Slippery” Hairpins*

We show that CUG repeats form “slippery” hairpins in their natural sequence context of the myotonin kinase gene transcript. This novel type of RNA structure is characterized by strong S1 and T1 nuclease and lead cleavages in the terminal loop and by mild lead cleavages in the hairpin stem. The latter effect indicates a relaxed metastable structure of the stem. (CUG)₅ repeats do not form any detectable secondary structure, whereas hairpins of increasing stability are formed by (CUG)₁₁, (CUG)₂₁, and (CUG)₄₉. The potential role of the RNA hairpin structure in the pathogenesis of myotonic dystrophy is discussed.

Eleven human diseases associated with the expansion of trinucleotide repeats have been identified so far. The progress of research in this new area has been discussed in several recent reviews (1–5). One of these diseases is myotonic dystrophy (dystrophia myotonica (DM)),¹ the most prevalent form of muscular dystrophy in adults with a global incidence of 1 in 8000. The molecular basis of this multisystemic disease, with a complex clinical picture, is the expanded CTG repeat in the 3′-UTR of the DMPK gene. The repeat expansions, which cause DM, range from 50 repeats in mildly affected patients to >2000 repeats in the most severe congenital cases (6–8).

Despite the fact that the nature of the underlying DM mutation has been known for the past 6 years, the molecular pathology of this disease is not understood. In particular, it has been difficult to find the molecular mechanism by which this specific mutation, located in the 3′-UTR of the DMPK gene, causes the dominantly inherited disease. One of several recent proposals links the inheritance pattern with the molecular effects observed at the RNA level. It takes advantage of the observation that dramatic decreases in both mutant and normal DMPK poly(A)⁺ RNAs, as compared with the primary transcripts, occur in the DM tissue (9). This suggests that the expanded CUG repeat may have a dominant effect either on the processing of both the normal and expanded transcripts or on their transport to the cytoplasm. Other authors observed only a decrease in the expanded poly(A)⁺ transcript (10). The specific (CUG)₅-binding proteins, discovered recently (11–13), may be involved in normal DMPK RNA processing, transport, and/or translation. These RNA-protein interactions may be impaired by the expanded DMPK transcript.

Understanding the molecular basis of the DM disease is hampered by the lack of knowledge of the RNA structure formed by the CUG repeats. To fill this gap and to provide a background for further studies on the role of RNA level effects in the pathogenesis of myotonic dystrophy, we have analyzed the structure of the repeat region of DMPK RNA. In this paper, we describe the properties of an unusual “slippery” RNA hairpin that contains a metastable stem. The stability of the hairpin increases with the repeat length, and we postulate that long stable hairpins are important factors in DM pathogenesis.

MATERIALS AND METHODS

DNA Templates for in Vitro Transcription—A plasmid containing 49 CTG repeats was obtained from Robert D. Wells. The alleles of the human DMPK gene containing 5, 11, and 21 CTG repeats were selected from our laboratory genomic DNA collection. Control DNA samples from healthy individuals were screened by the polymerase chain reaction (PCR) with primers described earlier (8). The length of radiolabeled polymerase chain reaction products was compared with the M13 sequencing ladder on a polyacrylamide gel to determine the number of CTG repeats present. Bands corresponding to the selected alleles were cut out from the gel, eluted with 200 μl of H₂O, reamplified, and sequenced using the cycle sequencing protocol (Life Technologies, Inc.).

The polymerase chain reaction products with confirmed nucleotide sequences were reamplified with the same reverse primer and a modified forward primer containing the T₁ RNA polymerase promoter: 5′-TAA-TACGACTCATAATGGGTGAGAGGTCTCTTGATCCGGGA. All amplifications and reamplifications were done under the same conditions: total volume of 5 μl, 3 pmol of each primer, 200 μM each dNTP, and 0.125 units of AmpliTaq polymerase (Perkin-Elmer) in a buffer containing 10 mM Tris (pH 8.3), 50 mM KCl, and 1.5 mM MgCl₂. Cycling conditions were 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 1 min and annealing at 57 °C for 1 min, and extension at 72 °C for 1 min.

In Vitro Transcription and RNA Labeling—The transcription reaction was performed as described earlier (14) in the presence of 1000 units/ml RNazin (Promega). The reaction was stopped by a phenol/chloroform extraction followed by ethanol precipitation. Transcripts were then purified on a denaturing 10% polyacrylamide gel. The RNA bands corresponding to full-size transcripts were identified by staining with Stains-all (Serva), excised, eluted from the gel (with 0.3 mM potassium acetate (pH 5.1), 1 mM EDTA, and 0.1% SDS), and precipitated with ethanol. They were stored at −80 °C under ethanol before use. In vitro transcriptions were phosphorylated with [γ-³²P]ATP (5000 Ci/mmol; Amersham Corp.) and T4 polynucleotide kinase (New England Biolabs Inc.). The labeled RNAs were purified by electrophoresis on a denaturing 10% polyacrylamide gel, localized on the gel by autoradiography, and recovered as described above. As revealed by polyacrylamide gel electrophoresis under nondenaturing conditions, (CUG)₅, (CUG)₁₁, (CUG)₂₁, and (CUG)₄₉ migrate as monomorphic species under conditions used for structure probing experiments.

Nuclease Digestions and Lead Cleavages—Prior to structure probing reactions, the labeled RNA carrier to obtain a final RNA concentration of 8 μM. Limited digestions with lead ions and S1 and T1 nuclease (Pharmacia Biotech Inc.) were carried out in the buffer and under the conditions specified in the legend to Fig. 1. All reactions were stopped by adding 7 μl urea and

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The abbreviations used are: DM, myotonic dystrophy (dystrophia myotonica); DMPK, myotonic dystrophy protein kinase; 3′-UTR, 3′-untranslated region.
**RESULTS**

**RNA Models and Structure Probes**—The number of CTG repeats in normal alleles of the DMPK gene varies between 5 and 37. In the Caucasian population, the great majority of chromosomes show three ranges of the repeat size (15). The most frequent allele contains five repeats; the next between 10 and 15; and the third from 20 to 37 repeats. The third group gives rise to the expanded pathogenic alleles (16). We have selected one representative of each group for detailed RNA structure analysis: (CUG)$_5$, (CUG)$_{11}$, (CUG)$_{21}$, and the expanded allele containing 49 repeats. In all four model molecules, the same natural sequences flanking the repeat region are present: 30 nucleotides at the 5'-side, GGCGUCGAGGG-UCCUUGUAGCAGCGGAAUG; and 35 nucleotides at the 3'-side of the repeat, GGGGAUCACAGACCAUUUCU-UUCUUUCGCGAGG.

All RNA structure probing reagents used in this study are well characterized. Lead ions, in the absence of strong binding sites from which highly specific and efficient cleavages of phosphodiester bonds may be performed (17–21), differentiate between rigid and flexible sugar phosphate backbones (22, 23). Rigid double-stranded RNA structures and single-stranded regions involved in stable stacking or hydrogen bond interactions are not cleaved. Flexible single-stranded regions are susceptible to cleavage, as a mechanism of the reaction, which begins with the activation of the ribose 2'-OH group by the lead ion and the attack of the 2'-O' nucleophile on the adjacent phosphate P atom requires a conformational change of the sugar phosphate backbone (17, 24). The structure and sequence specificity of $S_1$ and $T_1$ nucleases is well established (25). Lead ions and nucleases have been widely used in the structural analysis of a variety of natural RNA molecules and their variants obtained by transcription in vitro. Here they are used for the first time in the analysis of RNA structures, formed by the same trinucleotide motif repeated many times. This gives the unique opportunity to assess the structure specificity of the probing reagents, which is not affected by the ambiguities caused by irregular variations of the nucleotide sequence. All RNA structure probing reactions described in this study were carried out under very similar solution conditions, and important conclusions were based on comparisons of the results obtained with different model substrates and structure probes.

**CUG$_{49}$ Forms a Metastable Slippery Hairpin**—All three probes give similar patterns of hydrolysis in the repeat region of the DMPK mRNA fragment containing 49 CUG repeats (Fig. 1). The strong cleavage by the lead ions, $S_1$ nucleases, and $T_1$ ribonucleases is observed in the central part of the repeat sequence. Repeats at both the 5'- and 3'-sides of the highly reactive region are not digested by the nucleases and are very poorly cleaved by lead ions (Figs. 1 and 2a). Several 3'-terminal repeats and the first two repeats from the 5'-side are cleaved by lead ions more easily. This pattern of hydrolysis seems consist-
CUG Repeats Form Metastable Slippery Hairpins

FIG. 2. Melting of the hairpin structures (CUG)_{49} (a) and (CUG)_{11} (b) analyzed by lead cleavages at different temperatures. The incubation temperature is shown above each lane. The Pb(II) concentration was 0.5 mM in each reaction, and the incubation time was adjusted for each temperature to give the same ratio of the unreacted substrate and cleavage products: 20 min, 10 min, 5 min, 3 min, 90 s, 30 s, and 15 s, respectively, for reactions at 25–85 °C. Lanes C, incubation controls (without a probe); lanes L, formamide ladder; lanes T, limited T1 ribonuclease digest under semi-denaturing conditions. In (CUG)_{11}, only the G residues from the terminal repeats are indicated.

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ent with the presence of a hairpin structure that contains a large terminal loop spanning at least four repeats and, in agreement with the results of lead cleavages, a stem “breathing” at its base. This model, however, does not explain the observed asymmetry in the number of lead cleavages induced at both termini of the repeated sequence (Figs. 1 and 2a). A more likely explanation of these results is the presence of several alternatively aligned variants of the (CUG)_{49} hairpin in which different combinations of the neighboring central repeats are involved in the formation of smaller loops, and different terminal repeats form protruding ends at the base of the stem. In favor of this alternatively aligned, slippery RNA model are the following facts. 1) Both the enzymatic and lead-induced cuts in the hairpin loop tend to expand more toward the 5’-end of the repeated sequence from the central, most reactive 25th repeat. 2) More repeats located at the 3’-end of the repeated sequence show enhanced reactivity with lead ions. 3) Computer modeling (26) of the (CUG)_{49} structure predicts the loops of four or seven nucleotides as the most energetically favorable. The fact that only the lead ions, but not the nucleases, cleave the protruding terminal repeats is not surprising. These single-stranded repeats, adjacent to the hairpin stem, are very likely inaccessible to the S1 and T1 nucleases. The proposed structures of the alternatively aligned hairpins formed by (CUG)_{49} are shown in Fig. 3.

The characteristic feature of the (CUG)_{49} hairpin stem is its metastability. Melting of this structure monitored by lead cleavages is revealed by the disappearance of more intense loop cuts and by the increase of cleavage intensity in the stem (Fig. 2a). The loop cuts become weaker at the temperature 65 °C and are only slightly stronger than the background cleavages at 75 °C, at which also the stem cuts remain weak. On the other hand, a dramatic increase in the intensity of the stem cuts is observed at 85 °C. These observations could be explained by the formation of an intermediate structure from the hairpin to the highly reactive single-stranded form, e.g., an intermolecular duplex formed by the antiparallel single strands held together by interactions similar to those present in the hairpin stem.

At a low temperature, all weak cuts in the stem are of nearly equal intensity, with only a minor preference for cleavages at UpG (Fig. 2a). This preference tends to disappear at a temperature above 45 °C. Therefore, this stem should not be considered as composed of units in which two base pairs, C-G and G-C, are followed by the internal loop, formed by the U-U mispair. In light of our data, the stem structure seems to be better described as a well balanced system, a result of a compromise between the stabilizing effects of the G-C and C-G interactions and the destabilizing effect of the U-U interactions. For this reason, all base-base interactions present in the hairpin stem are shown by dots in Fig. 3 to indicate their specific nature, which results in the decreased stability of the stem structure.

(CUG)_{21} and (CUG)_{11} Hairpins Are Less Stable—Slippery hairpins are also present in DMPK mRNA fragments containing 21 and 11 CUG repeats. Here again, several terminal repeats that form protruding ends at the base of the stem show enhanced reactivity. Highly reactive are the centrally located repeats that form a loop. The loops include repeats 9–12 in hairpin variants of (CUG)_{21} and repeats 4–6 in the (CUG)_{11} hairpins. A major difference among the (CUG)_{11}, (CUG)_{21}, and (CUG)_{49} hairpins is the rigidity of their stem structure. This is clearly seen as a difference in the intensity of lead-induced cleavages in their loops and stems (compare Figs. 1 and 4). The stem cuts in (CUG)_{11} are the strongest, whereas those in (CUG)_{21} are more efficient than those in (CUG)_{49}. Probably both the extended stacking interactions and the increased number of hydrogen bond interactions contribute to the higher rigidity of the sugar phosphate backbone in longer hairpins. A difference is also apparent when the intensities of lead-induced cleavages in (CUG)_{21} are compared with those of S1 nuclease cuts in the same model molecule. The S1 nuclease is not capable of detecting the higher degree of stem structure relaxation in (CUG)_{21}, and only very weak S1 nuclease cuts can be detected after extended autoradiography (data not shown). However, in (CUG)_{11}, where the relaxation of the stem structure is still more profound and the lead cleavages become fairly strong, the S1 nuclease cleaves the stem poorly, with a preference for CpU > GpC. The same preference is observed among the strong S1 nuclease cuts within the hairpin loop. The preference of the lead cleavages generated in the stem is different. In (CUG)_{11}, the order of reactivity is CpU > UpG > GpC. In the more stable stems of (CUG)_{21} and (CUG)_{49}, this cleavage specificity

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changes to UpG > CpU > GpC (Figs. 2 and 4).

Melting experiments, similar to this shown in Fig. 2a for (CUG)$_{21}$ indicate that hairpins formed by (CUG)$_{21}$ (data not shown) and (CUG)$_{11}$ (Fig. 2b) are significantly less stable. The (CUG)$_{11}$ hairpin melts completely at $-55^\circ C$, whereas the (CUG)$_{21}$ hairpin melts completely at $-65^\circ C$.

(CUG)$_5$ and Flanking Sequences Are Single-stranded—The repeat region of (CUG)$_5$ is cleaved by lead ions with the order

![Fig. 3](image-url) "Slipped" variants of the (CUG)$_{49}$ hairpin. Structures 1–3 and/or 4–7, when taken together, are consistent with the sites of the observed loop cleavages and cleavages of the terminal repeats at the base of the hairpin stem. The reactive nucleotides are shown in boldface. Below each hairpin variant, the calculated free energy values are shown ($\Delta G$ expressed in kcal/mol at 20 °C). These values do not include the unknown contribution of the single U-U interactions.

![Fig. 4](image-url) Structure probing of DMPK mRNA fragments containing 21 CUG repeats (a) and 11 CUG repeats (b): probe concentration dependence. The reaction conditions were the same as specified in the legend to Fig. 1. Lanes C, incubation controls (without a probe); lanes L, formamide ladder; lanes T, limited T$_1$ ribonuclease digest under semi-denaturing conditions. Only the G nucleotides corresponding to terminal CUG repeats are indicated. Note that the end groups in the T$_1$ nuclease and the lead cleavage products are 5'-OH and 2'-3'-cyclic phosphate. The same are the end groups in the formamide ladder. The S$_1$ nuclease leaves the 5'-phosphate and 3'-OH at its cleavage site; therefore, the shorter products of S$_1$ nuclease cleavages migrate slower on polyacrylamide gel.
of reactivity CpU > UpG > GpC, which is characteristic for the single-stranded looped repeats in all hairpin-forming repeats analyzed here and for stem cuts in the least stable hairpin, (CUG)$_{11}$. In sequences flanking the (CUG)$_{2}$ repeat, all G nucleotides are good targets for T$_{1}$ ribonuclease, and only a few sites are resistant to both S$_{1}$ nuclease and lead ions (Fig. 5). This means that the flanking sequences are mostly if not entirely single-stranded. It should also be pointed out that the pattern of reactivity of both short sequences flanking the CUG repeats is practically identical in all model molecules analyzed in this study. This indicates that independent of the length of the repeated sequence (5, 11, 21, or 49 units), the 30 nucleotides at their 5’-end and the 35 nucleotides at their 3’-end all form the same structural environment for the repeats.

**DISCUSSION**

**Features of New Hairpin Structure**—The molecular mechanism of trinucleotide repeat genetic instability could be explained by the hairpins formed by DNA during replication (27). In several recent biophysical studies, the presence of hairpin structures was confirmed in (CTG)$_{n}$ oligomers (28–32), and the existence of similar RNA structures was postulated (30, 31). In this paper, we show that the (CUG)$_{n}$ hairpins indeed exist in the stems of (CUG)$_{49}$ and (CUG)$_{21}$ as well as in this study. This indicates that independent of the length of the repeated sequence (5, 11, 21, or 49 units), the 30 nucleotides at their 5’-end and the 35 nucleotides at their 3’-end all form the same structural environment for the repeats.

**Specificity of lead-induced cleavages in CUG repeats**

![Fig. 6. Specificity of lead-induced cleavages in CUG repeats showing four distinct lead cleavage patterns. Structure 1, cleavage pattern observed at 20°C in single-stranded (CUG)$_{n}$ in all hairpin loops, and in the stem of (CUG)$_{11}$; structure 2, distribution of cleavages in the stems of (CUG)$_{21}$ and (CUG)$_{49}$ at 20°C; structure 3, pattern observed in the stems of (CUG)$_{21}$ and (CUG)$_{49}$ at an elevated temperature; structure 4, specificity of cleavages in all melted structures at 85°C. Strong cleavages are shown by filled arrowheads, and weak cleavages by open arrowheads. The size of arrowheads represents relative cleavage efficiency within each group.](Image 333x581 to 538x729)

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drgen bond, which forces one of the two U nucleotides to adopt an extended trans-conformation of the backbone to accommodate the mismatch.

It has been postulated that the U-U pairs, which are exceptionally flexible, can interconvert between different pairing modes. They could also be the loci for the fraying of the double helix (34). However, in the model hexamer, the CGCG blocks appear largely unperturbed and rigid within the A form of RNA structure (34). We show here that the presence of the U-U pair after every two pairs of C-G and G-C in the (CUG)ₙ hairpins perturbs their stem structure. According to our data, the entire (CUG)ₙ hairpin stem is a relaxed metastable structure, and the postulated interconversion between different U-U pairing modes could be reflected by the distinct lead cleavage patterns (2, 3) observed in the stem (Fig. 6).

From the perspective of RNA structure probing methods, our results show a high performance of lead-induced cleavages in determining the morphology of the previously unknown RNA structure. The lead cleavage reaction, as easy to handle as the nuclease digestion, has this advantage over the enzymes: that active species (the deprotonated lead ion hydrates (18)) are much smaller and can penetrate folded RNA structures easier. The hairpin formed by the repeated sequence turned out to be an excellent model to show a clear picture of the structure specificity of the probing reagents. Repeats involved in structures of different types or of different strengths give distinct lead cleavage patterns and/or cleavage intensity.

Relevance to DM Pathogenesis—If we assume that the problem of the DM pathogenesis can be explained by the post-transcriptional RNA level effects and that the hairpins described here exist also in vivo, we can ask the following question. What is the special property of the transcript with 50 or more CUG repeats that could not be shown by transcripts containing 37 repeats or less? Two differences in the structure of the normal and expanded transcripts are obvious: one is the CUG hairpin length and the other is its stability. How could these factors possibly affect the post-transcriptional activities? The CUG repeat is located in the 3'-UTR of DMPK mRNA, which is coded by the last exon (exon 15) of the gene (37). The repeat is 223 nucleotides from the translation stop codon and 485 nucleotides from the polyadenylation site (6). A secondary structure model of this portion of the DMPK RNA containing 120 CUG repeats is shown in Fig. 7. In the GC-rich (64%) 3'-UTR, the CUG hairpin can impose structural perturbations over a long distance. This could include the polyadenylation site, the splice sites, and the coding sequence. It is also worth noting that in the case of long repeats, the computer program (26) draws either a single long hairpin or two or more shorter hairpin structures with practically unchanged calculated free energy. It remains to be determined experimentally whether or not such multiple hairpins in fact exist in transcripts from the highly expanded alleles.

As far as the specific proteins that bind to the CUG repeats is concerned, it remains to be seen whether these proteins bind preferentially to the single-stranded or double-stranded repeated motifs. Usually, the loop sequences in RNA hairpins define the binding site for RNA-binding proteins, whereas the stem may help to correctly position the protein by sequence-independent contacts with the backbone (38). In (CUG)ₙ hairpins, the problem is less clear, as both the loop and the stem contain the same repeated sequence motif. The specific CUG-binding proteins have been isolated based on their affinity for (CUG)₁₁ (11, 12) and (CUG)₁₃ (13), which, in light of our results, do not form stable hairpins. In case the proteins bind to the single-stranded regions, the long hairpins would not titrate them more effectively than the short hairpins unless there are multiple hairpins or the stability of the stem structure makes a significant contribution to the protein binding efficiency. If the proteins bind to the double-stranded repeats, their titration by the long CUG hairpins could be more easily explained. Having the CUG hairpin structure determined in vitro, experiments can now be designed to demonstrate that the hairpins exist in vivo, to establish the way they bind the proteins, and to show how the protein binding changes with the repeat expansions.

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