RNA–DNA Hybrids Containing Damaged DNA are Substrates for RNase H

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Abstract—During the replication of the lagging strand, RNA–DNA hybrids are formed and the RNA is subsequently degraded by the action of RNase H. Little is known about the effects of damaged DNA on lagging strand replication and subsequent RNA removal. The rates and sites of digestion by *E. coli* RNase H of RNA–DNA hybrids containing either a thymine glycol or urea site in the DNA strand have been examined. The cleavage patterns for duplexes containing thymine glycol or urea differ from that of a fully complementary duplex. There is one major product of the digestion of the fully complementary hybrid, but three products are formed in the reactions with the hybrids containing damaged DNAs. Cleavage is partially redirected to the position adjacent to the damaged sites. The overall rate of cleavage of these hybrids containing damaged DNA is comparable to that of the fully complementary duplex. These results indicate that the cleavage of RNA–DNA hybrids by RNase H is less selective when a damaged site is present in the DNA strand. © 2001 Elsevier Science Ltd. All rights reserved.

Damaged DNA is present in all cells and can lead to stable mutations, cell sickness, or cell death.[1] The presence of DNA damage in the leading strand cannot only lead to stable mutations, but can also cause pauses or stops in replication.[1] However, little is known about the effects of damaged DNA on lagging strand replication. In contrast to leading strand synthesis, lagging strand synthesis is forced to occur in a discontinuous process because DNA is synthesized exclusively in the 5′ to 3′ direction. Replication of the lagging strand requires not only the components to synthesize DNA, but also those to make and remove the RNA primers and to ligate the Okazaki fragments. Therefore, a damaged site in the replication fork on the lagging strand will be a potential substrate not only for DNA polymerases, but also for primase, RNase H, flap endonuclease 1 (Fen 1), and ligase. If primase recognizes a damaged DNA site, the resulting RNA–DNA hybrid will contain that damaged site. The removal of the RNA initiators made by primase occurs by a two-step process in which RNase H acts to cleave the RNA leaving a single RNA nucleotide extension.[2–6] This RNA nucleotide is subsequently cleaved by Fen 1 and the resulting gap is filled in by DNA polymerases and sealed by DNA ligase.[3–6]

Damaged sites could affect removal of RNA primers from RNA–DNA hybrids. Since RNase H interacts with the RNA–DNA hybrid downstream of the cutting site, a damaged site could remotely affect RNA removal.[2] RNase H specifically recognizes the RNA in RNA–DNA hybrids.[7–11] These hybrids have been shown to have the general features of A-form RNA-type structures, but with enough difference from RNA duplexes to allow their recognition by RNase H. The enzyme is sufficiently sensitive to structural differences that mammalian RNase HI can recognize RNA–DNA junctions in single stranded polynucleotides.[12] Although the effects of damaged sites on the structures of RNA–DNA hybrids are not known, it is reasonable to expect that the structural distortions may be comparable to those observed for DNA–DNA duplexes containing damaged sites.[13–16] The presence of a damaged DNA base in RNA–DNA hybrids could also affect the flexibility and intrinsic curvature of such duplexes. We have shown that the diffusion of DNA–DNA duplexes containing thymine glycol and urea bases is faster than that of undamaged duplex of the same length, suggesting that the presence of the damaged site increases the flexibility or curvature of the duplex.[17] The structural distortion, or excess mobility, due to the presence of damaged DNA may prevent effective interaction of the RNA–DNA hybrid with RNase H. If the hybrid is a poor substrate for RNase H there could be profound biological consequences due to a pause or stop of

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lagging strand replication. The damaged site in an RNA–DNA hybrid is unlikely to be repaired because RNA–DNA hybrids containing damaged DNA sites are poor substrates for any of the DNA repair enzymes tested so far.[18]

RNase H activity has been shown to be highly sensitive to the chemical modifications of the DNA strand in hybrid duplexes. Although there have been investigations of the ability of RNase H to recognize mismatched sites as well as several different modified bases in the DNA strand, the ability of RNase H to recognize damaged DNA sites has not been studied. RNA–DNA hybrids containing a mismatched site are 2- to 3-fold poorer substrates for the nonspecific nuclease activity of *E. coli* and mammalian RNase H than are the fully complementary RNA–DNA hybrids.[11,19] The incorporation of 6-thioguanosine into the DNA strand of an RNA–DNA hybrid decreased *E. coli* and human RNase H cleavage rates 10-fold.[20] Cleavage of either of the two phosphodiester bonds adjacent to the site containing 6-thioguanosine was particularly inhibited.[20] Crooke et al. have monitored the incorporation of N^2-, N^6-imidazolylpropyl adenine and 2,6-diaminopurine into the middle of a DNA strand of a 5 base pair hybrid target sequence.[7] Neither N^2-imidazolylpropyl adenine nor 2,6-diaminopurine inhibited initial endonucleolytic cleavage. Both base analogues inhibited subsequent exonucleolytic cleavage, especially adjacent to the modified base pair. N^6-Imidazolylpropyl adenine inhibited initial endonucleolytic cleavage and exonucleolytic cleavage adjacent to the modified base pair.

Studies have shown that the most prevalent mammalian RNase H, HI, has homology to yeast RNase H and to prokaryotic RNase HII.[21–23] *E. coli* RNase HII, the enzyme used here, has homology to human RNase HII, which is found in the nucleus.[22] It appears that the major RNase H of human cells is evolutionarily related to the minor RNase H of *E. coli* and vice versa. There is also an RNase HIII in *B. subtilis* that may have evolved from the *E. coli* RNase HI class of nucleases.[24] All of these enzymes seem to have similar mechanisms. We chose to work with the *E. coli* RNase H1, since it is the best characterized of the RNase H enzymes.

The damaged sites investigated here are thymine glycol and urea, and their structures are depicted in Figure 1. These damaged sites were examined since both are commonly occurring and both are stable under the conditions used to carry out the RNase H reactions. Thymine glycol and urea have different effects on the

Figure 1. The structures of undamaged thymine base and damaged thymine glycol and urea bases.

replication of the leading strand.[25] Thymine glycol is more easily bypassed than is urea, while miscoding of urea is much more likely than miscoding of thymine glycol.[25]

Thymine glycol and urea were incorporated into the duplex formed between d(CGCGATACGCC) and r(GGCGUAUCGCG) at position 6 in the DNA strand. This duplex was chosen because the effects of thymine glycol and urea on the structure and flexibility of the related DNA duplex have been studied previously.[26,27] The single dT of the purified DNA, d(CGCGATACGCC), was converted to thymine glycol by oxidation of the parent single-stranded DNA with 0.1 M $KMnO_4$ using the methods previously described.[13,25,28] Urea-containing DNA was prepared by alkaline hydrolysis of the single-stranded DNA containing thymine glycol as previously described.[13,25,28]

Since RNase H cleaves the RNA only when it is hybridized to DNA, the stabilities of the hybrid duplexes containing damaged sites were examined. Prior studies on DNA duplexes containing single damaged sites suggested that the melting temperatures of the hybrids containing a single damaged site under the conditions used in the RNase H experiments would be approximately 30 °C.[13–16,29] The thermal stability of the hybrids was examined by monitoring the optical absorbance at 260 nm as a function of temperature. The melting temperature, T_m, of a duplex is dependent on the concentration of the sample, since the formation of the duplex is a bimolecular reaction. The concentration used in the optical T_m experiments was twice that used in enzymatic RNase H experiments. This is the lowest concentration that is compatible with reliable melting temperature determinations. The melting temperatures of the undamaged hybrid, thymine glycol hybrid, and urea hybrid duplexes are 50, 28, and 29 °C, respectively. To ensure formation of the duplexes, RNase H reactions were carried out at 12 °C. Formation of duplexes was complete, without any traces of single-stranded material as shown by native gel electrophoresis (data not shown).

The time course of each RNase H reaction was monitored by denaturing PAGE. Cleavage reactions were carried out in 20 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 0.2 mM DTT, at pH 8.0. The reaction volume of 7 µL contained 0.15 pmol of RNA, 10 fmol of ^{32}P-labeled RNA, 0.2 pmol of DNA and 2 units of *E. coli* RNase H, which was obtained from Life Technologies (Carlsbad, CA). The reactions were initiated by the addition of RNase H. Both the enzyme and the reaction mixture were maintained at 12 °C during this addition. An excess of DNA was used to ensure all of the RNA was hybridized. Both DTT and EDTA needed to be present in the reaction mixture for reproducible results to be obtained. The reactions were stopped by the addition of urea, and the resulting reaction mixture was analyzed on a 20% denaturing polyacrylamide gel. The results obtained from a typical reaction are shown in Figure 2. Cleavage products were identified by comparison to a limited alkaline hydrolysis reaction.
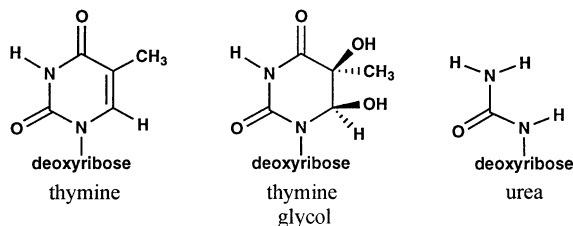
RNase H cleavage results in a terminal 3'-hydroxyl group, while limited alkaline hydrolysis results in a terminal 3'-phosphate. Therefore, the products of RNase H digestion have a lower mobility than those of limited alkaline hydrolysis due to the presence of one less negative charge. To confirm the identification of the RNase H digestion products we independently synthesized the major product of the digestion of the fully complementary hybrid duplex, r(GGCGUAU).

A qualitative examination of the gels indicated that the three reactions have similar rates and go to completion over the time period of the experiments. The relative rates of digestion of the three reactions were determined by measuring the intensity of the full length RNA band at each time point. Five independent runs of all three reactions were used to calculate the ratios of the rates of digestion relative to that of the fully complementary hybrid. Averaged ratios were 1.2 ± 0.2 for the thymine glycol sample and 1.2 ± 0.4 for the urea sample. The ratios of the rates of these three reactions show that the hybrids containing these two damaged sites are as good substrates for RNase H, or somewhat better, than the fully complementary duplex.

Since these reactions were performed at 12 °C, while reactions in vivo occur at 37 °C, we repeated these reactions at 25 °C. In the 25 °C experiments we found, as expected, that RNase H did not digest the hybrids containing thymine glycol or urea, presumably because fully stable hybrid duplexes were not formed at this temperature. The fully complementary RNA–DNA duplex was digested at 25 °C, while single-stranded RNA remained intact even after several days of incubation with RNase H.

Although damaged hybrids, under the given salt conditions, had a T_m that was lower than 37 °C, conditions in the cell can facilitate formation of stable RNA–DNA duplexes at these temperatures. Several factors can play a role in stabilizing such hybrids, including the length of the RNA–DNA hybrid and the concentration of divalent cations. Our duplexes are only of modest length (11-mers), but the duplexes of longer length, found in cells, will be more stable. Also, our latest results on the effect of ionic strength on the stability of dA-tract DNA duplexes[30] as well as previously published results[17,29,31,32] clearly support the possibility of increased duplex stability due to the presence of physiological cation concentration.

Although similar rates of digestion of the three hybrid duplexes were found, the three reactions have markedly different cleavage patterns. There was one major product of the digestion of the fully complementary duplex. This product is r(GGCGUAU) resulting from cleavage after U7. Only a small amount of longer or shorter products was formed, even when the reaction mixtures were incubated for a period of several days. In contrast, the duplexes containing thymine glycol or urea formed three major products resulting from cleavage after U5, U7, and G9. The initial product was the 9-mer oligoribonucleotide. Over the course of the reaction this product disappeared and the final products in each case were primarily the 7-mer and 5-mer oligoribonucleotides. In the case of the hybrid containing urea, the 5-mer formed the major product at the longest time points. This pattern is consistent with the known activity of RNase H in which initial endonucleolytic cleavage is followed by exonucleolytic cleavage in the 3' to 5' direction.[7] Since the 5' end of the RNA is radioactively labeled, progressively shorter pieces are observed as the reaction progresses. These data suggest that RNase H is less selective in the cleavage of the hybrids containing thymine glycol or urea than the fully complementary hybrid. Unlike the previously studied DNA base modifications, cleavage is redirected to a position adjacent to the damaged base pair, suggesting that thymine glycol and urea alter the hybrid structure to cause RNase H to be less selective as to cleavage site.

These results demonstrate that RNA–DNA hybrids containing thymine glycol and urea damaged sites in the DNA strand are substrates for RNase H. In fact, the hybrids with damaged sites appear to be as good, or slightly better, substrates for RNase H than the fully complementary hybrid and cleavage is redirected adjacent to the damaged sites. In contrast, other DNA base modifications that have been examined previously inhibit enzymatic activity, especially adjacent to the site of modification. Since RNase H requires a duplex substrate, the urea and thymine glycol damaged sites might inhibit RNase H activity in vivo by limiting duplex formation at 37 °C. Even if RNase H activity is unchanged, the altered cleavage pattern could have pronounced affects on DNA replication machinery.

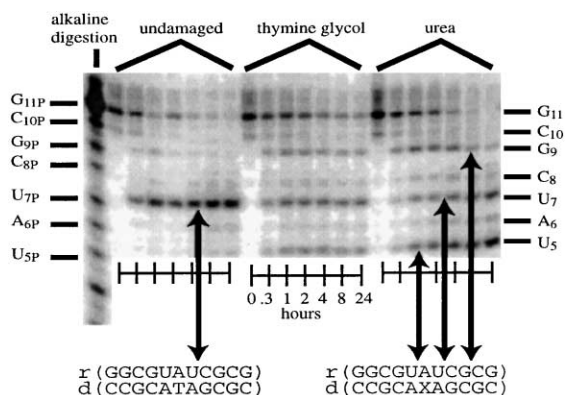**Figure 2.** The rates of the RNase H digestion of the RNA–DNA hybrids were monitored by denaturing gel electrophoresis on 15 cm ×40 cm×0.4 mm 20% acrylamide, 20:1 mono/bisacrylamide denaturing gels. The reactions were stopped after 0, 0.3, 1, 2, 3, 4, 8, and 24 h. The farthest left lane is an alkaline sizing ladder.[33] The next seven lanes are the reactions run with a fully complementary hybrid duplex formed with d(CGCGATACGCC), the middle seven lanes are with the DNA with a central thymine glycol, and the seven lanes on the right are with the DNA with a central urea.

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