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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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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control for the detection of a nuclear gene (Fig. 3C). With each tRNA-specific probe, Northern hybridization revealed a tRNA-sized transcript (*26*). These experiments demonstrate that the five tRNA genes shown in Fig. 1 are the only such genes in either the mtDNA or nDNA of *A. castellanii* and that they produce stable, tRNA-sized transcripts.

To determine whether these tRNAs have the same sequence as the genes that encode them, we used the oligonucleotide probes from the hybridization experiments of Fig. 3 as primers for reverse transcriptase dideoxy sequencing. This allowed us to determine the sequence of the 5′ half of each tRNA, except for the 5′ terminus itself. Comparison of these RNA sequence data with the corresponding DNA sequences shows a number of differences (Fig. 4). For each tRNA, the differences between the DNA and RNA sequences occur only at the positions of mismatches in the acceptor stem; in each case, the changes restore standard base pairing at these positions (Fig. 2). The $tRNA^{Pro}$ gene sequence predicts a tRNA with typical secondary structure and no mispairing in the acceptor stem; here, no differences were found between the DNA and RNA sequences (*26*).

These observations provide evidence for an RNA editing system in *A. castellanii* mitochondria, one that changes the sequence of tRNA molecules to make their final secondary structures conform to that of a conventional tRNA. Because of the importance in tRNA function of higher-order structure in the acceptor stem (*21*, *22*), these results imply that editing is required to produce functional tRNAs in this system. Other than the editing observed specifically at mismatch positions in the acceptor stem, no changes were observed in the ~40% of each tRNA sequence that could be directly surveyed in the experiments reported here, including the positions of mismatches in the dihydrouridine stem of $tRNA^{Asp}$ and $tRNA^{Met2}$ (at 10 and 25). Mismatches in the acceptor stem could potentially be corrected by changes in either the 5′ half or 3′ half of the stem; however, it appears that all of the editing necessary to restore correct base pairing occurs in the 5′ half (Fig. 2). The RNA editing characterized here involves both pyrimidine-to-purine (U → A and U → G) and purine-to-purine (A → G) changes; in this regard, it differs from other editing systems. The mechanism of editing presumably involves either base or nucleotide replacement, rather than base modification.

In the $tRNA^{Ala}$, U3 is edited to an A and not a G, even though a G3:U70 pair is a major determinant of alanine aminoacylation specificity in *Escherichia coli* $tRNA^{Ala}$ (*27*) and is highly conserved in $tRNA^{Ala}$ from various organisms (*28*). An A3:U70 pair has, however, been reported for $tRNA^{Ala}$ from *Bacillus subtilis* [H. Ishikura, K. Murao, and Y. Yamada, cited in (*29*)].

A question in this and other RNA editing systems is what determines the specificity of the editing. In the present case, the editing process must specify final nucleotide identity as well as position. In trypanosome mitochondria, the information for editing appears to reside in small guide RNAs, encoded in both maxicircle and minicircle DNAs, that are partially complementary to the unedited mRNA (*30*). The pattern of editing in the *A. castellanii* system suggests that the unedited tRNA itself may carry the information for editing, with the 3′ half of the acceptor stem functioning as an internal guide RNA to direct editing of the 5′ half. Such a mechanism could be considered a form of directed mismatch repair. The persistence of a G10:U25 pair

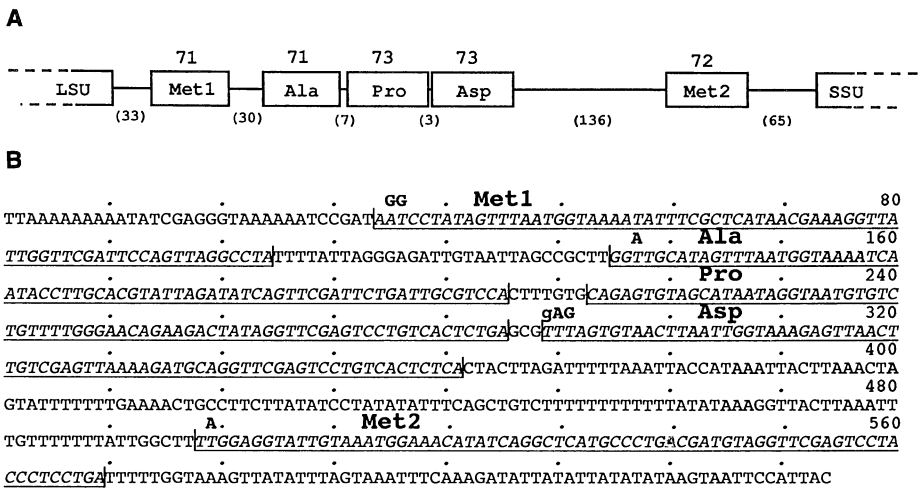

Fig. 1. Organization and sequence of the tRNA gene region located between the LSU and SSU rRNA genes in *A. castellanii* mtDNA. (**A**) Diagram showing the arrangement of tRNA genes, identified on the basis of anticodon sequence. Numbers above each boxed area show the length (in nucleotides) of each tRNA gene (none of which encodes a 3′ terminal CCA sequence); numbers underneath in parentheses indicate the lengths of intergenic spacers. The genes are all organized in the same transcriptional orientation (5′ to 3′, left to right). (**B**) Nucleotide sequence of the tRNA gene region (*34*). The sequence of only that portion of the mtDNA containing the five tRNA genes and flanking spacer regions is shown. Numbering begins immediately after the 3′ terminus of the LSU rRNA gene and concludes immediately before the 5′ terminus of the SSU rRNA gene [both termini have been determined experimentally (*26*)]. The RNA-like strand is shown. Coding sequences of the indicated genes are shown underlined and in italics. Confirmed (uppercase letters) and predicted (lowercase letter) editing sites (Fig. 2) are shown in bold above the sequence.

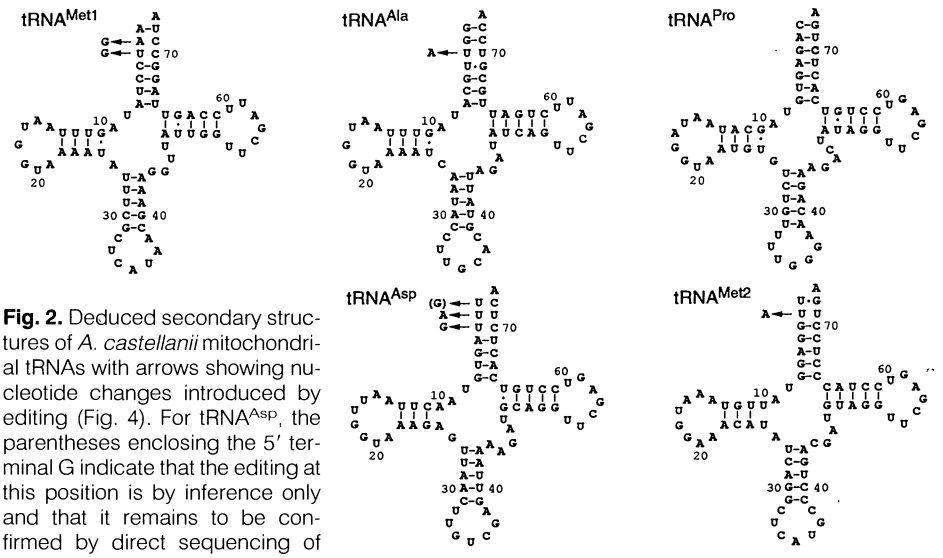

Fig. 2. Deduced secondary structures of *A. castellanii* mitochondrial tRNAs with arrows showing nucleotide changes introduced by editing (Fig. 4). For $tRNA^{Asp}$, the parentheses enclosing the 5′ terminal G indicate that the editing at this position is by inference only and that it remains to be confirmed by direct sequencing of the tRNA. The numbering of nucleotides within the tRNA sequences is according to the standardized scheme (*29*).

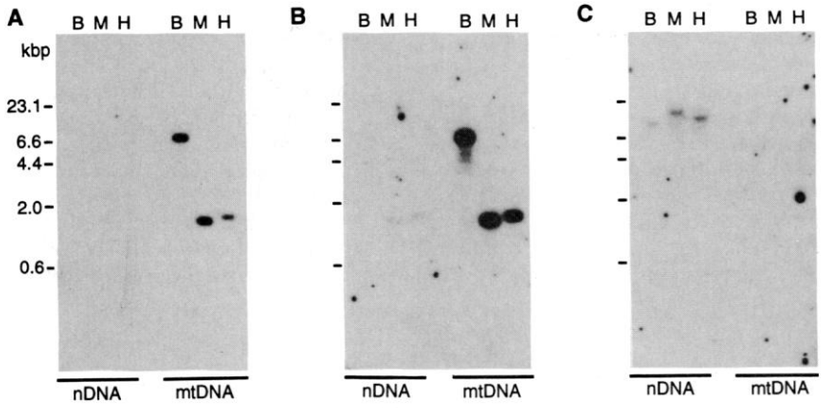

Fig. 3. Autoradiograms showing the results of Southern hybridization experiments (*35*) with tRNA gene-specific oligonucleotide probes. The same blot containing electrophoretically separated Bam HI (B), Mlu I (M), and Hind III (H) digests of nDNA and mtDNA was probed sequentially with 5′ ^{32}P–end-labeled oligonucleotides specific for either the mitochondrial tRNA^{Ala} gene (**A** and **B**) (*23*) or the nuclear myosin IB (MIB) heavy chain gene (**C**) (*25*). The blot in (B) is the same as that in (A) except that we exposed (B) threefold longer to permit detection of any weak nDNA hybridization signals. The migration positions of selected Hind III marker fragments of λ DNA are indicated. After we stripped the blot of bound oligonucleotide, we reprobed it with oligonucleotides (*23*) specific for the other three tRNA genes (Met1, Asp, and Met2) whose products showed evidence of editing, with results similar to those obtained for tRNA^{Ala} (*26*).

in tRNA^{Met1}, tRNA^{Ala}, and tRNA^{Pro} and of a U4:G69 pair in tRNA^{Ala} (assuming in the latter case that G69 is not edited to A) (Fig. 2) suggests that U:G and G:U pairs are not edited in this system. In fact, such pairs are relatively common in helical regions of RNA.

Additional evidence suggests that the editing process described here operates post- rather than co-transcriptionally. After longer exposures of film to sequencing gels, a ladder extending beyond the 5′ terminal stop is evident in each case. The sequences deduced from these ladders correspond to those of unprocessed tRNA precursors that are present at low concentrations in the mitochondrial RNA preparation (*31*). Because the *A. castellanii* mitochondrial tRNA genes are closely spaced (Fig. 1), it was possible after extended electrophoresis for us to read the pre-tRNA sequence from within one tRNA coding region into and through the one immediately upstream. In the case of pre-tRNA^{Met1} and pre-tRNA^{Ala}, comparison of the RNA and DNA sequences showed no differences (Fig. 5); positions edited in the mature tRNA appear unedited in the unprocessed precursor. Because the length and structure of the acceptor stem in pre-tRNA are important for processing by bacterial ribonuclease (RNase) P (*32*), the posttranscriptional maturation of *A. castellanii* mitochondrial tRNA precursors, including any coupling between processing and editing, should be investigated.

It is probable that many of the tRNA species encoded by *A. castellanii* mtDNA are altered by editing. We have sequenced a second cluster of five tRNA genes immediately upstream of the LSU rRNA gene in *A. castellanii* mtDNA. The derived tRNA secondary structures corresponding to four of these genes display a distribution of mismatch in the acceptor stem similar to that documented here. Of the ten *A. castellanii* mitochondrial tRNA genes sequenced so far, we predict the transcripts of eight of these to be edited at a total of 15 different sites (six are confirmed here). In all cases, the confirmed and predicted editing changes have the following specifications: (i) They affect one or more of the first three nucleotides of the 5′ half of the acceptor stem (positions 1, 2, 3, or a combination thereof). (ii) They occur opposite a pyrimidine in the 3′ half of the acceptor stem (positions 70, 71, 72, or a combination thereof). (iii) They involve either pyrimidine-to-purine or purine-to-purine changes. (iv) They result in the formation of a standard base pair (either A:U or G:C) so that the final secondary structure better conforms to that of a conventional tRNA. DNA and limited RNA sequence data for *A. castellanii* mitochondrial rRNA and protein-coding genes and rRNA secondary structure modeling give no indication of editing in these other classes of RNA (*26*).

The editing we describe here is the first documented case of mitochondrial tRNA editing, and the observed pattern suggests that the structural changes made by this editing system are required for biological activity. The editing we have detected is

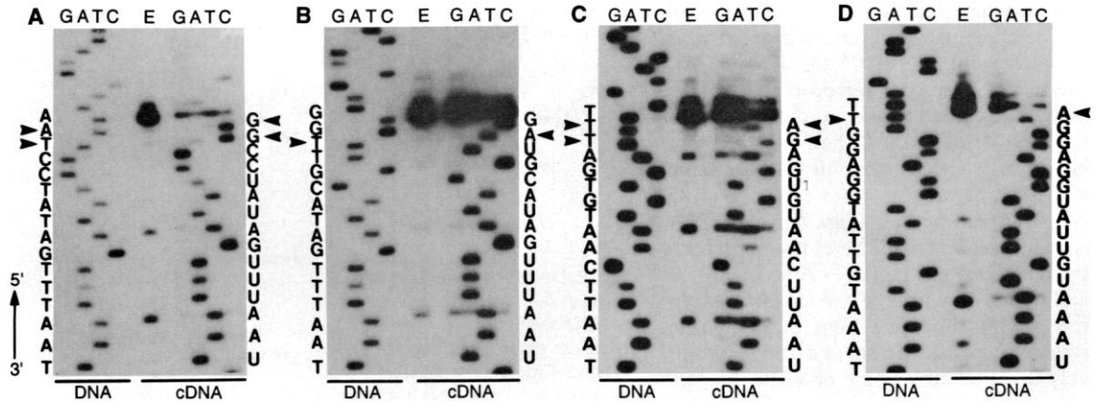

Fig. 4. Comparison of the sequences of tRNA genes and their mature products. Autoradiograms of sequencing gels display DNA and RNA (cDNA) sequences for selected portions of each gene and tRNA, as well as a primer extension lane (E). (**A**) tRNA^{Met1}, (**B**) tRNA^{Ala}, (**C**) tRNA^{Asp}, and (**D**) tRNA^{Met2}. The nucleotide sequence of the RNA-like strand of the DNA and the nucleotide sequence of the RNA (the complements of the sequences actually determined) are shown, reading 3′ to 5′ to facilitate direct comparison with the secondary structures of Fig. 2. Arrowheads indicate differences between the sequences of each tRNA and its gene. DNA and RNA sequences were obtained as described (*34, 36*). Sequencing reaction products and primer extension products were analyzed by electrophoresis on gels with 10% polyacrylamide and 7 M urea (50 cm by 33 cm by 0.025 cm) in TBE [50 mM tris, 50 mM boric acid, and 1 mM EDTA (pH 8.3)] for ~3 hours at 2000 V. X-ray film (XAR-5, Kodak) was exposed to the gels for 1 to 5 days with or without an intensifying screen.

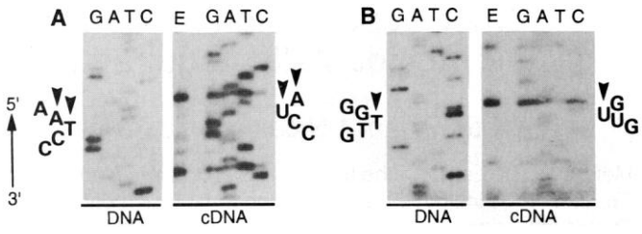

Fig. 5. Comparison of the sequences of the $tRNA^{Met1}$ and $tRNA^{Ala}$ genes and their corresponding precursor tRNAs. Autoradiograms of the sequencing gels display DNA and RNA (cDNA) sequences for selected portions of these genes and their pre-tRNAs, as well as a primer extension lane (E). (**A**) Pre-$tRNA^{Met1}$. (**B**) pre-$tRNA^{Ala}$. The nucleotide sequence of the RNA-like strand of the DNA and the nucleotide sequence of the RNA (the complements of the sequences actually determined) are shown, reading in the 3′ to 5′ direction to facilitate direct comparison with the secondary structures of Fig. 2. Arrowheads indicate positions of editing that occur in the mature tRNA transcripts (Fig. 4, A and B). To obtain the pre-$tRNA^{Met1}$ sequence, we used the $tRNA^{Ala}$-specific oligonucleotide as a sequencing primer; we used the $tRNA^{Asp}$-specific oligonucleotide to obtain the pre-$tRNA^{Ala}$ sequence. DNA and RNA sequences were determined as described (*34, 36*), except that the concentration of ddNTPs used for the RNA sequencing was one-fifth of that described (*36*). Reaction products were analyzed by electrophoresis for 5 to 6.5 hours at 2000 V on gels with 6% polyacrylamide and 7 M urea in TBE. The gels were dried onto 3MM paper (Whatman), and x-ray film (XAR-5, Kodak) was exposed to the gels for at least 14 days in the presence of an intensifying screen.

confined to the first three base pairs of the acceptor stem, and it is this region of the tRNA molecule that frequently provides major identity elements (*22*) and specific contact points (*21*) for the cognate aminoacyl-tRNA synthetase.

This finding of another RNA editing system that acts on tRNAs emphasizes that caution is required when inferring the sequence of either an RNA or a protein from the corresponding DNA sequence. It has become important to determine sequences at the RNA as well as the DNA level, and this is particularly true for mitochondria, where many RNA editing systems function. As deduced from gene sequences, mitochondrial tRNAs frequently depart from conventional structure (*19*). In some cases, inferred abnormalities have been confirmed by direct sequencing of the corresponding tRNAs (*19*); in many other instances, however, only DNA sequence has been reported. Thus, tRNA editing, like mRNA editing, may turn out to be more widespread in nature than we had considered until now.

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31. We can exclude the possibility that the ladders are the result of trace mtDNA contamination because template RNA samples were treated with deoxyribonuclease I (DNase I) before sequence analysis. Consistent with this interpretation, neither the mature tRNA ladder nor the ladder attributed to pre-tRNA was observed if the RNA preparation was treated with RNase A before sequencing (*26*).
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33. *Acanthamoeba castellanii*, strain Neff (American Type Culture Collection 30010) was maintained as a suspension culture at 30°C [R. J. Neff, S. A. Ray, W. F. Benton, M. Wilborn, *Methods Cell Physiol.* **1**, 55 (1964)]. Mitochondrial DNA was prepared by two alternative methods, depending on whether or not mitochondrial RNA was also required. Method 1 (DNA only): Total cellular nucleic acids were isolated from washed cells by conventional phenol-SDS extraction. DNA and small RNAs were separated from high molecular mass, salt-insoluble RNAs (mostly rRNA) by incubation in 1 M NaCl at 4°C for 16 hours. DNA was precipitated from the salt-soluble fraction by the addition of polyethylene glycol (PEG) 6000 (Sigma) to a final concentration of 10% and by incubation on ice for 60 min. After multiple phenol extractions, mtDNA was separated from nDNA and residual RNA by CsCl density-gradient centrifugation in the presence of dye [74 μg/ml; Hoechst 33258 (Sigma)]. Method 2 (DNA and RNA): Nuclei and mitochondria were isolated by differential centrifugation of a whole-cell homogenate by a modification of a procedure provided by E. A. Dawidowicz (Tufts University, Boston). All procedures were performed at 4°C. A 100-ml log-phase suspension culture was centrifuged at 900*g* with swinging bucket rotor (IEC 269; International Equipment Co., Needham Heights, MA) for 2 min, and the cells were washed twice with 0.5 volume of 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl [in phosphate-buffered saline (PBS)]. Cells were resuspended in 5 ml of homogenization buffer [10 mM tris-HCl (pH 7.6), 1 mM $ZnCl_2$, and 0.25 M sucrose] and disrupted in a tissue grinder (Dounce; Kontes, Vineland, NJ). The nDNA was isolated from the crude nuclear pellet (900*g* for 10 min) by phenol-SDS extraction and further purified by two rounds of CsCl density-gradient centrifugation after RNase A treatment. Mitochondria were recovered from the postnuclear supernatant (9000*g* with a fixed angle rotor for 20 min; IEC 870) and washed twice with 5 ml of 10 mM tris-HCl, 2 mM EDTA-Na_2, and 0.35 M sucrose (pH 7.7). Nucleic acids were isolated by phenol-SDS extraction. The mitochondrial nucleic acids were further treated with RNase A for those experiments in which only DNA was required or with DNase I for those experi-

ments in which only RNA was required.

34 GenBank accession number M97651 A 1 5-kb Mlu I fragment of *A. castellanii* mtDNA [method 1 of (*33*)], containing the 3′ terminal region of the LSU rRNA gene, five tRNA genes, and the 5′-half of the SSU rRNA gene, was subcloned into M13 vectors [J Messing, *Methods Enzymol.* **101**, 20 (1983)] from a 6 7-kb Bam HI insert that had been cloned initially into pAT153 [A J Twigg and D. Sherratt, *Nature* **283**, 216 (1980). A nested deletion series was generated [S. Henikoff, *Gene* **28**, 351 (1984)] and both strands of the DNA were sequenced [F Sanger, S. Nicklen, A. R. Coulson, *Proc Natl. Acad Sci U.S.A.* **74**, 5463 (1977); Sequenase, version 2 0 (U.S. Biochemicals, Cleveland, OH)] For some experiments (Figs 4 and 5), tRNA gene-specific oligonucleotides, 5′ end-labeled with ^{32}P, were used as sequencing primers.

35. After RNase A treatment, nDNA and mtDNA [method 2 of (*33*)] were hydrolyzed with restriction endonucleases, and the products (2.5 μg of nDNA and 2.5 ng of mtDNA per lane) were separated by electrophoresis in a 0 7% agarose gel in 40 mM tris acetate and 1 mM EDTA-Na_2 (pH 8.1) for 2 hours at 150 V DNA was transferred to GeneScreen Plus nylon membrane (DuPont Canada, Lachine, Quebec) according to the manufacturer's specifications. Before addition of probe, the blot was hybridized for 2 hours at 65°C in 30 ml of 5× saline sodium citrate (SSC; 1× is 0.15 M NaCl and 0 015 M sodium citrate), 20 mM sodium phosphate (pH 7 0), 10× Denhardt's solution [T. Maniatis, E F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), p 327], 7% SDS, and 10% dextran sulfate Overnight hybridization was with the addition of 100 ng of oligonucleotide probe, 5′ end-labeled with [γ-^{32}P]adenosine triphosphate (ATP) by T4 polynucleotide kinase The hybridization temperature was 6° to 7°C lower than the calculated dissociation temperature (T_d) of the oligonucleotide probe [R A. Zeff and J Geliebter, *Focus* **9** (no. 2), 1 (1987)] The blot was washed for a total of 60 min in 2× SSC and 1% SDS and for 15 min in 0.5× SSC and 0 1% SDS at a wash temperature 7° to 12°C below T_d For reprobing experiments, the blot was stripped of bound oligonucleotide by incubation in 1× SSC at 80°C for 10 min

36 Primer extension analysis and RNA sequencing were performed according to a published protocol [J Geliebter, *Focus* **9** (no. 1), 5 (1987)] but without actinomycin D Mitochondrial nucleic acids were treated with DNase I, and the resulting RNA (10 μg) was annealed to 5 ng of 5′ ^{32}P–end-labeled oligonucleotide (*23*) that served as a primer for extension by avian myeloblastosis virus (AMV) reverse transcriptase in the presence of deoxynucleoside triphosphates and either the presence (G, A, T, and C) or absence (E) of dideoxynucleoside triphosphates (ddNTPs) (Figs 4 and 5). In some experiments, we used terminal deoxynucleotidyl transferase to eliminate sequence ambiguities resulting from the internal pausing of reverse transcriptase [D. C. DeBorde, C. W. Naeve, M. L. Herlocher, H. F. Maassab, *Anal. Biochem.* **157**, 275 (1986)] For $tRNA^{Met1}$ and $tRNA^{Met2}$ (Fig. 4, A and D), the products of standard RNA sequencing reactions were extracted with phenol, precipitated three times with ethanol, dissolved in water, and heated at 80°C for 2 min. Samples were then cooled on ice and incubated with 40 U of terminal deoxynucleotidyl transferase (Promega) in the presence of 1 mM dATP, 100 mM cacodylic acid (pH 6.8), 1 mM $CoCl_2$, 0.1 mM dithiothreitol, and bovine serum albumin (0.1 mg/ml) in a 20-μl reaction volume at 37°C for 60 min Samples were precipitated with ethanol before we loaded them onto the sequencing gel.

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Tumor Suppression in *Xiphophorus* by an Accidentally Acquired Promoter

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Melanoma formation in the teleost *Xiphophorus* is caused by a dominant genetic locus, *Tu*. This locus includes the X*mrk* oncogene, which encodes a receptor tyrosine kinase. Tumor induction is suppressed in wild-type fish by a tumor suppressor locus, *R*. Molecular genetic analyses revealed that the *Tu* locus emerged by nonhomologous recombination of the X*mrk* proto-oncogene with a previously uncharacterized sequence, *D*. This event generated an additional copy of X*mrk* with a new promoter. Suppression of the new X*mrk* promoter by *R* in parental fish and its deregulation in hybrids explain the genetics of melanoma formation in *Xiphophorus*.

Melanoma formation in *Xiphophorus* has been studied since 1922 (*1*). Crossing of a tumor-free wild-type strain carrying *Tu* and *R* with a strain carrying neither locus produces progeny that develop tumors. The emergence of this phenotype is due to the loss of *R*-bearing chromosomes in the hybrids, which allows expression of the full oncogenic potential of *Tu* (*2*). We have addressed how such a potentially injurious locus was generated and maintained during evolution and how its transforming activity is suppressed in the feral populations.

We recently showed that X*mrk*, a gene encoding a receptor tyrosine kinase closely related to the epidermal growth factor (EGF) receptor, is the critical constituent of the *Tu* locus (*3*). X*mrk* is present in the *Xiphophorus* genome in two different copies. All examined specimens of the genus *Xiphophorus* and related species contain one X*mrk* gene that fulfills all the characteristics of a proto-oncogene (*4*, *5*). A second copy of X*mrk* is present only in individuals that carry a *Tu* locus. This second copy is a structural constituent of the *Tu* locus (*3*–*5*) and is the melanoma-inducing oncogene. The two genes reside within 2 centimorgans (cM) of each other on the sex chromosomes (*5*), with X- and Y-chromosomal alleles of the oncogene (*4*).

The proto-oncogene is differentially expressed during embryogenesis and is expressed at low levels in adults in normal epithelial tissues as well as in melanoma. Its expression is independent of *R*. The oncogene, however, is only expressed in melanoma (at high levels) and only when *R* is absent (*3*, *4*, *6*). Primer extension analyses have revealed that the X*mrk* oncogene and proto-oncogene have different transcription start sites (*4*, *7*) and that their transcripts differ in size by ~1 kb (*4*). These observations indicate that there may be differences in transcriptional control between the X*mrk* oncogene and proto-oncogene, possibly because of structural differences in the 5′ promoter region.

To investigate this potential structural difference, we cloned a full-length cDNA corresponding to the X*mrk* proto-oncogene from the *Xiphophorus* embryonal epithelial cell line A2 (*8*). Alignment of the clone with the previously isolated oncogene cDNA (*3*) revealed colinearity of the sequences starting 3′ of codon 10 in exon 1 (Fig. 1). The two sequences had no similarity 5′ of this region, however. This observation confirmed that the X*mrk* oncogene and proto-oncogene had different 5′ regions and thus possibly also different promoters and regulatory sequences.

To isolate the respective promoter regions, we first screened a genomic library of *Xiphophorus maculatus* with a DNA fragment comprising the 5′ end of the oncogene cDNA. A 5.3-kb clone (λX21-1) was isolated. This clone contained exon 1 of the X*mrk* oncogene as well as 5′ flanking sequences with TATA- and CAAT-like elements located at the expected distance from the transcription start site (Fig. 1). Surprisingly, the potential promoter se-

Table 1. Expression of the X*mrk* oncogene promoter in the absence (PSM cells) or presence (A2 cells) of the *R* locus. CAT conversion rates obtained with the oncogene promoter are shown relative to the basal CAT conversion rates seen with the internal standards in two independent experiments (*25*).

Experiment	Cells	CAT activity
1	PSM	8.96
	A2	1.42
2	PSM	36.28
	A2	5.56

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