RNA Editing of the Drosophila \textit{para} $\text{Na}^+$ Channel Transcript: Evolutionary Conservation and Developmental Regulation

Christopher J. Hanrahan,* Michael J. Palladino,* Barry Ganetzky$^2$ and Robert A. Reenan*

*Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, Connecticut 06030 and 
$^2$Laboratory of Genetics, University of Wisconsin, Madison, Wisconsin 53706

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ABSTRACT

Post-transcriptional editing of pre-mRNAs through the action of dsRNA adenosine deaminases results in the modification of particular adenosine (A) residues to inosine (I), which can alter the coding potential of the modified transcripts. We describe here three sites in the \textit{para} transcript, which encodes the major voltage-activated $\text{Na}^+$ channel polypeptide in Drosophila, where RNA editing occurs. The occurrence of RNA editing at the three sites was found to be developmentally regulated. Editing at two of these sites was also conserved across species between the \textit{D. melanogaster} and \textit{D. virilis}. In each case, a highly conserved region was found in the intron downstream of the editing site and this region was shown to be complementary to the region of the exonic editing site. Thus, editing at these sites would appear to involve a mechanism whereby the edited exon forms a base-paired secondary structure with the distant conserved noncoding sequences located in adjacent downstream introns, similar to the mechanism shown for A-to-I RNA editing sequences located in adjacent downstream introns, similar to the mechanism shown for A-to-I RNA editing.

These results demonstrate that $\text{Na}^+$ channel diversity in Drosophila is increased by RNA editing via a mechanism analogous to that described for transcripts encoding mammalian GluRs.
technique that identifies inosine-containing transcripts in Caenorhabditis elegans may eliminate the serendipitous nature currently associated with detection of RNA-editing sites (Morsey and Bass 1997, 1999).

Mechanistically, the editing of pre-mRNA appears to require the formation of stable, highly base-paired RNA secondary structures. Whereas nonspecific editing requires extended regions of complementary duplex dsRNA (Nishikura et al. 1991), specific editing appears to require more complex RNA secondary structures (Egebjerg et al. 1994; Hurszt et al. 1995; Herb et al. 1996; Maa et al. 1996). These secondary structures include a sequence called an editing site complementary sequence (ECS) found in downstream introns (Higuchi et al. 1993). Even though there may be numerous bulges, hairpins, and loops within the intervening secondary structure, the ECS and exon form an extended, energetically stable duplex RNA around the adenosines that undergo modification (Egebjerg et al. 1994).

Editing activity has been assayed in Drosophila embryonic nuclear extracts (Casey and Gerin 1995), but the only reports of RNA editing in Drosophila have been for the 4fRN P gene (Pertschek et al. 1997), the Dmcal A calcium channel gene (Smit h et al. 1996), and the chloride channel subunit (Semenov and Pak 1999). However, little is known about the mechanism, overall degree of editing, or developmental regulation of editing in these transcripts, and definitive proof of RNA editing vs. other possible explanations for transcript variability remains to be provided. We have discovered four editing sites in para transcripts, which encodes the major Na⁺ channel of the nervous system. Interestingly, MLE, a dsRNA helicase, appears to be involved in the editing process at one of the editing sites in the para transcript (Reenan et al. 2000). In this article, we describe the remaining three A-to-I RNA editing sites found in para, as well as their developmental regulation and evolutionary conservation. In addition, we provide strong evidence for an ECS-based mechanism involving RNA secondary structures acting at these sites.

MATERIALS AND METHODS

Preparation of RNA and genomic DNA: Whole RNA was prepared from four developmental stages of Drosophila melanogaster, as well as from D. melanogaster and D. viridis adults, by the LiCl/urea method and stored in ethanol at −80°C (Auffray and Rougeon 1980). Genomic DNA from adult D. viridis and D. melanogaster was prepared by homogenizing 100 adult flies and extracting the DNA with the Wizard genomic DNA purification kit (Promega, Madison, WI).

Reverse transcription-PCR: Reverse transcription (RT)-PCR reactions were performed as described by the manufacturer using MMLV-RT (Gibco BRL, Gaithersburg, MD), and the addition of RNasin (Promega). Standard hot start PCR reactions were performed with cDNA from the RT reaction or genomic DNA as template. Using a Robocycler (Stratagene, La Jolla, CA), 40 cycles of PCR were performed with the following parameters: denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, and extension at 72°C for variable intervals depending on the expected size of the product. When the PCR product was presumed to be >1.5 kb, Ex Taq (Takara) polymerase was used for reactions, which were performed as indicated by the manufacturer. All reactions were 50 μl total volume.

Preparation and cloning of PCR products: After phenolchloroform extraction and ethanol precipitation, PCR products were cut with restriction enzymes for ligation. Cut products were gel purified and ligated into the pBluescript SK+ vector (Stratagene). Plasmids were transformed into XL1-Blue cells (Stratagene) and isolated by the alkaline lysis miniprep protocol (Ausubel 1987). Restriction enzyme digests were performed to determine the editing status of each clone.

Genomic and cDNA sequence: Colonies that contained insert DNA to be sequenced were replated on LB agarose with ampicillin. DNA was purified with the Wizard Plus miniprep DNA system (Promega) according to the manufacturer’s directions. Sequencing was performed on an ABI automated sequencer (University of Connecticut Health Center Core Facility) and data were analyzed using the ABI Seqed program.

Evolutionary comparison and RNA secondary structure predictions: Genomic DNA was sequenced for regions of D. melanogaster and D. viridis. Sequences were aligned and compared in overlapping 50-bp increments. Subsequently, RNA secondary structures were predicted using the mfold program of the Wisconsin GCG software. The “forc” function of the mfold program was used to force base pairing of the Fsp site for comparison between D. melanogaster and D. viridis. LoopDloop version 1.2d63 by D. G. Gilbert (1992) was used to convert mfold data into printable structures.

Generation of trangenic Drosophila: The primers Split1 and XAFP were used to amplify the genomic region of the FSP editing site by PCR. These primers generate an amplification product that, when digested with EcoRI and XbaI, includes 1472 bp upstream of the edited adenosine extending through 178 bp downstream of the edited adenosine. The PCR products were subcloned into pBluescript (Stratagene) and subjected to sequencing. Sequence-confirmed clones from this region were then cloned into pCasPeR-hs (obtained from C. Thummel, University of Utah) cut with EcoRI and XbaI. These constructs were grown in Escherichia coli XL1-blue (Stratagene) and subjected to QIAprep plasmid purification (QIAGEN, Chatsworth, CA). Constructs were then injected into embryos from a transposase overproducer and w+ progeny were obtained via standard transformation procedures (Park and Lim 1995). For detection of transcripts from the FSP transgene, the RTHS primer was used in first-strand cDNA synthesis and PCR was performed using the HSKn and FSPnot primers. Products were subjected to restriction enzyme analysis with BanI or directly sequenced using the FSP-S primer. Cognate para cDNAs were amplified using the PRP8 primer in first-strand cDNA synthesis and the FSPnot and FASP primers for PCR.

Oligonucleotides: RT primers were LR1, 5′-TCGGTGTTGACCAAATGCACAGCG-3′, or PRP8, 5′-CGCGAAGGAGCAAGTTGTCCG-3′. Fsp site cDNA primers for both D. melanogaster and D. viridis were Fsp-S, 5′-CCGGAGCTCGTATAGCAATTGCAAAGG-3′, and Fsp-B, 5′-CGGATCTGATTTGAACTAC-3′. Fsp i—i— and i—i— splice-form-specific primers were: Fa-Mim, 5′-CCGATCCGGTTCATGACTGAC-3′, and Fa-Pim, 5′-CCGATCCGGTTCATGACTGAC-3′, respectively, replacing Fsp-B for cDNA amplification. Sp and Sf site cDNA primers for D. melanogaster were Pore11, 5′-CCGAGCTCCTTGTCTGTTTGAATGG-3′, and Pore12, 5′-CGGATCCATGATTGGTGAAGAC-3′. For D. viridis, Sf-S, 5′-CCGAGCTCAAGAGCTACTTGTCTGTCTCG-3′, replaced Pore11. The intron upstream of the Fsp site was amplified with Fsp-S and either Fds (D. melanogaster), 5′-CGGGATCCCGTTG.
CCCTTTCGCGCCGC-3', or FupsB (D. virilis), 5'-CGGATCCGACTCCTGACCCACTCGAC-3'. The intron downstream of the Ssp site was amplified with Pore12 and Ssp-S, 5'-CGGAGCTCGTGGCTAGGCTGCTGACC-3'. The intron upstream of the Sfc site was amplified using SUFR-1, 5'-CGGAGCTCGTGTAACCACGATCTGCAC-3', and SUFR-2, 5'-CGGAGCTCCACCATGAACAG-3'. The intron downstream of the Sfc site was cloned using Sfc-S and Sfc-B, 5'-CGGATCCTTTTAAGATCGAGAAT-3'.

**RESULTS**

We have identified a total of four A-to-I RNA-editing sites within Drosophila para transcripts, three of which are described in detail here (Figure 1). The editing sites are named for restriction enzyme recognition sequences for each (Table 2). A total of 131 cDNAs were analyzed in genomic DNA but edited cDNAs have the sequence despite their proximity (Figure 1). These sites were originally discovered through sequence analysis of cDNAs. Edited transcripts (%)

### TABLE 1

<table>
<thead>
<tr>
<th>Editing site</th>
<th>D. melanogaster</th>
<th>D. virilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ssp</td>
<td>21 ± 2 (131)</td>
<td>30 ± 5 (45)</td>
</tr>
<tr>
<td>Sfc</td>
<td>43 ± 5 (131)</td>
<td>29 ± 6 (45)</td>
</tr>
<tr>
<td>Fsp</td>
<td>69 ± 3 (168)</td>
<td>0 (44)</td>
</tr>
</tbody>
</table>

*Percentage ± SEM (number of clones).
TABLE 2

Independence of editing at Ssp and Sfc sites

<table>
<thead>
<tr>
<th>Editing status</th>
<th>Exp. freq. of occurrence (%)</th>
<th>Obs. freq. of occurrence (%)</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ssp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>45</td>
<td>45 ± 7</td>
<td>59</td>
</tr>
<tr>
<td>E</td>
<td>34</td>
<td>34 ± 5</td>
<td>45</td>
</tr>
<tr>
<td>Sfc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>12</td>
<td>11 ± 2</td>
<td>14</td>
</tr>
<tr>
<td>E</td>
<td>9</td>
<td>10 ± 3</td>
<td>13</td>
</tr>
</tbody>
</table>

Exp., expected; freq., frequency; obs., observed.

1 U, unedited; E, edited.
2 Calculated on basis of Table 1.
3 Frequency ± SD.
4 131 total clones analyzed.

Alternative splicing decisions do not affect RNA editing: Alternatively spliced exons are present at several different locations throughout the para transcript resulting in the potential to encode at least 192 Na channel isoforms in D. melanogaster and at least 128 in D. virilis (Thackeray and Ganetzky 1995). RNA editing further increases transcript diversity of para. However, since both processes may require conserved intronic elements, alternative splicing decisions may influence editing or vice versa. We hypothesized that certain splice forms might be preferentially edited at the Fsp site, which lies 200 bp upstream of an intron involved in the alternative splicing of exons i and a (O'Dowd et al. 1995; Thackeray and Ganetzky 1995). For example, exon-i-containing splice forms may be edited significantly more than splice forms lacking exon i. Using chi-square analysis for goodness of fit, we found no preference for editing of any splice form near the Fsp editing site regardless of splice form abundance (Table 3). These results suggest that specific editing occurs independently of alternative splicing at the Fsp site.

Editing of para occurs in two developmentally regulated patterns: To determine whether editing of the para transcript is developmentally regulated as is editing of GluR transcripts (Lomeli et al. 1994), we analyzed para cDNAs representing transcripts from each stage of development. The Ssp and Sfc sites show only minimal editing (1-2% of cDNAs) at all stages from embryos through the third larval instar. However, a dramatic 20- to 40-fold increase in editing of the Ssp and Sfc sites occurs at pupation, with the frequency of editing approaching adult levels (Figure 2A). The Fsp site showed a different pattern of editing during development, exhibiting higher starting levels in the embryo (15%), and increasing less than fourfold during the pupal stage to adult levels (Figure 2B). Thus, specific editing at the three sites in the para transcript examined here is developmentally regulated.

Evolutionary comparisons reveal putative ECS elements within introns downstream of the Ssp and Sfc sites: If A-to-I editing were occurring in para transcripts via a mechanism analogous to that for mammalian GluR transcripts, we hypothesized that each editing site would have a corresponding ECS within the downstream intron (Higuchi et al. 1993; Egebjerg et al. 1994; Herb et al. 1996). It has been shown previously that exonic regions of para are highly conserved between D. melanogaster and D. virilis, whereas intronic regions are divergent (Thackeray and Ganetzky 1995). Therefore, if an intron contains within it an ECS complementary to a conserved editing site, this should be apparent as a conserved sequence surrounded by a highly divergent flanking intronic sequence.

Because editing at the Ssp and Sfc sites was conserved between D. melanogaster and D. virilis (Table 1), intronic regions surrounding these sites were cloned and sequenced in these two species. We generated an identity profile based on aligned sequences (Figure 3). As expected, exonic regions were nearly identical, whereas intronic sequences varied considerably, generally exhibiting <50% identity. However, we discovered regions of high sequence identity between D. melanogaster and D. virilis within the introns downstream from each editing site. For the Ssp site, a 40-bp region located 240 bp downstream of the exon-intron boundary was identical

TABLE 3

Editing of para splice forms

<table>
<thead>
<tr>
<th>Splice form</th>
<th>Exp. freq. of occurrence (%)</th>
<th>Clones (n)</th>
<th>Edited transcripts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i+ a+</td>
<td>45</td>
<td>36</td>
<td>72</td>
</tr>
<tr>
<td>i+ a-</td>
<td>36</td>
<td>42</td>
<td>69</td>
</tr>
<tr>
<td>i− a+</td>
<td>11</td>
<td>38</td>
<td>59</td>
</tr>
<tr>
<td>i− a−</td>
<td>8</td>
<td>54</td>
<td>74</td>
</tr>
</tbody>
</table>

Exp., expected; freq., frequency.

1 Constitutive exon, shaded box; alternative exon i, striped box; alternative exon a, open box.
2 Calculated on basis of Thackeray and Ganetzky (1995).
3 Due to low abundance splice forms lacking alternative exon i were selected for (see materials and methods).
4 Null hypothesis (all forms are edited equally) is not rejected (0.9 > P > 0.1, χ² for goodness of fit).
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The essential feature of an ECS (data not shown).

RNA secondary structure predictions indicate base pairing between editing sites and putative ECSs: In addition to an ECS, editing of mammalian GluR transcripts requires formation of an extended RNA secondary structure that aligns the distant ECS with the region encompassing the edited adenosine (Higuchi et al. 1993; Egebjerg et al. 1994; Lomeli et al. 1994; Herb et al. 1996). To determine whether the putative ECSs would be capable of base pairing with their respective exonic counterparts in the context of a larger RNA secondary, we used a computer program to generate secondary structures in these regions for the para pre-mRNA sequence (Zuker 1989). For the Ssp site, the predicted secondary structures for both species appear strikingly similar despite the divergence of intron sequences (Figure 4). For instance, they each have an extended repeat capable of forming a hairpin of duplex RNA ≥24 bp long. Both structures also contain a large A-rich loop (60–70% adenosines over 53 or 73 nucleotides in melanogaster and virilis, respectively). However, the most striking aspect of these structures involves the specifically edited adenosine at the Ssp site. It is contained within a relatively long region of duplex RNA in which the editing site base pairs with the 40-bp conserved intronic segment, confirming its identity as an ECS. For the Sfc site, the predicted structure in D. virilis also juxtaposes the editing site with the predicted ECS. However, a similar structure was not predicted when the D. melanogaster sequence was used in this analysis. Nonetheless, when base-paired structures are generated in the two species. For the Sfc site, a stretch of 62 bp found 1036 bp downstream of the exon-intron boundary was nearly identical (61/62 nucleotides) between D. melanogaster and D. virilis. On the basis of their conservation and location, these two intronic regions appear to be good candidates for ECSs. Moreover, a preliminary analysis reveals that these regions of conservation are complementary to the sequences surrounding the exonic editing sites and are thus capable of forming base-paired duplexes in this region, the essential feature of an ECS (data not shown).

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Editing of the Fsp site occurs in D. melanogaster, but not in D. virilis: In contrast with editing at the Ssp and Sfc sites, which is conserved between D. melanogaster and D. virilis, editing at the Fsp site appears to occur only in D. melanogaster (Table 1). To examine this region in more detail we cloned and sequenced genomic DNA flanking this editing site from both species, including a nearby upstream intron (Thackeray and Ganetzky 1995). We were unable to find any intronic regions that were highly conserved between the two species. Because the Fsp site is edited in D. melanogaster, we predicted that this would require an ECS as for the other edited sites. The upstream intron is only 58 bp from the Fsp site and we examined the possibility that it or the exon
itself contained the corresponding ECS. Computer-generated RNA secondary structures using the D. melanogaster sequence predicted formation of a stable RNA duplex in the Fsp region (Figure 6A). Surprisingly, the adenosine to be edited in the melanogaster sequence is predicted to lie within an extended hairpin that forms entirely from exonic sequences. Nonetheless, the predicted local RNA secondary structure for the Fsp site compares favorably with other known editing sites (Figure 6C). If editing at the Fsp site also occurred in D. virilis, an RNA secondary structure analogous to that in melanogaster would be expected for the corresponding virilis sequence. To test this possibility, we attempted to generate the analogous structure in D. virilis (see materials and methods) and obtained a structure that differs markedly from the structure observed in D. melanogaster, consistent with our failure to find editing of the Fsp site in D. virilis (Figure 6A). The computed free energies for the two structures differ (D. melanogaster = -1620 kcal/mol vs. D. virilis = -538 kcal/mol), with the D. melanogaster structure predicted to form a much more stable structure. Furthermore, we generated local structures using a pairwise alignment of the sequence surrounding the edited adenosine. Even using these local comparisons, the D. virilis structure was less stable compared with the melanogaster structure (Figure 6C). These RNA secondary structure predictions are consistent with the lack of conservation of RNA editing at the Fsp site.

Predicted sequences are capable of directing RNA editing of the FSP site in vivo: A valid test of RNA secondary structure predictions would be to assay RNA editing on substrates removed from the context of the entire para primary transcript. To this end, we generated transgenic flies via germline transformation capable of expressing an FSP editing site minimal construct. We subcloned the region of the FSP site genomic DNA shown in Figure 7A into a heat-shock-inducible transformation vector and obtained numerous stable transgenic fly lines. Since the transgene contains the intron upstream of the edited exon as well as part of the upstream exon, we were able to monitor processing of the transgene by the splicing. The FSP transgene produced transcripts in which the intron was removed efficiently under non-induced conditions. By restriction analysis of RT-PCR products from numerous transgenic lines, we observed efficient RNA editing of the FSP transgene in all lines (Figure 7B). Direct sequence analysis of RT-PCR products of both cognate para and transgene transcripts revealed that the transgene is edited faithfully and specifically; only the single adenosine is edited in both para and transgene products (data not shown). Thus, all the sequences necessary to direct RNA editing at the FSP editing site are contained within the transgene, which includes all the sequences shown in the predicted structure (Figure 6A).

DISCUSSION

RNA editing is a mechanism for diversifying the protein products encoded by a given gene. Despite the ubiquitous presence of editase activity, the number of known targets is limited. We report here three A-to-I RNA-editing sites in para sodium channel transcripts of Drosophila. A large number of independent cDNAs revealed identical modifications, whereas such changes were never observed in genomic DNA, eliminating other possibilities such as the presence of allelic polymorphisms or polymerase errors. Characterization of editing frequencies in adult flies, as well as the developmental regulation, evolutionary conservation, and predicted RNA secondary structures are all consistent with the process of A-to-I RNA editing.

Flanking sequences near the para editing site support editing by an ADAR-based mechanism. The 5' nucleotide neighboring each edited adenosine is consistent
RNA Editing of para Transcripts

Figure 4.—Comparison of putative RNA secondary structures for the Ssp site in para orthologues. The RNA secondary structure program in the GCG software package predicted secondary structures from the D. melanogaster or D. viridis genomic sequence downstream of the Ssp editing site. The two structures are similar in their overall appearance. They both have long regions of duplex RNA and bulges and loops that are in similar positions. Most importantly, the predicted ECS (uppercase letters; see Figure 3) base pairs with the exonic region containing the editing site. The boldface lines indicate the location of the intron-exon boundaries.
with editase preferences described by Polson and Bass (1994; A = T → C → G). That is, two of the sites (Ssp and Sfc) have the most preferred 5’ neighbor, an adenosine, while the third site (Fsp) has a 5’ cytosine. None of the sites have the least preferred 5’ neighbor, a guanosine. Also, duplex formation surrounding the editing sites conforms to editing structures described for other known A-to-I RNA-editing sites. The Ssp and Sfc editing site adenosines base pair with uracil (Figures 4 and 5B), which occurs with the GluR-B Q/R site (Figure 6C; Higuchi et al. 1993). However, there is no absolute requirement that the edited adenosine be base paired; in fact, the adenosines edited in GluR-5 and GluR-6 Q/R sites are present within a small bubble (Herb et al. 1996). In vitro experiments have demonstrated that the local duplex affects the efficiency of editing. Introduction of a cytosine opposite the edited adenosine in the GluR Q/R sites increases the efficiency of modification by recombinant ADAR1 (Herb et al. 1996; Maas et al. 1996), while introduction of guanosine opposite the GluR-B Q/R editing site does not (Higuchi et al. 1993). Interestingly, the Fsp adenosine, which is edited at the highest frequency of any para editing site, forms a mismatch with cytosine similar to the GluR R/G and HDV sites (Figure 6C; Lomeli et al. 1994; Casey and Ger in 1995). These observations are consistent with a Drosophila ADAR activity mediating editing of these sites.

Initially, the location of one editing site suggested experiments that might reveal a correlation between editing and alternative splicing. The Fsp site is upstream of alternatively spliced exons. We suspected that the Fsp site might be edited more frequently in one splice form than another. This hypothesis proved unsubstantiated by experimental evidence (Table 3).

Although the Ssp and Sfc sites are 2000 bp apart in pre-mRNAs, analysis of a large number of cDNAs (n = 131) spanning both of these editing sites revealed that editing occurred independently (Table 2). One interpretation of this result is that the editing activity is present in all tissues in which para is expressed and the sites themselves determine the intrinsic level of modification independently of one another. Another interpretation is that these sites are edited in a spatially regulated manner. In this model, some tissues would perform one edit, while some perform both in a manner that mimics expected ratios. This could be accomplished by multiple editases or isoforms, each editing a different site in a spatially distinct manner.

**Developmental regulation:** The presence of three unique editing sites has allowed us to compare the regulation of editing during development. Two of the sites (Ssp and Sfc) appear to be tightly regulated in a similar manner, while the third site (Fsp) has a different editing profile (Figure 2A and B). There are several possible interpretations of these observations. First, there could be different accessory factors involved in the editing of each site. In the case of the Fsp site, which is edited throughout development, either no additional regulatory factors would be required or they would be constitutively expressed. In contrast, the Ssp and Sfc sites would require accessory factors or the accessory factor expression would be induced during pupation. Alternatively, a repressor of RNA editing that acts at these sites specifically may be expressed early in development. Second, different enzymes or isoforms may recognize different secondary structures. In this case, editing at the Fsp site involves an enzyme that recognizes its limited secondary structure, while the Ssp and Sfc sites might utilize an enzyme whose binding and activity requires more extensive secondary structures. The different enzymes may have tissue-specific distributions that would add further diversification to the expression of edited proteins.

**Evolutionary comparisons:** Evolutionary conservation of the Ssp and Sfc sites suggests that A-to-I RNA editing of the para transcript provides a selective advantage to the organism. Evolutionarily conserved intronic elements that maintain significant complementarity to exonic sequences containing the Ssp and Sfc editing sites further support this conclusion. In contrast, the lack of conservation of a cis-element for the Fsp site correlates with the absence of editing in D. virilis. This correlation is further strengthened when the estimated 61–65 million years of divergence between the two species is considered (Beverley and Wilson 1984). In addition, the Fsp editing site occurs in one of the least conserved portions of the para protein (R. Reenan, unpublished observation) and thus the absence of RNA editing in this region in D. virilis may simply reflect reduced selective constraints in this region of the protein.

An evolutionary comparison of editing frequencies
Figure 6.—RNA secondary structures generated near the Fsp editing site. (A) RNA secondary structure predicted for D. melanogaster by the mfold program (GCG software). Note the extensive regions of duplex RNA and the location of the Fsp site within an extended hairpin. The intron-exon boundary is indicated. Shaded regions indicate base pairs that were forced to occur with the mfold “force” function for comparison of D. melanogaster and D. virilis sequence. (B) RNA secondary structure formed when boxed base pairs from Figure 6A were forced to occur with D. virilis sequence. (C) Local RNA secondary structure comparison of Fsp in D. melanogaster and D. virilis, as well as to other known A-to-I RNA-editing sites. Adenosines that are edited are shown in white.

in adult flies is intriguing. At the Ssp site the editing frequency is similar between D. melanogaster and D. virilis, while the editing frequency at the Sfc site is slightly higher in D. melanogaster. These data may reflect intronic sequence differences that would alter the RNA secondary structure of editing site substrates. The similarity of predicted RNA secondary structures, both globally and locally, for the Ssp site is consistent with similar editing frequencies. For the Sfc site, the large size of the intron downstream of the Sfc site, without significant selective pressures, provides ample opportunity for sequence changes that may alter RNA secondary structure or even
that conserved intronic regions of > 30 bp are rare. Most importantly, the conserved candidate ECSs are capable of forming extended imperfect duplexes with the regions of the edited adenosines and these local structures resemble the structure of known ADAR substrates (Figures 4–6). It has been shown for vertebrate GluR R/G sites that the most highly conserved nucleotides in the editing site are those participating in base pairing interactions within the hairpin known to be important for RNA editing (Aruscavage and Bass 2000). In all cases presented here, the para editing site/ ECS pairings predicted are almost completely conserved including mismatched pairs and bulges. In addition, a recent study of ADAR1 shows that an important determinant of ADAR specificity may be the presence of nearby loops or bulges that act as helix ends (Lehmann and Bass 1999). The presence of such helical defects in one defined substrate was shown to limit ADAR1 activity to certain A residues in a substrate that would otherwise be edited more extensively. Such helical defects are present in all the para substrates predicted in this study and may serve the purpose of positioning a Drosophila ADAR in a manner similar to that seen for ADAR1. These criteria make these conserved sequences excellent candidates for ECSs, not only by definition, and taken together suggest that we have predicted energetically favorable RNA secondary structures that are utilized for A-to-I RNA editing of para.

In addition, for the FSP site, we have shown that RNA editing of a minimal substrate occurs in vivo. The transgene that was constructed in this instance contains the predicted secondary structure and some additional upstream intron and exon sequences (Figure 7). Interestingly, for this editing site, a contrast is seen with all other known editing sites. While all other reported mammalian ion channel editing sites require downstream intronic ECSs, the FSP editing construct contains no downstream intron sequences. In fact, while the predicted ECS for the FSP site is downstream, it lies entirely within the coding region of the same exon as the edited adenosines. Efficient editing of the transgene supports the predicted secondary structure and shows that downstream intronic sequences are necessary. Moreover, the absence of RNA editing in D. virilis is also supported by this evidence since a similar secondary structure for this species is predicted to differ significantly from the predicted D. melanogaster structure. In particular, changes are predicted in exactly the region of the virilis structure near the edited adenosines and similar changes at known sites of RNA editing have been shown to disrupt RNA editing in vitro.

**Functional significance:** Our discovery of RNA editing of a Na⁺ channel transcript follows the trend of A-to-I edited ligand- and voltage-gated ion channels including the first and most extensively studied GluR subunits, the serotonin receptor 2C, and the squid K⁺ channel (Higuchi et al. 1993; Kohler et al. 1993; Burns et al. 1997; Patton et al. 1997). In contrast with these other
edited channels, the functional significance of para editing must be inferred. Evolutionary conservation of para editing suggests that Sps and Sfc editing sites are biologically important. The Sfc site is edited in both D. melanogaster and D. virilis and has also been observed in M. domestica (R. Reenan, unpublished observation).

The functional consequences of all three editing sites are intriguing considering the structural properties of the Na\(^+\) channel. First, the Fsp site is contained within the first cytoplasmic domain, which is known to contain several PKA phosphorylation sites (Murphy et al. 1996; Smith and Goldin 1996, 1997). Although the Fsp site itself does not create or abolish a phosphorylation site, the charge change introduced by editing (Q-to-R) may affect regulation by phosphorylation. Histidine is encoded by the D. virilis genome at this position. Thus, in D. virilis, this position may be positively charged depending on local pH. In D. melanogaster, editing changes the coding potential from an uncharged Q to a positively charged R residue, which may be functionally equivalent to the D. virilis edited histidine. Second, the Sfc site is contained within a short cytoplasmic linker between homology domains III and IV, a region known to be important for inactivation of the Na\(^+\) channel. More importantly, the edited Sfc site introduces the serine (N-to-S) of a consensus PKC phosphorylation site (Kemp and Pearson 1990; Keneally and Krebs 1991). The functional changes introduced by phosphorylation of this region have been studied in rat brain Na\(^+\) channels. For example, phosphorylation of a protein kinase C (PKC) site, whose serine is seven amino acids from the Sfc site, is required to slow inactivation of the Na\(^+\) channel (Numann et al. 1991; West et al. 1991).

In addition, this PKC phosphorylation site is required for reduction in peak sodium currents induced by cAMP (Li et al. 1993). The addition of a second PKC phosphorylation site by RNA editing of the Sfc site may prolong the slow inactivation associated with phosphorylation at the adjacent genomic encoded site.

We have documented the existence, evolutionary conservation, and developmental regulation of editing at three sites in para. More important, we can now use the power of Drosophila genetics for in vivo analysis of editing substrates as well as temporal and spatial analysis of editing.

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