

# The *mle<sup>napts</sup>* RNA Helicase Mutation in *Drosophila* Results in a Splicing Catastrophe of the *para* Na<sup>+</sup> Channel Transcript in a Region of RNA Editing

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## Summary

The *mle<sup>napts</sup>* mutation causes temperature-dependent blockade of action potentials resulting from decreased abundance of *para*-encoded Na<sup>+</sup> channels. Although *maleless* (*mle*) encodes a double-stranded RNA (dsRNA) helicase, exactly how *mle<sup>napts</sup>* affects *para* expression remained uncertain. Here, we show that *para* transcripts undergo adenosine-to-inosine (A-to-I) RNA editing via a mechanism that apparently requires dsRNA secondary structure formation encompassing the edited exon and the downstream intron. In an *mle<sup>napts</sup>* background, >80% of *para* transcripts are aberrant, owing to internal deletions that include the edited exon. We propose that the Mle helicase is required to resolve the dsRNA structure and that failure to do so in an *mle<sup>napts</sup>* background causes exon skipping because the normal splice donor is occluded. These results explain how *mle<sup>napts</sup>* affects Na<sup>+</sup> channel expression and provide new insights into the mechanism of RNA editing.

## Introduction

RNA helicases comprise a large and ubiquitous family of proteins that have been implicated in processes ranging from mRNA stability, transport, and splicing to regulation of protein translation. Several reports of knockout mutations of RNA helicase genes in organisms from yeast to mammals indicate their essential roles in normal development (Lee et al., 1998; Machesky et al., 1998; Zaffran et al., 1998). However, little evidence exists for definitive roles and specific substrates for RNA helicases in vivo. The *maleless* (*mle*) locus in *Drosophila* encodes an ATP-dependent double-stranded RNA (dsRNA) helicase that is involved in dosage compensation (Kuroda et al., 1991). Curiously, the *napts* allele (for *no action potential*) of *mle* was originally isolated on the basis of its temperature-sensitive (ts) paralytic phenotype, which is associated with a temperature-dependent block in nerve conduction at restrictive temperatures (Wu et al., 1978). The behavioral and electrophysiological phenotypes of the *mle<sup>napts</sup>* mutation are nearly indistinguishable from those of *para<sup>ts</sup>* mutations, which are conditional mutations in the gene encoding the primary

type of Na<sup>+</sup> channel expressed in the *Drosophila* nervous system (Loughney et al., 1989; Hong and Ganetzky, 1994). Numerous genetic experiments strongly suggest that the phenotypic defects of *mle<sup>napts</sup>* result from an unconditional decrease in the expression of *para*-encoded Na<sup>+</sup> channels and that this deficit in wild-type Na<sup>+</sup> channel expression confers a ts paralytic phenotype on mutant flies (Ganetzky and Wu, 1982a, 1982b; Ganetzky, 1984; Loughney et al., 1989). The most compelling genetic evidence is that the paralytic phenotype of *mle<sup>napts</sup>* mutants can be completely rescued by the addition of a single extra dose of *para*<sup>+</sup> (Stern et al., 1990).

Although these results indicate that *para* expression is aberrant in an *mle<sup>napts</sup>* background, the exact molecular mechanism by which this occurs has remained uncertain. Genetic and molecular studies revealed that *napts* is an unusual allele of the *mle* locus (Kernan et al., 1991). *mle* is one of several autosomal genes required for dosage compensation—the mechanism by which the transcriptional activity of the single X chromosome in *Drosophila* males is doubled relative to that of each X chromosome in females (Kelley and Kuroda, 1995). Null mutations of *mle* are male specific lethals, owing to inadequate expression of X-linked genes. Although *para* is X linked, several lines of evidence indicate that *mle<sup>napts</sup>* does not perturb dosage compensation of X-linked genes in general or *para* in particular.

These observations suggested that Mle must have some other non-sex-specific regulatory role that is disrupted by the *mle<sup>napts</sup>* mutation. In support of this interpretation, Mle is found in the nuclei of both male and female cells (Kuroda et al., 1991). However, in male nuclei Mle is predominantly localized to the X chromosome, whereas in female nuclei no such preferential association is observed. Thus, Mle was proposed to increase expression of *para*<sup>+</sup> in both sexes by a mechanism that is independent of dosage compensation. The demonstration that Mle is a dsRNA helicase related to known splicing factors raised the possibility that the protein could act at the level of *para* RNA processing (Kernan et al., 1991).

Here, we report the discovery that the *para* transcript undergoes adenosine-to-inosine (A-to-I) RNA editing like that first observed for mRNA encoding mammalian glutamate receptor subunits (GluRs). What is known about the mechanism of this type of editing in other systems highlights the possibility that the *mle<sup>napts</sup>* defect is exerted through an involvement in RNA editing. RNA editing of GluRs and serotonin 5-hydroxytryptamine 2C receptor (5-HT<sub>2C</sub>R) in the mammalian brain introduces changes in the coding potential of messages via hydrolytic deamination of adenosine (A) residues to inosine (I) (Bass, 1997; Rueter and Emeson, 1998). Altered coding is due to the base-pairing properties of inosine, which resemble those of guanosine (G). Editing of two distinct sites (the Q/R and R/G sites) in transcripts from several *GluR* genes has profound functional consequences. For instance, the Q/R site controls Ca<sup>2+</sup> permeability, while the R/G site affects rates of receptor desensitization (Kohler et al., 1993; Lomeli et al., 1994).

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As elucidated for *GluR* transcript editing sites, the mechanism of A-to-I editing requires a *cis* region of the primary transcript that extends into the intron downstream of the exonic editing site (Higuchi et al., 1993; Herb et al., 1996). This intronic region contains an editing site-complementary sequence (ECS) that base pairs with the exonic sequences surrounding the edited adenosine to form a dsRNA substrate for the editing enzyme, an adenosine deaminase that acts on RNA, or ADAR (Bass, 1997; Bass et al., 1997). Furthermore, the region between the ECS and the exonic editing site may contain extended inverted repeat hairpins or more extensive secondary structures with significant stretches of duplex dsRNA. Formation of this large scale dsRNA structure brings the ECS and the editing site sequences into appropriate juxtaposition for efficient editing. It is presumed that subsequent to editing, the dsRNA structure must be resolved before splicing can take place.

As is the case for *GluRs*, we find that the *para* transcript also contains a putative ECS in the intron downstream of the edited exon and that this region is predicted to form an extensive dsRNA secondary structure. Because of the stability of such an extended dsRNA structure, its resolution is likely to require the activity of an enzyme such as the ATP-dependent dsRNA helicase encoded by *mle*. We have investigated this possibility by detailed analysis of *para* transcripts in wild-type and *mle<sup>napts</sup>* mutant backgrounds. The *mle<sup>napts</sup>* mutation results in the occurrence of a "splicing catastrophe" in the region of the editing site: the majority of *para* transcripts in an *mle<sup>napts</sup>* background contain internal deletions encompassing the edited exon. These deletions result from exon-skipping events caused by the utilization of inappropriate splice donors upstream of the edited exon. The pattern of exon-skipping events can be readily explained if normal splicing requires resolution of the predicted dsRNA secondary structures and if this step is impaired in an *mle<sup>napts</sup>* background. These results fully explain the *mle<sup>napts</sup>* phenotype and demonstrate that in addition to its function in dosage compensation, the Mle dsRNA helicase plays a role in RNA editing.

## Results

### *para* cDNAs Show Sequence Changes Indicative of RNA Editing

Sequence analysis of a set of partial *para* cDNAs revealed a region of 12 nucleotides within which several purine transitions (A-to-G) were observed. These types of changes have been seen in transcripts that undergo A-to-I RNA editing. Since these partial cDNAs were obtained by RT-PCR, polymerase error might account for the observed sequence changes arising during reverse transcription or amplification of the cDNAs. Another possible explanation was that these sequence variants were genomically encoded and introduced by alternative splicing. To test these possibilities, we cloned and sequenced the corresponding genomic DNA (n = 12) as well as a large number (n = 77) of partial cDNAs that span the region of suspected editing. The results of this analysis are compatible only with the interpretation of A-to-I RNA editing. First, A-to-G transitions are found only in cDNAs, not in genomic DNAs cloned utilizing

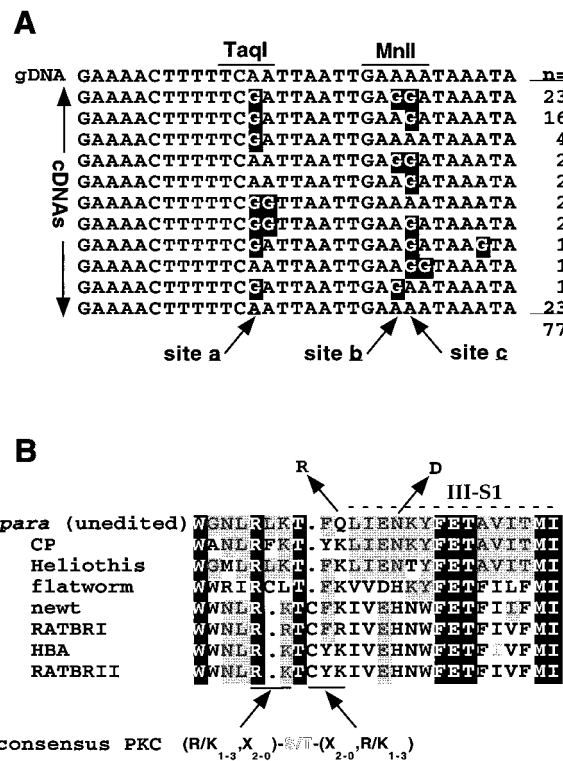


Figure 1. *para* cDNA and Polypeptide Variants Resulting from RNA Editing

(A) Sequence analysis of 77 *para* cDNAs from a wild-type background is summarized and compared with genomic sequence of the same region of *para*. The number of cDNAs with the given sequence is shown. TaqI (TCGA) and MnII (GAGGA) sites, which are generated by RNA editing, are indicated above the sequence.

(B) Amino acid sequence alignment of *para* and other Na<sup>+</sup> channel genes is shown for the region of RNA editing. III-S1 refers to the first transmembrane domain (S1) of the third homology domain. Na<sup>+</sup> channels in the comparison are Colorado potato beetle (CP), tobacco budworm (*Heliothis*), flatworm (*Bdelloura candida*), newt (*Cynops pyrrhogaster*), rat brain I, HBA (human), and rat brain II. The *para* sequence listed is the completely unedited version. Arrows indicate the amino acid changes introduced by editing.

the same PCR primers under the same conditions (see Experimental Procedures; Figure 1A). Second, genomic sequence analysis confirmed the absence of alternative exons in this region of the *para* transcript. PCR across the relevant genomic region using several different primer pairs always resulted in the production of single bands, and sequence analysis revealed that these PCR products contain only the known *para* sequence, with an adenosine residue located at all presumptive editing positions. Third, the nucleotide changes within the edited region occurred independently of each other, and several more rarely modified sites were found (Figure 1A). Fourth, the sequence changes introduced restriction sites for TaqI and MnII. Restriction analysis of a small number of cDNAs from another species, *Drosophila simulans* (estimated 1 million year divergence), revealed the same cDNA sequence changes occurring at frequencies similar to those observed in *Drosophila melanogaster*. Fifth, when analyzing a particular rare (<2% of total *para* cDNAs) *para* splice variant involving alternative splicing downstream of the RNA editing site,

we found that editing was dramatically less frequent in this form (11% versus 70%). This rules out polymerase error during the reverse transcriptase step of RT-PCR or artifacts due to the method of RNA isolation as sources for the observed changes. Finally, the same frequency of cDNA modification was observed when high-fidelity thermostable polymerases were used in PCR and editing was assayed by cloning and restriction analysis. Thus, these changes do not reflect alternative splicing, sequence polymorphisms, or polymerase artifacts; they are consistent with all of the criteria for sites of A-to-I RNA editing.

Seventy percent of characterized *para* cDNAs undergo some form of editing, with the most frequent modifications occurring at three sites called *a*, *b*, and *c* (Figure 1A). Although editing at site *b* is in the third position of a codon and is silent, editing at the other two sites introduces amino acid changes in a conserved segment of the Na<sup>+</sup> channel near and just within the III-S1 transmembrane domain (Figure 1B). Editing at site *a* results in a Q/R (CAG-to-CGG) substitution that introduces a basic residue, creating a strong consensus protein kinase C (PKC) phosphorylation site (R/K<sub>1-3</sub>, X<sub>2-0</sub>) – S\*/T\* – (X<sub>2-0</sub>, R/K<sub>1-3</sub>) (Kemp and Pearson, 1990; Kennelly and Krebs, 1991). Moreover, in a number of other invertebrate and vertebrate Na<sup>+</sup> channels, the genome encodes a basic residue at this position that resides in a strong PKC consensus sequence. The amino acid change introduced by editing at site *c* results in an N/D (AAT-to-GAT) substitution within the III-S1 transmembrane domain. This residue is histidine (H) in almost all other invertebrate and vertebrate Na<sup>+</sup> channels (Figure 1B).

#### *para* Editing Sites and Putative ECSs Are Evolutionarily Conserved

We used an evolutionary approach to investigate the functional significance of editing of *para* transcripts. We reasoned that if this editing is evolutionarily conserved, it has been preserved by natural selection and, therefore, must be biologically important to the organism. To this end, we assayed RNA editing of *para* transcripts from *Drosophila virilis*, a species that diverged from *D. melanogaster* 60–80 million years ago (Powell, 1997). Preliminary sequence analysis of a limited number of cDNAs from *D. virilis* confirmed the conservation of edited adenosine residues in the corresponding region of the *D. virilis para* transcript. A more detailed analysis of editing was performed on a large number of *D. virilis* cDNAs by restriction analysis with TaqI (see Figure 1A). Editing at position *a* in *D. virilis* occurs at nearly the same frequency as in *D. melanogaster*, 53.4% ± 6.2% versus 57.4% ± 5.0%, respectively. Sequence analysis of the corresponding genomic region from *D. virilis* confirmed the presence of adenosine residues at all of the conserved editing sites and the lack of sequence changes at these sites in cloned genomic DNA. We conclude that RNA editing of *para* is not unique to *D. melanogaster* but has been conserved over a long evolutionary period and is therefore likely to be of biological significance.

The editing of *para* transcripts described above results in A-to-G changes in cDNAs. These changes are the same type as those observed in mammalian *GluR-B* transcripts at the Q/R and R/G editing sites. Editing of *GluR-B* transcripts requires the enzymatic deamination of A to I, whose base-pairing properties resemble

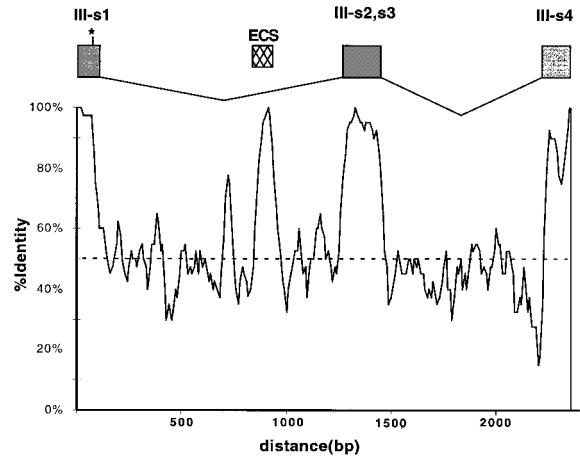


Figure 2. Plot of Sequence Identity between *D. melanogaster* and *D. virilis* Editing Sites

Sequence analysis was performed on cloned genomic DNA of *D. melanogaster* and *D. virilis* in the region of the RNA editing site. The sequences were then aligned and plotted as percent identity (see Experimental Procedures). Boxed regions indicate exonic sequences and the presumptive ECS. An asterisk indicates the location of the RNA editing site. III-s1, -s2, -s3, and -s4 refer to homology domain III, transmembrane domains s1–s4.

G. Consequently, we propose that the *para* transcript also undergoes A-to-I editing by a mechanism similar to that occurring in *GluR-B* transcripts. This mechanism involves base pairing between the edited exons and ECSs contained within the downstream intron. In addition, other portions of the intron sequence near the ECS form large scale RNA secondary structures that bring the edited exon and ECS into proper alignment, creating a substrate for the presumptive editing enzyme ADAR. If *para* transcripts undergo editing by a similar mechanism, the existence of a sequence complementary to the edited exon in the downstream intron is predicted. Furthermore, because editing is conserved between *D. melanogaster* and *D. virilis*, the ECS should also be conserved in these species.

To investigate these predictions, we compared the *D. melanogaster* and *D. virilis* genomic DNA sequences through the region of RNA editing (Figure 2). As shown, *para* coding sequences are highly conserved between the two species, approaching 100% identity at the nucleotide level. In contrast, intron sequences are not highly conserved between these species and rarely exceed 50% identity. However, we observed an exception in the intron immediately downstream of the edited exon. Here, we found a region extending over 87 bp whose level of sequence identity approaches that of exonic sequences between the two species (94%). In addition, examination of this conserved intronic sequence reveals that it contains extensive “complementarity” with the edited exon and thus corresponds to a potential ECS.

#### The Intron Downstream of the Edited Exon May Form an Extensive RNA Secondary Structure

If the mechanism of editing of *para* transcripts is similar to that of mammalian *GluR* transcripts, then the downstream intron should be capable of forming an extended dsRNA secondary structure that juxtaposes the *para*

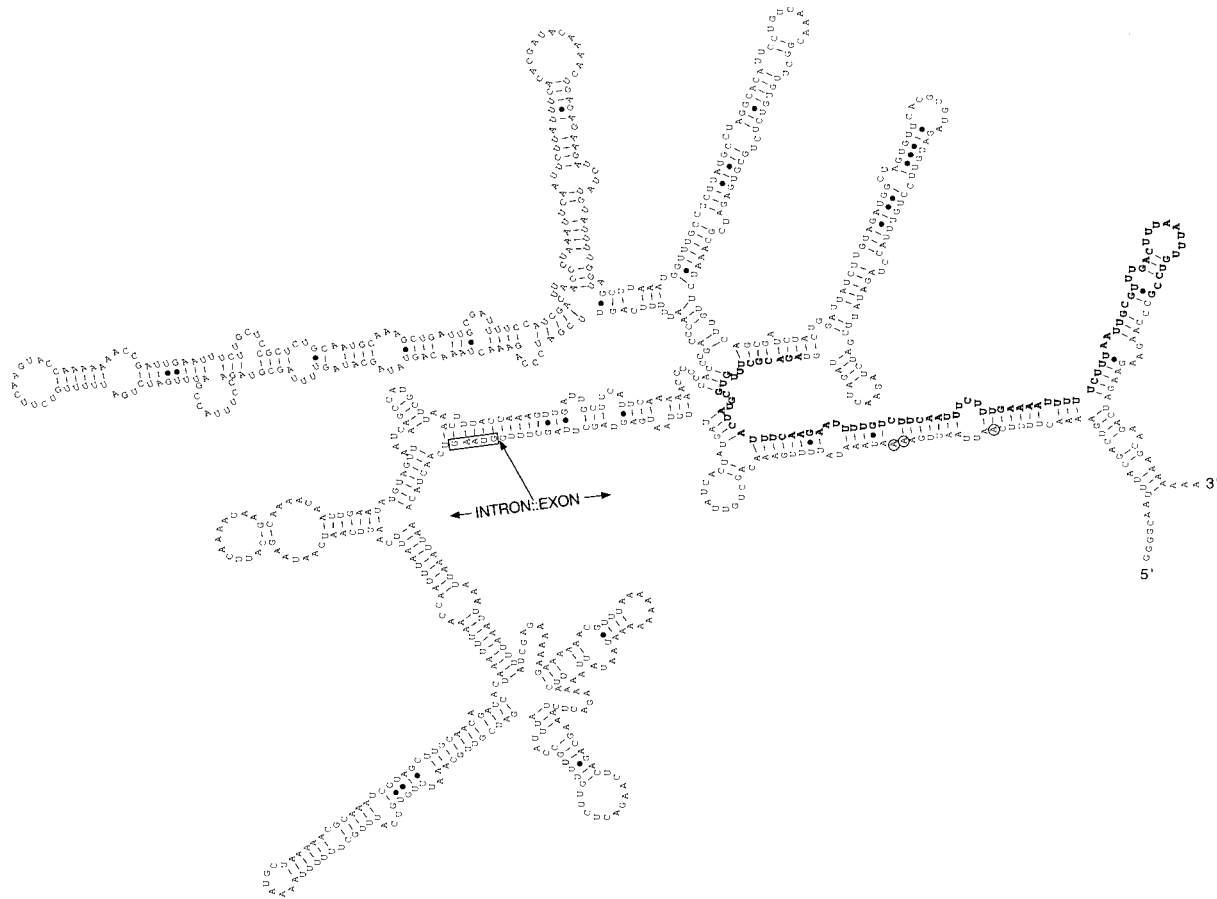


Figure 3. RNA Secondary Structure of the *para* Transcript in the Region of the RNA Editing Site

Exonic and intronic sequences were used to predict an RNA secondary structure for the editing site based on conserved sequences (see Experimental Procedures); 5' and 3' ends of the transcript are indicated. An arrow indicates the position of the 5' intronic donor site, and the ECS is indicated by bold text. The most frequently edited adenosines are circled. The 5' splice donor is indicated by the boxed GUAAG.

editing site and the ECS. We utilized RNA secondary structure prediction programs (see Experimental Procedures) to obtain hypothetical RNA secondary structures using the entire edited exon and downstream intronic sequences of *D. melanogaster* as input sequence. The program predicted a complex, highly base-paired RNA secondary structure for this sequence (Figure 3). The salient feature of this structure is that it brings the putative ECS into register with the edited exonic sequences. Although the sequence of the downstream intron of *D. virilis* differs substantially from that of *D. melanogaster* outside of the ECS, the computer program also predicted an extensive dsRNA secondary structure for the *D. virilis* sequence that aligns the editing site with the corresponding ECS (data not shown). The *D. melanogaster* and *D. virilis* RNA structures also share a high level of "double-strandedness" (64% and 67% base pairing, respectively), although the overall secondary structures bear little resemblance to one another. The key structural feature in common is the alignment of editing sites with intronic ECSs (Figure 4). The *D. melanogaster* and *D. virilis* structures are nearly identical near the editing sites; edited adenosine residues are in identical positions. Moreover, the edited adenosine

residues are contained in regions of dsRNA duplex that bear striking similarity to the proposed structures for the 5-HT<sub>2c</sub>R and GluR-B,5,6 Q/R editing sites (Rueter and Emeson, 1998).

#### The Sequence Encompassing the Predicted RNA Secondary Structure Is Sufficient to Direct RNA Editing In Vivo

If the above model for editing of *para* transcripts is correct, the sequences needed to direct this process should reside entirely within the edited exon and the downstream intron. To test this model, we generated a minimal expression construct for assaying RNA editing of *para* transcripts in vivo. This construct encompassed a portion of the genomic region of *para* from 229 bp upstream of the editing site to 174 bp downstream from the end of the predicted ECS. This genomic segment essentially encodes only that portion of the *para* transcript represented in the RNA secondary structure shown in Figure 3. This segment was placed into a heat shock-inducible P element vector and introduced into *D. melanogaster* via germline transformation. This minimal construct contains the normal 5' splice donor site for the editing exon (Figure 5A) but lacks the normal 3'

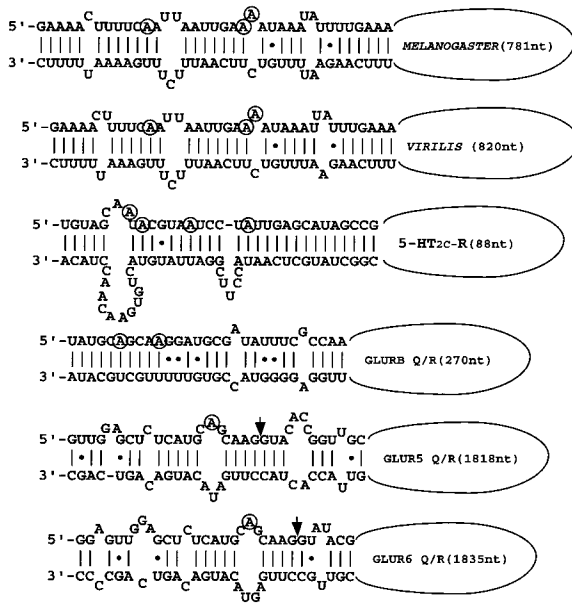


Figure 4. Detailed Comparison of the dsRNA Substrate in the Edited Region of the *para* Transcript with Other Known Edited Transcripts. Localized base-paired structures are shown in the vicinity of edited adenosines for the *D. melanogaster* and *D. virilis* editing sites, the GluR-B,5,6 Q/R sites, and the serotonin receptor (5-HT<sub>2C</sub>R). Edited adenosines are indicated by circles. Numbers of nucleotides omitted from the predicted secondary structures are indicated inside looped regions. GluR and 5-HT<sub>2C</sub>R structures are adapted from Figure 2, Rueter and Emeson (1998).

acceptor site. Nevertheless, RNAs transcribed from this transgene are spliced. Sequence analysis revealed that all transcripts derived from the transgene splice from the normal 5' donor site to one of two cryptic acceptor sites; one within the *para* intronic sequences and the other in the actin 3' untranslated (UTR) sequences (Figures 5A and 5B). What is most important is that high levels of editing were found in RNA transcribed from the transgene as assayed by restriction analysis of RT-PCR products (Figure 5B). In the endogenous *para* transcript, multiple adenosine residues at this location undergo editing. To address whether editing of RNA from the minimal *para* construct faithfully reproduced that of the endogenous gene, direct sequence analysis of RT-PCR products was performed. These comparisons revealed that editing of the mini-*para* transcript within this exon was indistinguishable from that occurring in the same region of the much larger native *para* transcript despite the difference in context (Figure 5C). We have proposed that RNA editing of *para* requires the formation of a large dsRNA secondary structure that brings an evolutionarily conserved ECS into alignment for base pairing with the edited exon. The results described in this section demonstrate that all the sequence information required for faithful editing of the *para* transcript is contained entirely within that region of the RNA predicted to form the dsRNA secondary structure. These results provide direct evidence for the functional significance of this RNA segment in vivo and support the conclusion that A-to-I editing of the *para* transcript involves an ECS-based mechanism analogous to that described for mammalian *GluR* transcripts.

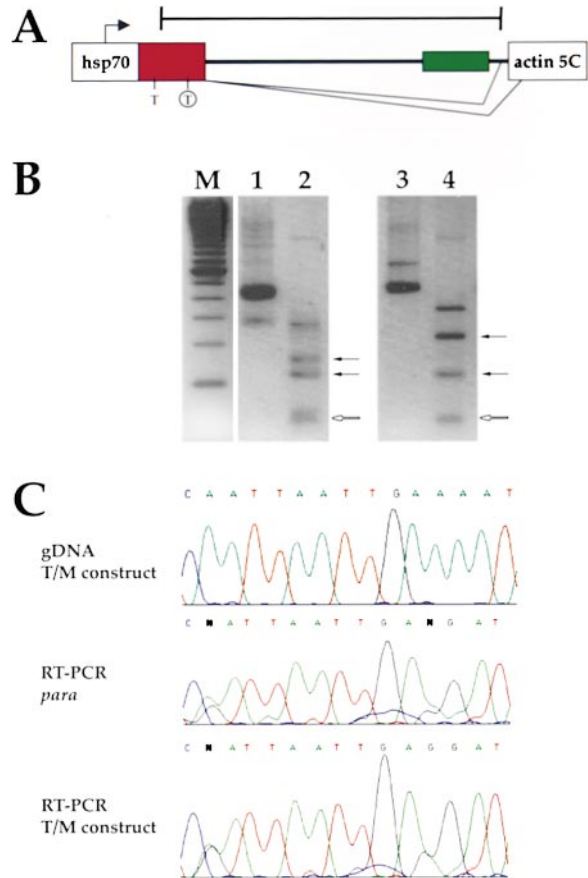


Figure 5. A Minimal *para* Transgene Faithfully Undergoes RNA Editing

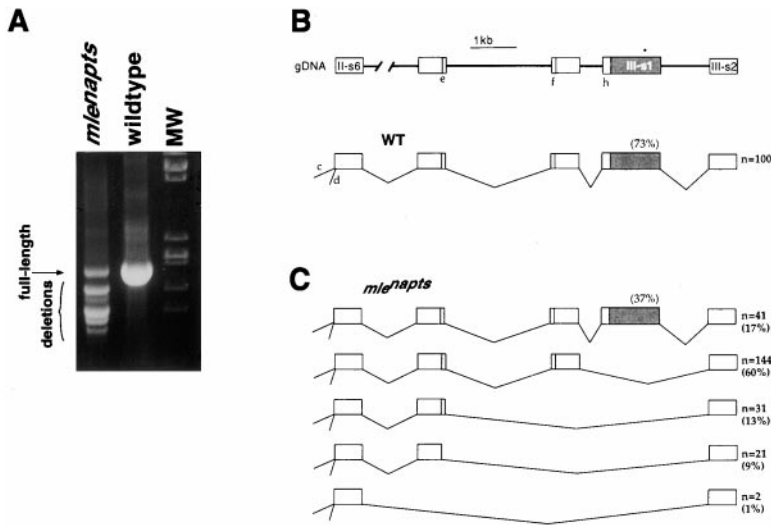
(A) Depiction of sequences used in the pCasPeR-hs/act:::para/T/M transgene. The two observed 3' splice acceptor sites are shown. TaqI restriction enzyme sites are indicated by T. Circled T represents TaqI site introduced by RNA editing. The bracket indicates the extent of sequence shown in Figure 3 RNA secondary structure. Red box indicates *para* exon. Line indicates intronic sequences. ECS is indicated by green box.

(B) Restriction analysis of *para* and transgene RT-PCR products. Lanes 1 and 3 are undigested RT-PCR products of transgene and *para*, respectively. Lanes 2 and 4 are RT-PCR products digested with the enzyme TaqI for both transgene and *para*, respectively. M is 100 bp ladder molecular weight marker. Closed arrows indicate positions of bands generated by restriction at the TaqI site introduced by RNA editing. Open arrow indicates control band for TaqI digestion (see Experimental Procedures).

(C) Direct sequence analysis of *para* and transgene RT-PCR products. PCR products of genomic DNA from transgenic construct (gDNA T/M construct) and RT-PCR products of transgene and cognate *para* transcripts were subjected to direct automated sequence analysis (see Experimental Procedures). Presence of mixed adenosine (green) and guanosine (black) sequence signals are indicative of RNA editing.

### The *para* Transcript Undergoes a Splicing Catastrophe in an *mle<sup>napts</sup>* Background

The results of the above transgenic experiments suggest that extensive dsRNA structures such as that shown in Figure 3 may actually form in vivo. Because these structures may be extremely stable, their resolution seems unlikely to occur spontaneously but rather is energy dependent and enzyme catalyzed. This notion



**Figure 6.** Splicing Catastrophe of *para* RNA in *mle<sup>napts</sup>* Background Generates Deleted Transcripts

(A) Agarose gel showing RT-PCR products derived from *para* transcripts in the region of the RNA editing site amplified from wild-type and *mle<sup>napts</sup>* backgrounds. Note the depletion of full-length *para* products in the *mle<sup>napts</sup>* background and the appearance of a ladder of smaller-sized products.

(B and C) Analysis of *para* cDNAs from wild-type ( $n = 100$ ) (B) and *mle<sup>napts</sup>* ( $n = 239$ ) (C) backgrounds in the region of the RNA editing site (indicated by asterisk). cDNAs were characterized into size classes for the *mle<sup>napts</sup>* samples, and then several representatives of each class were subjected to DNA sequence analysis. Numbers in parentheses over the III-s1 exon are the percent of total transcripts that are edited in each background. "c," "d," "e," "f," and "h" refer to alternative exons in *para* (Thackeray and Ganetzky, 1994). II-s6 refers to homology domain II, transmembrane domain s6. III-s1 and -s2 refer to homology domain III, transmembrane domains s1 and s2.

immediately raised the question of whether some aspect of the RNA editing process is disrupted by *mle<sup>napts</sup>*, whose paralytic phenotype has been attributed to a reduction in expression of *para*-encoded  $\text{Na}^+$  channels and which is a mutation of a gene encoding an ATP-dependent dsRNA helicase.

To address this question, we performed RT-PCR on RNA isolated from wild-type and *mle<sup>napts</sup>* flies using primers spanning the editing sites (Figure 6A). In contrast to the products obtained using RNA from wild type, which forms a single band of the predicted size, only a minority (<20%) of *para* RT-PCR products from an *mle<sup>napts</sup>* background represent full-length transcripts for this region. The majority of amplification product from *mle<sup>napts</sup>* mutants is detected as a ladder of discrete, smaller molecular weight bands that represent transcripts deleted for differing extents of this region. Similar analyses using different primer sets did not uncover any differences between wild type and *mle<sup>napts</sup>* in product size or abundance for any other region of the *para* transcript (data not shown).

To elucidate the precise nature of the aberrant *para* transcripts, a library was made of the RT-PCR products from wild-type and *mle<sup>napts</sup>* backgrounds, and the cDNAs were sorted into size classes. Several representatives of each size class were sequenced. From this analysis, we determined that the cDNA products obtained from an *mle<sup>napts</sup>* background contained deletions representing specific exon-skipping events (Figure 6B). All of the deletions begin at inappropriate upstream splice donor sites, and they all terminate at a common 3' splice acceptor site corresponding to the usual acceptor site of the intron downstream of the edited exon. Most often, a single exon is skipped. An additional one to three exons are skipped with decreasing frequencies, owing to less frequent utilization of more distal 5' donor sites. The consequence of this pattern of exon skipping is that all of the deleted transcripts skip the exon in which RNA editing occurs. A plausible explanation for this result emerges from the observation that the appropriate splice donor

is located between the editing site and the downstream intronic ECS and is thus contained within the proposed dsRNA intermediate required for RNA editing (Figure 3). If resolution of this structure were delayed or impaired in an *mle<sup>napts</sup>* mutant background, the correct splice donor would be inaccessible to the splicing machinery, thereby inducing the utilization of inappropriate upstream donor sites in order for splicing to proceed. Because all of the deleted *para* transcripts from an *mle<sup>napts</sup>* background would lack essential coding regions or cause translational frameshifting, only about 20% of *para* transcripts in an *mle<sup>napts</sup>* background are expected to encode functional  $\text{Na}^+$  channels.

Immediately upstream of the editing exon are several regions in which alternative splicing occurs (c/d, e/f, h; see Figure 6B) (Thackeray and Ganetzky, 1994). In each case, for a given class of *para* cDNA deletions, the alternative splicing events occurring upstream of the 5' deletion donor occur at their expected frequencies (data not shown). However, the usage of 5' donor sites in deletion events does not follow their expected usage from alternative splicing in a wild-type background. For instance, the e<sup>-</sup>/e<sup>+</sup> competing 5' donor sites are normally utilized at frequencies of 73%/27%. However, the e<sup>-</sup> donor site is used in only 41% of deletion events, and the e<sup>+</sup> donor site in the remaining 59%. In short, the changes in splicing that occur in an *mle<sup>napts</sup>* background do not appreciably affect other splicing decisions that occur outside of skipped regions.

#### The Frequency of Edited *para* Transcripts Is Aberrant in an *mle<sup>napts</sup>* Background

Because of the exon-skipping events described above, only 17% of *para* transcripts in an *mle<sup>napts</sup>* background are full length. We sequenced a set of RT-PCR-generated cDNAs ( $n = 44$ ) representing this set of full-length transcripts to determine if editing occurred normally in those transcripts that were properly spliced. Overall, the level of editing was decreased in these transcripts from

an *mle<sup>napts</sup>* mutant (36% versus 70% in a wild-type background). However, in several transcripts, more adenosine residues were edited than in a wild-type background. Thus, even among those transcripts that are correctly spliced, some aspect of the editing process is aberrant in an *mle<sup>napts</sup>* background, though the effect is not profound.

#### Comparison of *mle<sup>napts</sup>* with *mle* Null Mutations

The *mle<sup>napts</sup>* mutation causes ts paralysis and action potential failure in both males and females, demonstrating that its effect is independent of dosage compensation. Null mutations of *mle* are male lethal because of a failure in dosage compensation leading to inadequate expression of X-linked genes. However, homozygous *mle* females are viable and do not manifest any behavioral or electrophysiological defects. These results imply that although *mle<sup>napts</sup>* is fully recessive to *mle<sup>+</sup>*, it encodes an altered protein whose effect on Na<sup>+</sup> channels is more severe than the complete absence of the protein. To investigate whether this is also true at the molecular level, we analyzed several other *mle* genotypes for their effects on *para* transcripts.

Examination of *para* RT-PCR products derived from females homozygous for *mle<sup>γ203</sup>*, a null allele of *mle*, revealed no detectable deletions caused by exon skipping. Analysis of *para* transcripts from *mle<sup>napts</sup>/mle<sup>+</sup>* heterozygotes yielded similar results (data not shown). Thus, there is complete concordance between the behavioral phenotype of *mle<sup>napts</sup>* and the occurrence of the splicing catastrophe of the *para* transcript. Furthermore, with respect to anomalous splicing of the *para* transcript, the effect of the protein encoded by the *mle<sup>napts</sup>* allele is recessive to the wild-type protein and more severe than complete absence of the protein.

These results can be explained in terms of the model presented in Figure 7. According to this model, the Mle helicase is required to resolve the dsRNA editing structure prior to splicing. The mutant protein encoded by *mle<sup>napts</sup>* binds to the dsRNA substrate but fails to resolve the structure triggering the occurrence of aberrant splicing and exon skipping. In addition, binding of the mutant protein prevents other functionally redundant helicases from acting. In the complete absence of the Mle helicase, these other helicases, which may be part of the splicing machinery itself, could compensate for the loss of Mle.

#### Discussion

RNA helicases have been implicated in almost every area of RNA metabolism both in prokaryotes and eukaryotes. Although genetic studies have revealed the essential nature of this class of proteins, little data exist on specific functions or target molecules in vivo. Evidence reported here indicates that the product of the *Drosophila mle* locus, a dsRNA helicase, is required for resolution of an RNA secondary structure that forms in the primary transcript of the *para* locus. This transcript encodes a Na<sup>+</sup> channel polypeptide, and the secondary structure is associated with A-to-I RNA editing like that reported for the mammalian GluRs and 5-HT<sub>2c</sub>R. Although the requirement for dsRNA secondary structures has been

demonstrated for the known mammalian editing sites, involvement of dsRNA helicases in the editing process has not been addressed. In the *mle<sup>napts</sup>* mutant, the apparent failure to resolve the dsRNA editing structure causes the majority of *para* transcripts to undergo inappropriate splicing that results in the production of internal deletions because of exon skipping. These deleted transcripts encode nonfunctional Na<sup>+</sup> channels and fully account for the decrement in *para* Na<sup>+</sup> channels in *mle<sup>napts</sup>* mutants previously inferred from genetic and electrophysiological studies.

#### A-to-I Editing of *para*

We report here the discovery of a site within the *para* transcript that undergoes A-to-I RNA editing like that seen in mammalian GluRs, always producing A-to-G transitions in cloned cDNAs. Three adenosine residues within 12 nucleotides are modified at high frequency at this site, resulting in two amino acid changes in the predicted edited protein product. We have ruled out possibilities other than editing and have shown that this editing has been conserved in the distantly related species *D. virilis*. Editing of *para* also resembles that observed in the mammalian serotonin receptor (5-HT<sub>2c</sub>R), where four adenosine residues within 13 nucleotides undergo modification, resulting in numerous cDNA isoforms (eleven distinct RNA species) and concomitant predicted amino acid changes (three amino acid residue changes resulting in seven detected receptor isoforms) (Burns et al., 1997; Niswender et al., 1999). Like 5-HT<sub>2c</sub>R, RNA editing of *para* transcripts at each modified nucleotide can occur independently. Eleven *para* cDNA species resulting from editing have been detected, and these transcripts differ most often at two amino acid positions, generating four potential Na<sup>+</sup> channel isoforms. It was shown for 5-HT<sub>2c</sub>R that the individual modified adenosines within the editing site were acted upon differentially by distinct editases. Thus, *para* RNA editing may also result from the action of multiple *Drosophila* RNA editases. Although the functional significance of RNA editing of *para* remains speculative, the Q/R edit creates a strong consensus PKC phosphorylation site. It is interesting that a large number of other Na<sup>+</sup> channels, both invertebrate and vertebrate, have a potential PKC site at this location (Figure 1). In fact, many channels encode a basic K residue at the same position as the Q/R site. Since K is encoded by AAA and AAG codons, they must be encoded by the genome and cannot be introduced by RNA editing. Thus, the *para* Q/R edit introduces a basic residue, generating a PKC site, whereas most other channels encode a PKC site constitutively.

#### Conserved Exonic/Intronic dsRNA

##### Secondary Structures

The mechanism for A-to-I RNA editing has been elucidated through study of the *GluR* genes and a class of enzymes called ADARs (Bass, 1997; Rueter and Emerson, 1998). Adenosines destined to become modified are included in RNA secondary structures that are then acted upon by an ADAR, hydrolytically deaminating the targeted A to I in a highly specific manner. For GluRs, the edited exonic sequences form a base-paired secondary structure with a complementary downstream intronic

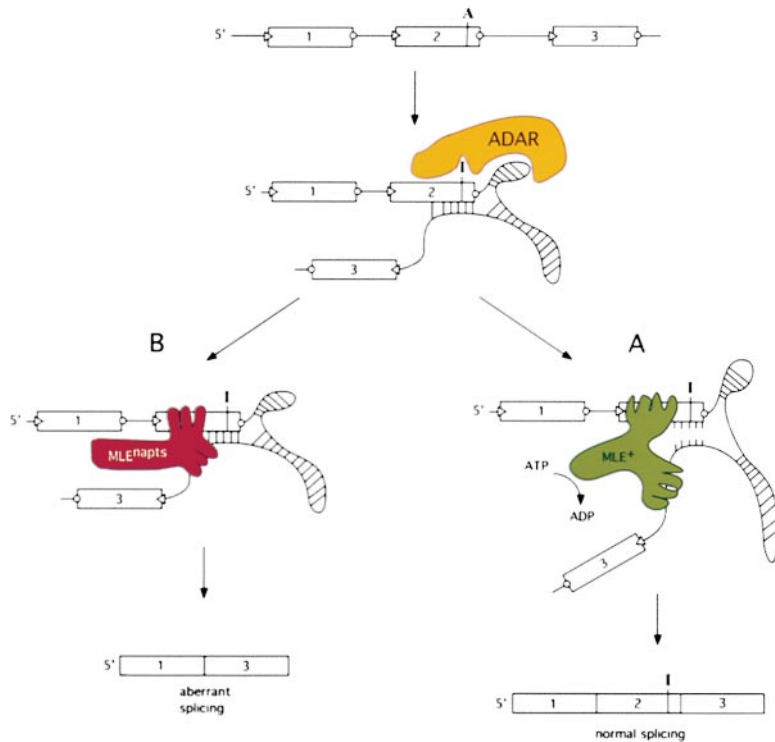


Figure 7. A Proposed Model for the Occurrence of Exon Skipping following RNA Editing of the *para* Transcript in an *mle<sup>napts</sup>* Background

We hypothesize the formation of a dsRNA editing structure that brings the putative ECS into register with the edited exon. An ADAR-editing enzyme (in yellow) is shown binding to elements of this structure and converting A to I. The wild-type Mle protein (in green) is shown as having a role in binding to and unwinding the dsRNA secondary structure (A). Normal constitutive splicing joins the exons in a sequential fashion. When the Mle<sup>napts</sup> protein (in red) fails to resolve the secondary structure, it remains bound to the structure, and splicing proceeds around the trapped splice donor, skipping the edited exon (B). Exons 1, 2, and 3 are indicated by boxed regions. Introns are indicated as lines. Circles represent 5' splice donor site, triangles represent 3' acceptor sites.

sequence (ECS). We analyzed RNA editing in *D. virilis* and found that editing was conserved. We reasoned that conservation of RNA editing between the distantly related species could help identify *cis*-acting (possibly intronic) sequences necessary for RNA editing. This analysis revealed an extensive region of intronic sequence downstream of the RNA editing site that was highly conserved between the two species and complementary to the region of the editing site, as expected for a putative ECS (Figure 2). Additionally, computer programs that predict RNA secondary structures align this putative ECS with the edited exon within a large dsRNA secondary structure (Figure 3). The modified adenosines are contained in regions of this secondary structure that locally resemble predicted structures for the *GluR* and 5-HT<sub>2C</sub>R edited sites to a striking degree (Figure 4). Also, ADARs have been shown to act preferentially upon adenosine residues with a specific 5' neighbor preference (A = T > C > G), indicating that a *Drosophila* ADAR is a likely candidate for the observed editing. Moreover, we have shown via characterization of a minimal *para* transgene that faithful RNA editing occurs in transcripts containing only those sequences that were predicted on the basis of evolutionary conservation and computer-based structural analysis to form a large dsRNA secondary structure (Figure 5). All known ADAR-dependent, specific editing events have been shown to require an RNA secondary structure and a sequence that functions as an ECS. We propose that such a secondary structure does, in fact, form in this limited region of the *para* transcript and that the conserved intronic segment whose sequence is complementary to the editing exon indeed functions as an ECS. Although all of our data

support this model, direct evidence that the putative ECS in the *para* sequence actually functions in a manner analogous to that of the ECS in *GluR* transcripts will require site-directed mutagenesis of this sequence and the corresponding exon.

#### Role for the Mle dsRNA Helicase

Given the indirect evidence for a potentially large and energetically stable RNA secondary structure in this region of the *para* transcript, it was of interest to examine the effect of the *mle<sup>napts</sup>* mutation on the fate of *para* transcripts. The Mle protein is an ATP-dependent dsRNA helicase that is highly similar in amino acid sequence and biochemical activity to human RNA helicase A (HRA) (Lee and Hurwitz, 1993; Zhang et al., 1995). The *mle<sup>napts</sup>* allele was originally isolated on the basis of its paralytic phenotype and subsequently shown to have a synergistic, lethal interaction with *para* mutations. These results and a variety of other genetic data led to the conclusion that the phenotypic effects of *mle<sup>napts</sup>* are mediated via a reduction in *para*-encoded Na<sup>+</sup> channels. Our analysis of *para* transcripts in an *mle<sup>napts</sup>* background revealed the occurrence of a splicing catastrophe occurring in a *napts* background in the edited region. The aberrant transcripts all result from exon-skipping events that delete the RNA editing exon as a result of splicing between an inappropriate upstream splice donor site and the usual 3' splice acceptor site immediately downstream of the edited exon. Less often, an additional one to three upstream exons, in addition to the edited exon, are also deleted from the *para* transcript, as increasingly distant splice donor sites are utilized with decreasing frequencies.

Other examples of exon skipping have been reported in a variety of systems as a result of mutations in 5'

splice donor site consensus sequences (Krawczak et al., 1992). In these cases, the exon 5' to the mutant donor site is skipped. The simplest explanation of the exon skipping caused by *mle<sup>napts</sup>* is that the 5' splice donor site directly downstream of the edited exon is sequestered in a large RNA secondary structure (see Figure 3) and is therefore inaccessible to the splicing machinery. This interpretation is consistent with a body of experimental data demonstrating that utilization of splice donor and acceptor sites in vivo and in vitro can be compromised by their inclusion within RNA secondary structures, resulting in the consequent occurrence of exon skipping (Solnick, 1985; Solnick and Lee, 1987; Eng and Warner, 1991; Goguel et al., 1993; Liu et al., 1995; Lin and Rossi, 1996; Chabot et al., 1997).

Mle and its closest relative, HRA, have been shown to exhibit stand-alone RNA helicase activity—the ability to unwind dsRNA duplexes in a 3' to 5' direction (Lee and Hurwitz, 1992; Lee et al., 1997). This implies that Mle is capable of functioning independently of the splicing machinery. In addition, Mle (and HRA) contains two amino-terminal repeats of a dsRNA-binding motif (DSRBM) (Gibson and Thompson, 1994). Mammalian ADARs also possess two or three DSRBMs at their amino termini (Kharrat et al., 1995). Thus, the binding of two different DSRBM-containing proteins in *Drosophila*, an unidentified ADAR homolog and the Mle protein, could converge at one dsRNA secondary structure at the *para* locus.

Taken together, these observations support a model in which the wild-type Mle protein is necessary in order to resolve the RNA secondary structure in *para* primary transcripts after editing is completed, thereby making the 5' donor site downstream of the edited exon accessible to the splice machinery (Figure 7A). An alternative, less parsimonious, explanation is that *mle<sup>napts</sup>* indirectly affects *para* processing by altering the expression of other genes whose products act on the *para* transcript. However, the specificity of the genetic interactions between *mle* and *para*, together with the data presented here, is most consistent with a direct effect.

A puzzling result from previous genetic studies confirmed by our present molecular analysis is that, with respect to its effect on Na<sup>+</sup> channels, the *mle<sup>napts</sup>* mutation is more extreme than *mle* null alleles. We propose that *mle<sup>napts</sup>* encodes an altered RNA helicase that correctly recognizes and associates with its dsRNA substrate but then fails to execute the resolution step (Figure 7B). Association of the mutant *mle<sup>napts</sup>* protein with target sites could prevent these sites from being accessed by other RNA helicases. However, the complete absence of the wild-type or mutant Mle in *mle* null homozygotes would allow RNA helicases encoded by other helicase genes to access the substrate and resolve the structure before splicing occurs. Alternatively, the splicing machinery itself may be capable of resolving the structure, albeit more slowly, and the complete absence of Mle is less detrimental than a stalled or malfunctioning helicase bound to the transcript.

In conclusion, we have shown that *para*-encoded Na<sup>+</sup> channels in *Drosophila* undergo RNA editing by a mechanism that appears to be analogous to that observed for mammalian *GluR* transcripts. The splicing catastrophe that befalls the *para* transcript in *mle<sup>napts</sup>* mutants because of an apparent failure to complete the editing

process reveals a role of a dsRNA helicase in this mechanism and explains the heretofore puzzling mystery of how a mutant helicase could cause a specific reduction in Na<sup>+</sup> channels.

## Experimental Procedures

### *Drosophila* Stocks

Wild type in this study is Canton S. *mle<sup>napts</sup>* and *mle<sup>γ203</sup>* are described in Kernan et al. (1991). *D. simulans* (stock number 14021-0251.102) and *D. virilis* (stock number 15010-1051.0) were obtained from the National *Drosophila* Species Resource Center at Bowling Green State University. *mle<sup>napts</sup>* heterozygotes were generated by crossing homozygous *mle<sup>napts</sup> cn* and *In(2LR)CyO/Sc* flies and recovering the F1 Cy progeny for the molecular studies described below. The stock used as a source for transposase in transformation studies is Bloomington Stock Center number 4368; y<sup>1</sup> w<sup>1</sup>; Ki<sup>1</sup> P{ry + t7.2 = Delta2-3}99B.

### Cloning of cDNAs and RT-PCR Analysis

All RT-PCR analysis was performed using whole adult RNA using a modification of the LiCl/Urea procedure described in Auffray and Rougeon (1980), which was stored as an ethanol precipitate at -80°C. Genomic DNA from adult flies was prepared using the QIAamp tissue kit (Qiagen). First-strand cDNA synthesis was performed with Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL) at 42°C using LR4 or PRP11 as primers.

PCR amplifications were performed in a Robocycler (Stratagene). Various polymerases were used to rule out polymerase error as a source for cDNA modifications. PCR products were generated using the RL3, FR5 primer combination and Taq polymerase (Promega), ExTaq (PanVera), or Deep Vent polymerase (NEB) and then subjected to TaqI and MnlI restriction digestion. The results were identical for each polymerase: PCR products generated from genomic DNA were not cut by either enzyme, indicating no modification, whereas PCR products generated from first-strand cDNAs were cleaved at the sites predicted to be modified at a characteristic frequency regardless of the polymerase used. Cloned cDNAs for sequence analysis were also amplified with the RL3, FR5 primer combination. Full-length cDNAs from an *mle<sup>napts</sup>* background were generated using the Edsac-I, FR5 primer pair. The low abundance alternative splice-form cDNAs described in the Results were amplified using the Edsac-I, SORFII primer combination.

The PCR products shown in Figure 5 were generated using the RL3, WORFII primer combination. Other regions of *para* were amplified in wild-type and *mle<sup>napts</sup>* backgrounds using the LR2, LR1 and LR3, RL6 primer combinations. These regions cover the remainder of the *para* open reading frame not spanned by the RL3, FR5 primer pair. No difference in product size or abundance was seen in either genetic background using these other primer combinations. All cDNAs were cloned into pBluescript-SK(+) (Stratagene) digested with SacI and BamHI restriction enzymes and transformed into XL1-blue (Stratagene). ssM13 DNA templates were generated from pBluescript clones using a protocol from the Sequenase Version 2.0 kit manual (Amersham). DNA for automated sequence analysis was prepared using the Wizard prep plasmid kit (Promega).

### Primers

Sequence in lowercase corresponds to the complete *para* coding sequence (GenBank accession number M32078). Sequence changes or additions to add or generate restriction enzyme sites for cloning are indicated in uppercase. Location of the 5' nucleotide of the primer in relation to the reported *para* cDNA is given in parentheses. When sequences have been added at the 5' end, the number refers to the first 5' *para* nucleotide. Forward primers: Edsac-I, 5'-TCGTCGAGctgcgcttctggcaagg-3' (4111); LR2, 5'-TCTAGAcgttgccgcatagacaatgacag-3' (255); LR3, 5'-ttggatc(a/c)ttttcacactcaatctgtctattgg-3' (4907); RL3, 5'-tcttcgatccctctgcgagc-3' (2715); TIBS, 5'-GGAATTcgccagcaaggaggattagtc-3' (3928); and T/Mseq, 5'-cttagccggtgacgatgactgcgagc-3' (4093). Reverse primers: FR5, 5'-aaa(t/g)gatccaaatgatgaagaa-3' (4918); LR1, 5' 5'-tcgtgtgaccacaatgacagcg-3' (2768); LR4, 5'-gctaataactcgctgcatcttgg-3' (6710); PRP8,

5'-cggaagagcagtgtccg-3' (5491); PRP11, 5'-CCGAGCTccttggcttggaaatggc-3' (5086); RL6, 5'-aggcctggcctcagacatccgc-3' (6676); Sbl1u, 5'-cgaatcagagccaacacc-3' (4388); SORFII, 5'-CGGGATC Cactttcatgccctcccag-3' (4524\*); TIRM, 5'-cttgaagcggagcgaacac-3' (4352); WORFII, 5'-GCGGATccctatgcctgcatac-3' (4523); \* the SORFII primer is in the same location as the WORFII primer but in a previously unreported alternative splice form); KPACT, 5'-GGGGTACCGTACTTGCCTCTGGCGGGC-3'; and RTACT, 5'-CGCACTTGCACTTTCGCTGCTGC-3'.

#### Sequence Analysis and Secondary Structure Predictions

Sequence analysis in Figure 1 was performed manually using the Sequenase Version 2.0 kit (Amersham) on ssM13 DNAs generated from pBluescript clones. The Edsac-I primer was used for sequencing, and only the adenosine and guanosine termination reactions were performed. The sequencing reactions were analyzed on a 6% glycerol-tolerant denaturing gel (Amersham) and allowed the detection of A-to-G modifications occurring in a region extending from 30 bp 5' to the TaqI editing site to ~300 bp downstream of the same site.

Sequences of genomic DNAs are a consensus of three independent clones in each case and were generated by automated sequencing (University of Connecticut Health Center Core Facility). Homology plot (Figure 2) was generated by comparing the percent nucleotide (nt) identity in a sliding 50 nt window moving in 10 nt increments. RNA secondary structures were analyzed using the mfold and plotfold programs of the Wisconsin GCG software package. Data sets were converted to secondary structures using Loop-Dloop Version 1.2a63 by D. G. Gilbert (1992).

#### Generation and Analysis of Transgenic *Drosophila*

The primers TIBS and TIRM were used to amplify the genomic region of the editing site by PCR. These primers generate an amplification product, which, when digested with EcoRI and XbaI, includes 230 bp upstream of the edited adenosines extending through 174 bp downstream of the proposed ECS. The PCR products were subcloned into pBluescript (Stratagene) and subjected to sequencing. Sequence-confirmed clones from this region were then cloned into pCasPer-hs/act (obtained from C. Thummel, University of Utah) cut with EcoRI and XbaI. These constructs grown in *Escherichia coli* XL1-blue (Stratagene) and subjected to QIAprep plasmid purification (QIAGEN). Constructs were then injected into embryos from a transposase overproducer, and w<sup>+</sup> progeny were obtained via standard transformation procedures (Park and Lim, 1995). RT-PCR analysis of editing was performed on transgenic lines as follows. RNA was isolated from transgenic lines, and separate first-strand cDNAs were generated using specific primers for *para* (PRP-8) or the actin 3' UTR sequence of the transgenic construct (RTACT). These cDNAs were subjected to PCR using the TIBS and Sbl1u primer combination for *para* or the TIBS and Kpact primer combination for the transgene construct. The TIBS primer is common to both *para* and the transgene and is 109 bp upstream of the first TaqI site (uncircled T in Figure 5). Sbl1u and KPACT were chosen to generate similar-sized products from the transgene and *para*. RT-PCR products were digested with TaqI and electrophoresed on a 2.3% agarose gel. The upstream TaqI site served as an internal control for complete TaqI digestion. These RT-PCR products as well as the genomic transgene product generated by PCR using the TIBS and KPACT primers were subjected to direct automated sequence analysis using the T/M-seq primer, which is 58 bp upstream of the TaqI site introduced by editing (circled T in Figure 5).

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