DNA was then sonicated and banded in CsCl gradients.

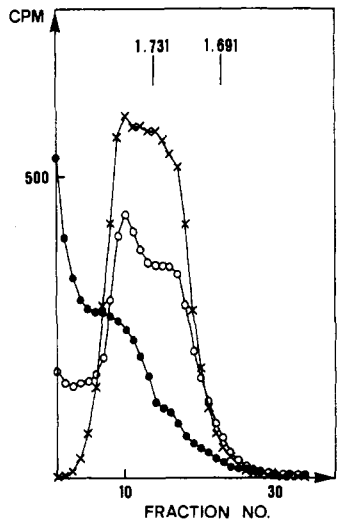

FIGURE 4: Density gradient analysis of DNA from cells lysed in dilute alkali. The cells were incubated with aphidicolin (10 μg/mL) for 60 min with added [^{3}H]thymidine and BrdU during the last 30 min. The cells were either immediately lysed (●) or lysed after incubation in fresh medium for 60 min (○) or 24 h (×). After cell lysis and sonication the DNA was banded in neutral CsCl gradients. 1.731 and 1.691 denote the location and density (g/mL) of double-stranded DNA markers.

amount of single-stranded DNA is reduced, and at 24 h we could not detect any of the single-stranded DNA. One can detect only the double-stranded DNA. Hence, the labeled single-stranded DNA shows a transient appearance.

Recovery of DNA Synthesis. To examine the recovery of DNA after treatment with aphidicolin, cells were incubated in the following way: Cells were treated with aphidicolin for 60 min, washed free of drug, and then incubated for 15 min with [^{3}H]thymidine and BrdU. Alternatively, the cells were incubated for 60 min in fresh medium before the incubation with [^{3}H]thymidine and BrdU.

After cell lysis in dilute alkali, half of the sample was treated with nuclease S_1 whereas the other half was not. Separation of the DNA was performed in CsCl (Figure 5). In cells examined 15 min after termination of the drug treatment, single-stranded DNA is detected. The picture is very similar to that obtained during drug treatment. In cells incubated for 60 min in fresh medium, the results differ however. The single-stranded DNA has now largely disappeared. The separation now shows mainly double-stranded DNA.

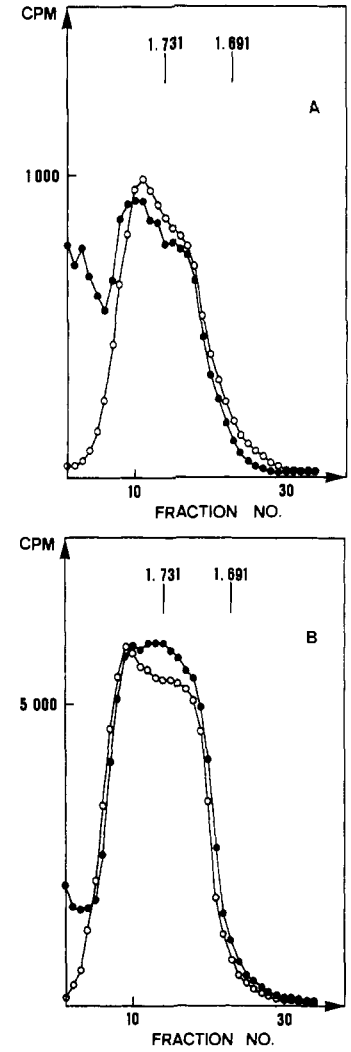

FIGURE 5: Recovery of DNA synthesis. Cells were treated with aphidicolin (10 μg/mL) for 60 min and then either immediately incubated for 15 min with [^{3}H]thymidine and BrdU (A) or incubated with thymidine and BrdU after incubation of the cells in fresh medium for 60 min (B). After cell lysis and sonication, half of the sample was treated with nuclease S_1 (○), and the other half was not (●). The samples were then separated in neutral CsCl gradients. 1.731 and 1.691 denote the location and density (g/mL) of double-stranded DNA markers.

Discussion

In this paper we have examined DNA labeled in cells treated with aphidicolin. The label appears in a high molecular weight DNA, i.e., DNA with the size of parental DNA. To examine the DNA, we use a procedure to lyse cells in dilute alkali to induce a partial DNA denaturation. During the uncoiling of DNA, single-stranded DNA replication intermediates smaller than 20 kb are released. The remaining DNA appears as double-stranded high molecular weight DNA (Erixon, 1980; Ahnström & Erixon, 1981; Lönn & Lönn, 1986). The DNA labeled during aphidicolin treatment is part of the high molecular weight DNA.

When human melanoma cells, grown in medium with density label, are incubated with aphidicolin and lysed at neutral pH, a density shift in the DNA cannot be detected. However, if cells from the same sample are lysed in dilute alkali, we can detect a slight density shift of double-stranded DNA. Furthermore, there appears now also another DNA population that sediments at the bottom of the gradient. This DNA population is sensitive to nuclease S_1 and therefore comprises single-stranded DNA. When the cells are washed free of aphidicolin and then incubated in fresh medium, the single-stranded DNA disappears. Hence, this DNA shows a transient appearance. The gel electrophoretic separations show that this DNA appears as high molecular weight DNA both immediately after the labeling period and after incubation of the cells in fresh medium. Furthermore when cells recovering from aphidicolin treatment are analyzed, one can detect initially single-stranded DNA that is not detectable when the cells are analyzed 60 min after drug treatment. Hence, this single-stranded DNA shows a transient appearance although it is part of what is usually called parental DNA.

Mitochondrial DNA synthesis proceeds uninterrupted in the presence of aphidicolin. Mitochondrial DNA is synthesized by a strand displacement mechanism via intermediates that range from several hundred nucleotides to 15 kb, the unit length of mammalian mitochondrial DNA (Clayton, 1982). In our experiments, the gel electrophoretic analysis of aphidicolin-treated cells shows very few, if any, labeled DNA molecules of this size. Furthermore when the DNA labeled in aphidicolin-treated cells is postincubated in fresh medium for 24 h, one can detect only high molecular weight DNA and

no 15-kb DNA (mitochondrial size). Therefore, it is very unlikely that the transient single-standed DNA represents mitochondrial DNA.

Earlier when we examined cells treated with aphidicolin, we interpreted the incorporation of label as due to events occurring during the joining of preformed large DNA replication intermediates and the joining of adjacent replicons (Lönn & Lönn, 1983). This may still be one explanation, but the present data open also another possibility, i.e., the occurrence of the strand separation in human cells (Gaudette & Benbow, 1986).

There exist high levels of single-stranded DNA during DNA synthesis of *Xenopus laevis* embryos. In these cells the number of replication forks is underrepresented whereas single-stranded DNA is abundant. This had led Gaudette and Benbow (1986) to propose an alternative mechanism of DNA replication which does not involve formation of replication forks (the strand-separation hypothesis). According to this hypothesis, the DNA strands separate, and the resulting single-stranded DNA chains serve as substrate for attachment of enzymes necessary for DNA synthesis. The attachment does not necessarily occur at the fork at each end of the region that has separated but anywhere along the single-stranded DNA. This mechanism would allow rapid DNA synthesis. This hypothesis is supported by the finding that replication forks are underrepresented in *X. laevis* embryos. Instead, single-stranded DNA is abundant. The amount of single-stranded DNA was inversely correlated with the length of S phase during embryogenesis.

In this paper we have shown that in cells treated with aphidicolin one can detect single-stranded DNA. This supports the strand-separation hypothesis for DNA synthesis, indicating that this mechanism may also exist in human cells. Also, a mechanism for strand separation must exist that is independent of functional DNA polymerase α.

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Structure of M1 RNA As Determined by Psoralen Cross-Linking†

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ABSTRACT: The RNA moiety of ribonuclease P from *Escherichia coli* (M1 RNA) has been photoreacted with 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT) and long-wave UV light (320–380 nm) in a buffer containing 60 mM Mg^{2+}, where the RNA moiety acts as a true catalyst of tRNA processing. Limited specific digestion and two-dimensional gel electrophoresis yield fragments cross-linked by HMT. By photoreversal of the isolated cross-linked fragments and enzymatic sequencing of the fragments, the positions of the cross-links have been elucidated. This method allows us to locate the cross-link to ±15 nucleotides. Further assignments of the exact locations of the cross-links have been made on the basis of the known photoreactivity of the psoralen with different bases. Nine unique cross-links have been isolated in the M1 RNA including four long-range interactions. The short-range interactions are discussed here in detail.

Maturation of biologically active tRNA[1] in both eucaryotes and procaryotes involves the processing of a long RNA transcript. This processing includes both the cleavage of the RNA transcript at specific sites and chemical modification of specific bases. RNase P from *Escherichia coli* is a ribonucleoprotein complex that cleaves tRNA transcripts specifically at the 5′ end of the tRNA molecule. The structure of the RNA of this ribonucleoprotein complex (M1 RNA) is of great interest

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[1] Abbreviations: A, adenine; ATP, adenosine 5′-triphosphate; C, cytidine; EDTA, ethylenediaminetetraacetic acid; G, guanidine; HMT, 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen; RNA, ribonucleic acid; RNase, ribonuclease; Tris, tris(hydroxymethyl)aminomethane; tRNA, transfer ribonucleic acid; U, uracil.

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