Belokoń *et al.* (*41*). Nitrobenzyl-L-lactic acid (*10*) was prepared by reaction between L-lactic acid and *o*-nitrobenzyl bromide in the presence of two equivalents of vinyl magnesium bromide in tetrahydrofuran (THF) at 60°C. For synthesis of *o*-nitrobenzyl–protected glycolic acid, ethylene glycol was first monoalkylated with *o*-nitrobenzyl bromide, and then the hydroxyl group was oxidized with pyridinium dichromate. Bromination of the mono-*o*-nitrobenzyl–protected ethylene glycol (*42*) followed by reaction with *N*-NVOC-L-cysteine (*43*) and *N*-NVOC-L-homocysteine gave nitrobenzyl-protected *N*-NVOC-L-hydroxyethylcysteine and *N*-NVOC-L-hydroxyethylhomocysteine, respectively (NVOC, nitroveratryloxycarbamyl). The hydroxyl group of *N*-NVOC-L-*allo*-threonine was protected as the *tert*-butyl-dimethylsilyl (TBDMS) derivative. Removal of the TBDMS group after acylation of the protected amino acid to pdCpA was effected with a mixture of acetic acid–H_2O-THF (3.1·1). The nitro analog of glutamic acid was synthesized by D Suich (*44*).

48 K. Miura *et al.*, *Cancer Res.* **77**, 45 (1986)

49. Suppression efficiencies were determined by scintillation counting of Ras immunoprecipitated with monoclonal antibody 1F6 (which was prepared by standard methods and shown not to affect the intrinsic GTPase activity of cellular H-Ras)

50 GAP-mediated GTPase activity was measured by a modification of the procedure described by Han *et al.* (*45*). Immunopurified Ras (100 nM) was incubated for 15 min at 25°C with 50 μCi of [α-^{32}P]GTP (410 Ci/mmol; Amersham) in buffer I [50 mM Hepes (pH 7.4), 200 mM sucrose, 1 mM $MgCl_2$, 5 mM EDTA, 0.2% NaN_3, and 1 mM DTT]. The [α-^{32}P]GTP-bound Ras was purified on a PD10 column (Pharmacia LKB). The GTPase reaction was initiated by the addition of 10 mM $MgCl_2$ in the presence or absence of recombinant GAP (total assay volume was 1 ml) Incubation time was 10 min at 30°C Ras was collected by filter binding (0.2-μm pore size; Millipore) and released from the filter by incubation with 20 mM EDTA, 1% SDS, 4 mM GTP, 4 mM GDP, and 4 mM GMP at 65°C for 5 min The bound guanine nucleotides were resolved by chromatography on PEI plates as described (*46*) The labeled nucleotides were quantitated by scintillation counting The percent GTP remaining was calculated as [(counts per minute of GTP)/(counts per minute of GTP + counts per minute of GDP)] × 100. Results are representative of duplicated experiments.

51 Autophosphorylation activity of Ras was assayed at 37°C in the presence of 10 μM [γ-^{32}P]GTP in 100 μl of reaction buffer containing 50 mM tris-HCl (pH 7 5), 10 mM MgCl, 1 mM DTT, and 1 mM NaN_3. At various times, portions (10 μl) were removed and quenched with 100 μl of cold quenching solution [50 mM tris-HCl (pH 7.5), 2 mM GTP, 2 mM GDP, 5 mM potassium inorganic phosphate, 5 mM EDTA, and 1 mM DTT]. The sample was filtered through a nitrocellulose membrane filter (Schliecher and Schuell, Keene, NH) and washed six times with 1 ml of rinsing buffer [50 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM NH_4Cl, and 1 mM 2-mercaptoethanol]. The radioactivity retained on the filter was measured by liquid scintillation counting.

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Reduction in Size of the Myotonic Dystrophy Trinucleotide Repeat Mutation During Transmission

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Myotonic dystrophy (DM) is an autosomal-dominant disorder that affects 1 in 8000 individuals. Amplification of an unstable trinucleotide CTG repeat, located within the 3′ untranslated region of a gene, correlates with a more severe DM phenotype. In three cases, the number of CTG repeats was reduced during the transmission of the DM allele; in one of these cases, the number was reduced to within the normal range and correlated at least with a delay in the onset of clinical signs of DM. Haplotype data of six polymorphic markers in the DM gene region indicate that, in this latter case, two stretches of the affected chromosome had been exchanged with that region of the wild-type chromosome.

The most prevalent inherited neuromuscular disease in adults, myotonic dystrophy (DM), is a multisystem disorder that is characterized by progressive muscle weakness and myotonia (*1*). This disease is variable both in severity and age of onset; those most severely affected express the disease congenitally. The genetic defect in DM is thought to be an amplified trinucleotide CTG repeat located in the 3′ untranslated region of a gene, which putatively encodes a serine-threonine protein kinase (*2–4*). The number of CTG repeats in normal individuals, although quite polymorphic, is both mitotically and meiotically stable within a lineage. From 5 to 30 repeat copies are observed in normal alleles (*2, 4*). In contrast, the number of CTG repeats on DM chromosomes can be unstable and become extremely large. Mildly affected patients have 50 to 80 CTG repeats, whereas severely affected individuals have 2000 or more copies (*4*). Amplification of the CTG repeat has been proposed to be the molecular basis in this disease for genetic anticipation, a phenomenon in which the severity of an inherited disease increases in successive generations of an affected family (*5*).

In our Canadian DM kindreds, positive correlation was observed between increased numbers of CTG repeats and earlier age of disease onset (*6, 7*). One of these kindreds (Fig. 1) was haplotyped for a total of 18 restriction fragment length polymorphisms (RFLPs) at 12 loci, located between the gene encoding apolipoprotein C2 (APOC2) and the marker p134C, although only four markers are shown (*8*). This kindred possesses the most common DM haplotype of our affected population, one that occurs in approximately 30% of DM kindreds tested, and is quite rare among normal chromosomes: the calculated frequency is less than 0.000015 (*9*). We have not observed this haplotype on more than 2000 normal chromosomes analyzed to date.

Genetic data indicated that the affected chromosome of individual II-8 (Fig. 1) represented the most common DM haplotype. The DNA of individual II-8 was analyzed by genomic Southern (DNA) blot and polymerase chain reaction (PCR) with primers flanking the CTG repeat region (*2*). The extent of his expanded DM chromosome was in the range of 1.5 to 3.0 kb (Fig. 2A), and the heterogeneity is typical of DM individuals assayed by PCR (Fig. 2B). This was consistent with both the genetic and clinical data. Clinical symptoms of hand weakness and myotonia were originally noted in II-8 from the mid-to-late teens, and a myopathic facies, bitemporal narrowing, and early pattern baldness were apparent from photographs from that age.

The daughter of II-8 (III-10, Fig. 1) inherited her father's DM chromosome. However, no CTG trinucleotide repeat amplification was detected on Southern blot analysis (Fig. 2, A and B). Repeat blood samples were obtained and the initial findings were confirmed. Unlike her father, III-10 showed no evidence of CTG heterogeneity (Fig. 2B). Genomic DNA was amplified with the use of primers immediately flanking the CTG repeat (Fig. 2C). Two alleles, one with 13 and one with 22 CTG repeats, were detected in III-10. The 22-repeat allele was maternally derived. A reduction in the number of CTG repeats down to the normal number and an increase in their stability had occurred on her DM chromosome. The number of CTG copies in the unaffected (non-DM) allele of II-8 is the same as that observed for the paternally derived DM allele in III-10.

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Individual III-10 was recently examined at the age of 17 years. She reported no symptoms of DM. A complete clinical examination showed normal muscle strength in the face, neck, hands, wrist, legs, and feet and no percussion myotonia. Slit lamp examination was normal. Electromyograph (EMG) studies revealed weak myotonic discharges, but no evidence of myopathic changes was observed. These EMG findings, although not diagnostic of DM, may suggest that the disease could appear later on. At a similar age, her father had been clearly affected by DM. This would support the proposal that an amplification of the trinucleotide repeat is associated with disease onset and conversely a reversal of the amplification leads to a delay in onset or perhaps restoration to the normal phenotype.

The reversion in the number of CTG repeats in III-10 to the normal range may be explained by gene conversion. In this event originally described in fungi (*10, 11*), the DNA sequence of one gene or allele is used as a template to convert a related gene or allele. To determine the extent of DNA sequence altered in transmission of the DM chromosome from II-8 we analyzed polymorphisms in the regions surrounding the CTG repeat. The 1.4-kb Bam HI fragment that includes the CTG repeat (*2*) was amplified by PCR from the genomic DNA of individuals II-8 and III-10. These fragments were cloned and sequenced to detect sequence polymorphisms that would delineate the altered region. One base pair substitution (DMK G/T) was found, in an intervening sequence located 649 bp telomeric to the CTG repeat region (Fig. 3A). An allele-specific oligonucleotide, based on this DNA sequence polymorphism, was used to analyze this region by PCR (*12*) (Fig. 3B). This sequence polymorphism was informative: a T is found in the DM allele of II-8, whereas in the paternally derived (DM) allele of III-10 and the paternal unaffected allele a G is found.

Other RFLPs within and near the DM gene were also examined (*13*). One such polymorphism, Hha I, is located in an intervening sequence approximately 7.2 kb telomeric to the CTG repeat region (Fig. 3A). The genotype data for this RFLP in III-10 is informative and shows that in her inherited paternal chromosome, this region was derived from the father's unaffected allele (Fig. 3B). This would suggest that the altered portion of the DM chromosome was at least 7.2 kb in length. Two additional RFLPs, one an insertion polymorphism (DMK, Bgl I) (*2*) and the second a Hinf I RFLP, were also informative. These map between the Hha I RFLP and the polymorphic CTG repeat region (Fig. 3A), and are both located within introns. Unlike the flanking polymorphic markers, these regions were not derived from the paternal unaffected allele (Fig. 3B).

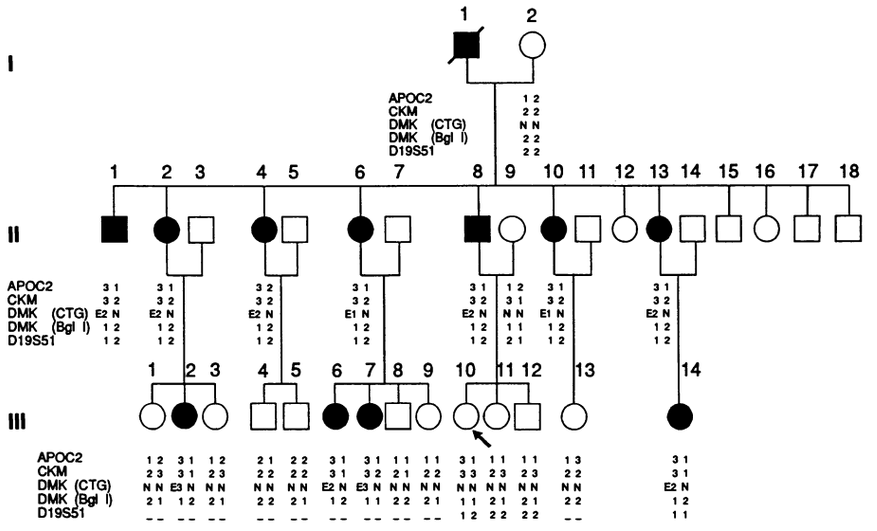

Fig. 1. Pedigree of an extended DM kindred, with chromosome haplotype data generated from the RFLP analysis of four 19q13.3 loci. These loci include three identified genes, encoding APOC2, the muscle-specific isoform of creatine kinase (CKM), the myotonic dystrophy protein kinase (DMK), and one locus (D19S51) detected by p134C. The genetic order of the loci is (19cen)-APOC2-CKM-DMK(CTG)-DMK(Bgl I)-D19S51-(19qter). DNA probes used to detect these loci are as described (*2, 25, 26*). Filled symbols represent individuals affected with DM. For locus DMK, the CTG amplification associated exclusively with DM individuals is detected by probing Eco RI genomic digests with pGB2.6 (*2*). The following classification, based on genomic DNA Southern blot, indicates the extent of CTG amplification above wild type: E0, no visible amplification; E1, up to 1.5 kb; E2, 1.5 to 3.0 kb; E3, 3.0 to 4.5 kb; E4, 4.5 or more (*2*). The non-DM allele is represented by "N." The probe pGB2.6 also detects a normal insertion polymorphism with the use of Bgl I genomic digests (identified as DMK, Bgl I). The arrow indicates the individual of interest who exhibits no CTG amplification but, nevertheless, has inherited a DM chromosome with the most common DM haplotype. Dashes indicate genotype not determined.

DNA sequencing studies of multigene families such as the globins, the immunoglobulins, and oxytocin-vasopressin have led to proposals that events similar to gene conversion have maintained homogeneity within a gene family (*14*). The recombinant pattern within the DM region of individual III-10 is analogous to discontinuous gene conversion tracts, which are thought to be the result in yeast of independent mismatch repair (*15*) or two independent gene conversion events (*16*). As a gene conversion event can only be determined by examination of all meiotic prod-

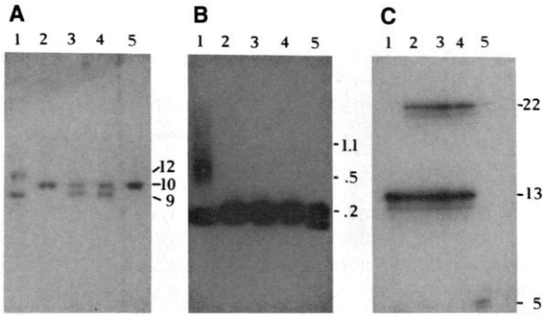

Fig. 2. CTG repeat length heterogeneity and exact CTG repeat copy numbers. In all three panels (**A** to **C**), lane 1 contains DNA from DM individual II-8; lane 2 contains DNA from his daughter III-10; lanes 3 and 4 contain DNA from the other unaffected offspring of II-8 (III-11 and III-12); lane 5 contains the DNA from the unaffected spouse of II-8 (II-9). Estimated molecular weights of the fragments are given in kilobases. (A) Southern blot analysis (*2*) of Eco RI digested genomic DNA showing loss of CTG amplification in one generation. Individual II-8 (lane 1) shows a CTG amplification (2 kb) above the range seen in non-DM individuals. (B) Southern blot analysis of products from PCR of genomic DNA (*2*). In II-8 (lane 1) the PCR-amplified region is heterogeneous in length. (C) Polyacrylamide gel electrophoresis of ^{32}P-labeled PCR products generated with the use of primers flanking the CTG repeat. The unaffected allele of II-8 consists of 13 CTG repeats. His DM allele could not be visualized by this technique, due to heterogeneity and an increased number of CTG repeats. All three offspring have alleles with 13 and 22 repeats, and II-9 has alleles with 5 and 22 repeats. This confirms that a decrease in the number of CTG repeats occurred during the transmission of the DM chromosome from II-8 to III-10.

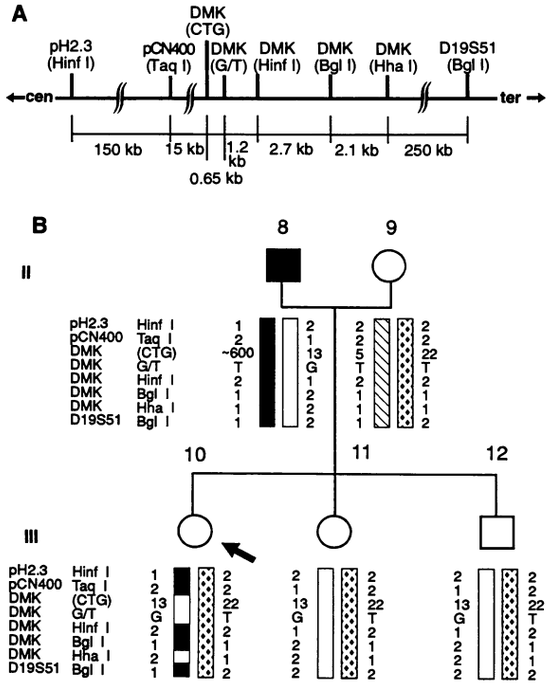

Fig. 3. (**A**) A partial map of the chromosomal region including the DM gene, indicating relevant polymorphic markers used to generate DM chromosome haplotype data. The order of the polymorphic markers is shown with an estimate of the distance between them (kilobases). (**B**) Pedigree of the nuclear family with haplotype data from seven RFLPs and one DNA sequence polymorphism. The polymorphic markers used in this study are listed on the left. The DM chromosome of II-8 is shown as a solid black bar, and his unaffected chromosome is an open bar. Individual III-10 has inherited only portions of the DM chromosome from her father.

ucts, we infer from comparisons with fungi that gene conversion has occurred here. However, we cannot discount reciprocal crossovers as an alternative explanation.

Similar "patchwork" patterns in mammalian genomes are rarely described in the literature, although one has been noted in human α-globin (*17*) and fetal globin gene clusters (*18*). In this latter study, the DNA sequence of the fetal globin allele $^{A}\gamma$-S showed evidence of conversion from the $^{G}\gamma$ fetal globin gene, and the converted regions were not continuous.

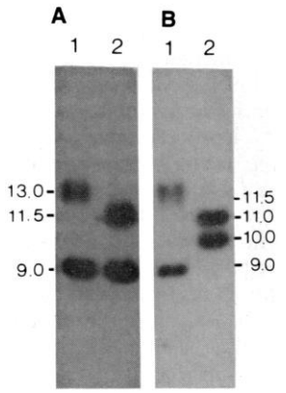

Fig. 4. Reduction in CTG repeat length in two additional DM individuals. The genomic Southern blot (*2*) indicates that a decrease in CTG repeat numbers has occurred during the transmission of the DM allele from (**A**) an affected father (lane 1) to his son (lane 2) and (**B**) an affected father (lane 1) to his daughter (lane 2). The estimated molecular weights of the fragments are given in kilobases.

Genetic analysis of our DM kindreds has also revealed two additional cases with an observed decrease in CTG repeat length from affected parent to child (*19*). In one of the kindreds, the transmitted DM allele of the affected father (Fig. 4A, lane 1) showed a decrease from 3.0 kb to 1.5 kb in his son (lane 2). At age 23 the father had shown clear evidence of wasting of the facial and bitemporal muscles, baldness, and ptosis. The son at age 30 showed less involvement than had his father at the age of 23, and the disease in the son appears to be following a milder course. The child in the second kindred (Fig. 4B) is too young for us to draw conclusions about age of onset and severity. She shows a relatively smaller reduction (0.5 kb) in size of the repeat (lane 2).

Fragile X syndrome, like DM, is characterized by an unstable trinucleotide repeat (CGG) (*20–22*). In Fragile X, it was previously demonstrated that a reduction in the number of repeats in the maternal allele can occur, from a full to a premutation state, when transmitted (*23*). As in Fragile X, the above individuals with DM show a reduction of triplet repeats, although the number of copies remains greater than the normal population range (*2*, *4*).

Transmission of a DM allele with a reduced number of triplet repeats is in these cases likely the result of mosaicism of the CTG repeats in the affected parent's germline. This is in contrast to our first case described (III-10), where the genetic alterations are not limited to the CTG repeat domain (Fig. 3B): many additional genetic differences were observed between the DM genes of III-10 and her father. Meiotic instability could explain the variability in numbers of CTG repeats, but not the sequence difference observed outside the CTG region.

Our results suggest a discontinuous gene conversion event in the DM allele of an affected individual that resulted in the reduction in the number of trinucleotide CTG repeats to within the normal population range in his daughter. A reduction in triplet repeat number to within the normal range was also reported in a DM kindred by Shelbourne *et al.* (*24*). We have sequenced the entire coding region of the DM gene of individual III-10, and detected no additional rearrangements. Individual III-10 certainly shows little or no indication of DM at an age when her father had obvious muscle involvement. This suggests that variation in the number of CTG repeats is a cause of variability in the clinical phenotype of myotonic dystrophy. This interpretation is supported by a second DM individual who showed a decrease in disease severity corresponding to a reduced number of CTG repeats. The significance of the weak myotonic discharges in III-10 remains unclear. They may foretell the eventual onset of DM, which would raise questions as to the genetic basis of DM given that the number of CTG repeats in this individual falls within the normal range. Further observation of this patient will be of interest.

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8. Gel electrophoresis, Southern blotting, hybridization probing, and autoradiography have been described for the markers APOC2 (*25*), CKM (*25*), and p134C (*26*). Experimental details of Southern blot analysis to determine the extent of the CTG repeat amplification (Eco RI RFLP) and identify the 1-kb insertion (Bgl I RFLP) are as in (*2*).
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13. The marker pH2.3 (Hinf I) was isolated during the generation of a 19q13.3 chromosome walk (*27*). DMK (CTG), DMK (Bgl I), and D19S51 have been described (*2, 26*). Markers pCN400 (*28*), DMK (Hinf I), and DMK (Hha I) are all PCR-based RFLPs (*29*).
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30. We thank G. Shutler for comments on the manuscript, S. Baird for assistance with the figures, A MacKenzie for discussions, S. Leblond for technical assistance, and P. Jacob and I. MacDonald for the EMG and ophthalmologic studies. Supported by grants to R.G.K. from the Muscular Dystrophy Associations of Canada and the United States, the Medical Research Council of Canada, and the Canadian National Centres of Excellence Genetic Disease Network. M.S.M. is supported by a Medical Research Council postdoctoral fellowship and C.T. is supported by an Ontario Ministry of Health genetic fellowship.

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Editing of Transfer RNAs in *Acanthamoeba castellanii* Mitochondria

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With the discovery of RNA editing, a process whereby the primary sequence of RNA is altered after transcription, traditional concepts of genetic information transfer had to be revised. The known RNA editing systems act mainly on messenger RNAs, introducing sequence changes that alter their coding properties. An editing system that acts on transfer RNAs is described here. In the mitochondria of *Acanthamoeba castellanii*, an amoeboid protozoan, certain transfer RNAs differ in sequence from the genes that encode them. The changes consist of single-nucleotide conversions (U to A, U to G, and A to G) that appear to arise posttranscriptionally, are localized in the acceptor stem, and have the effect of correcting mismatched base pairs. Editing thus restores the base pairing expected of a normal transfer RNA in this region.

RNA editing (*1*) constitutes an additional step in the pathway of genetic information transfer. Examples of RNA editing include U addition or deletion in trypanosome mitochondrial RNAs (*2*), C addition in slime mold (*Physarum polycephalum*) mitochondrial RNAs (*3*), and C → U substitution in mitochondrial (*4*) and chloroplast (*5*) RNAs of flowering plants. Not confined to the organelles of eukaryotic cells, RNA editing also affects transcripts of certain nuclear genes, specifically the mRNAs for mammalian apolipoprotein B (*6*) and a mouse glutamate receptor subunit (*7*). These editing systems act on transcripts of protein-coding genes, altering internal nucleotides in the reading frame and occasionally creating new initiation (*8, 9*) and termination (*6, 10*) codons. The consequences of editing range from a complete restructuring of a transcript, with >50% of the final sequence resulting from the editing process (*11*), to a single-nucleotide change in an individual codon (*7*). However, even single-nucleotide editing may alter the properties of the encoded protein (*7*), and in those systems in which it occurs, mRNA editing can be an obligatory step in the production of a functional protein (*12*).

Editing is also seen occasionally in noncoding regions of mRNA molecules (*9, 13*) and has been observed in intron sequences (*14*). To date, however, there is little evidence of editing in RNAs other than mRNAs, with only one reported example (of uncertain function) of apparent ribosomal RNA (rRNA) editing (*15*). The editing of two mammalian cytoplasmic tRNAs, bovine selenocysteine (*16*) and rat aspartate (*17*), has been inferred from the failure to find a gene corresponding precisely in primary structure to the sequenced tRNA. The biological significance of the editing in these examples (both involving pyrimidine transitions) is unclear because most of the apparent changes are localized in single-stranded regions of the tRNA; moreover, the alterations that occur in the rat $tRNA^{Asp}$ are actually away from (rather than toward) a highly conserved nucleotide pattern in the anticodon loop. Here, we document a form of tRNA editing with biological implications in the mitochondria of *A. castellanii*.

In sequencing the mitochondrial DNA (mtDNA) of *A. castellanii*, we found a cluster of five tRNA genes located between the large subunit (LSU) and small subunit (SSU) rRNA genes in the same transcriptional orientation (Fig. 1A). From the DNA sequence of this cluster (Fig. 1B), the secondary structures of the putative tRNA products of these genes could be modeled (Fig. 2). One of these, $tRNA^{Pro}$, has all of the structural features expected of a conventional tRNA (*18*). The other four are also normal, except that all contain one or more mismatched base pairs in the acceptor stem (specifically, pairs 1, 2, 3, or a combination thereof, counting from the 5′ end). Whereas deviations from the prototypical tRNA structure (*18*), including mispairing in normally helical regions, are rather common in mitochondrial tRNAs (*19*), the clustering of irregular features in the acceptor stems of these *A. castellanii* mitochondrial tRNAs is unprecedented. In three cases ($tRNA^{Asp}$, $tRNA^{Met1}$, and $tRNA^{Met2}$), the predicted mispairing in the acceptor stem is so extensive that it would appear to preclude formation of a stable helix extending up to and including the 5′ terminus (Fig. 2). Because the seven base-paired acceptor stem helix is important in the definition of the three-dimensional structure of tRNA (*20*) and the first three base pairs of the acceptor stem engage in specific interactions with aminoacyl-tRNA synthetases (*21, 22*), we investigated whether these aberrant mitochondrial tRNA genes are actually expressed.

Genomic distribution and transcription were examined in a series of Southern (DNA) and Northern (RNA) hybridization experiments with oligonucleotide probes (*23*) complementary to an interior portion of each tRNA sequence (from the 5′ end of the anticodon loop to the 5′ end of the TΨC loop). *Acanthamoeba castellanii* mtDNA is cleaved by Bam HI into four fragments (*24*), and the probes hybridized only to the (completely sequenced) 6.7-kb Bam HI fragment that contains the cluster of five tRNA genes described here (Fig. 3A). This indicates that these particular tRNA sequences are not represented elsewhere in the *A. castellanii* mitochondrial genome, a conclusion verified by additional hybridizations with the same oligonucleotides to Mlu I and Hind III digests of *A. castellanii* mtDNA (Fig. 3A). Parallel probe experiments with highly purified *A. castellanii* nuclear DNA (nDNA) revealed only minor hybridization bands that could be attributed to trace residual contamination with mtDNA (Fig. 3B). A myosin gene-specific probe (*25*) served as a positive

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