The *stoned* Locus of *Drosophila melanogaster* Produces a Dicistronic Transcript and Encodes Two Distinct Polypeptides

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ABSTRACT

The *stoned* gene of *Drosophila melanogaster* is required for normal neuronal function in both adult and larva. We have identified DNA sequences that lie within a genetic region that is known to include the *stoned* gene and that also reveal restriction site variations in two *stoned* lethal mutants. This genomic region contains a single transcription unit coding for an ~8.4-kb transcript. The transcript is preferentially expressed in the head of adult flies. The isolation and sequencing of cDNA and genomic clones reveals that *stoned* appears to encode a dicistronic mRNA, although the possible existence of other forms of mRNA cannot be excluded. Antibody cross-reactivity shows that two proteins are translated from the *stoned* locus *in vivo*. Both open reading frames (ORFs) encode novel proteins. The protein encoded by the first ORF contains four tandemly repeated motifs, and one domain of the protein encoded by the second ORF shows similarity to a family of proteins (AP50s) associated with clathrin assembly protein complexes.

THE *stoned* locus of *Drosophila melanogaster* was originally identified as a potential neurological gene by GRIGLIATTI and his colleagues (1973) when two *stoned* alleles were isolated in a screen for temperature-sensitive paralytic mutants. These mutants show severe debilitation at restrictive temperature but are more coordinated, if somewhat sedentary, at permissive temperature. A third behavioral mutant allele, stn^C, was isolated in a separate screen and was found to be stress sensitive (HOMYK 1977; HOMYK and SHEPPARD 1977). Along with the three behavioral mutant alleles, a number of lethal alleles at the *stoned* locus have been identified (MIKLOS *et al.* 1987; PETROVICH *et al.* 1993), one of which was isolated in a screen for *P* factor-induced lethal mutants (ZUSMAN *et al.* 1985), and another was recovered after the crossing of two long term laboratory stocks (SCHALET 1986).

Alterations in the transient components of the electroretinograms (ERGs) of the stn^{ts1}, stn^{ts2} and stn^C confirmed that this locus has a role in the normal functioning of the nervous system (KELLY 1983; HOMYK and PYE 1989; PETROVICH *et al.* 1993). A mosaic analysis using a *stoned* lethal allele has shown that wild-type *stoned* gene expression is required in regions of the embryo that generate the nervous system (PETROVICH *et al.* 1993).

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Further evidence that the *stoned* gene product is involved in neural function derives from the allele-specific interaction of stn^{ts} mutants with *shibire* (*shi*) and *dunce* (*dnc*) mutants (PETROVICH *et al.* 1993). In both cases the double mutant results in synthetic lethality or severely reduced viability. The *shibire* gene product, dynamin (CHEN *et al.* 1991; VAN DER BLIEK and MEYEROWITZ 1991), is known to be involved in endocytosis and synaptic vesicle recycling (POODRY and EDGAR 1979; KOSADA and IKEDA 1983; KOENIG and IKEDA 1989; MASUR *et al.* 1990), whereas the *dunce* locus encodes a cAMP phosphodiesterase (BYERS *et al.* 1981) and is thought to act in short term memory acquisition by altering synaptic facilitation and potentiation (ZHONG and WU 1991). Hence the interaction between the stn^{ts} mutations and the *dnc* and *shi* mutations points to a likely synaptic role for the *stoned* gene product.

The complementation patterns of the various *stoned* alleles are, for the most part, straight forward, in that all of the lethal alleles fail to complement each other. However, the pattern of complementation between the behavioral alleles and the lethal alleles is more complex. Thus the ERG defects associated with the stn^C mutation are partially complemented by some, but not all, of the lethal alleles, whereas the reduction in viability associated with the stn^{ts} alleles is enhanced by those lethal alleles that partially complement stn^C. Finally, the stn^{ts} and stn^C mutations complement each other. These data have been interpreted as indicating that the *stoned* locus encodes a polypeptide with two distinct functional domains (PETROVICH *et al.* 1993).

Genetic analysis has placed the *stoned* locus at the

base of the *X* chromosome, in polytene region 20 (MIKLOS *et al.* 1987). This euchromatin/heterochromatin boundary region has been the subject of considerable investigation. A number of lethal complementation groups as well as deficiencies have been used to define the genetic fine structure of this region of the genome (SCHALET and LEFEVRE 1976; MIKLOS *et al.* 1987). The complementation groups within cytological region 20 have been described, as have deficiencies that define the genetic position of the *stoned* locus. However, these deficiencies do not separate the *stoned* gene from two closely linked loci (20Bb and 20Ca) defined by the lethal mutations *l(1)13E3* and *l(1)EA41* (MIKLOS *et al.* 1987), nor is the proximal/distal order of these genes defined. As well as these deficiencies, there exist a number of lethal *stoned* alleles, some of which might disrupt the coding sequence (MIKLOS *et al.* 1987; PETROVICH *et al.* 1993). A combination of *stoned* mutations and region 20 deletions has been used in this study to isolate clones that identify the *stoned* gene.

The genetic complexity of the *stoned* locus, together with its interaction with a number of other neurological genes, poses some interesting questions relating to its structure and function. In this report we describe cloning and sequence analysis that reveals the dicistronic nature of the *stoned* gene and show evidence for both translation products. We interpret these findings in relation to the complementation pattern of the *stoned* mutant alleles. Analysis of the structure of the protein product of the second open reading frame (ORF) suggests a possible role for the *stoned* locus in membrane trafficking and allows for an explanation of the interaction between *shibire* and *stoned* mutants.

MATERIALS AND METHODS

Drosophila techniques: All of the *stoned* alleles are as previously described (PETROVICH *et al.* 1993). The wild-type and deficiency strains used in this study are as described in LINDSLEY and ZIMM (1992), MIKLOS *et al.* (1987) or HEALY *et al.* (1988).

In all cases, Southern blots of DNA derived from the *stoned* mutants were compared with those generated from the progenitor strain, or at least strains bearing the progenitor *X* chromosome.

Separation of heads and bodies, the preparation of soluble protein fractions from these tissues, and the preparation of DNA and RNA are as previously described (KELLY 1990; PHILLIPS *et al.* 1992)

Libraries and library screens: The mini-library used in this study was kindly provided by Dr. G. MIKLOS and has been described previously (MIKLOS *et al.* 1988). Three genomic libraries were used as a source of the overlapping λ genomic clones. The libraries were derived as follows: from Oregon-R (M. GOLDBERG) producing the "G" clones, an Oregon-R-derived isochromosomal strain (J. TAMKUN), the "E" clones, and a Canton-S-derived library (Clontech) the "C" clones.

The cDNAs were isolated from an oligo-dT and random primed Drosophila head cDNA library in the λZAP vector (Stratagene). Five clones were isolated using the insert from DmH95 and known as p95Z3 to p95Z7. Four clones were isolated using pG14-4.8 as a probe and known as p4.8Z3, p4.8Z4, p4.8Z6 and p4.8Z7. No cDNAs that cross-hybridized to DmH61 were found. Four of the five p95Z clones were presumed to be separate isolates based on the different sizes and/or orientation of the cDNA clone within the Bluescript plasmid. All of the p95Z clones contained the same terminal *Eco*RI site, suggesting the absence of protective *Eco*RI modification during the construction of the library.

Mapping and identification of insertions in *stoned* mutants: All molecular biological methods were as described in SAMBROOK *et al.* (1992). All extant *stoned* alleles were screened for DNA lesions by Southern blotting using hybridization probes spanning the region from coordinates −5.2 to +12.6. The DNA lesions in stn^{PH1} and $stn^{13\text{-}120}$ were mapped more accurately using several restriction enzyme digests and hybridization probes that spanned the sites of insertion. The restriction maps that were deduced for the inserted fragments were then compared to the restriction maps of known transposable elements (LINDSLEY and ZIMM 1992).

Sequencing: The sequencing of the two longest cDNA clones, p95Z7 and p4.8Z3, along with the genomic clone p61, was carried out on both strands using the method of SANGER *et al.* (1977) and T7 polymerase (Sequenase, USB). The single-strand template of p4.8Z3 appeared to contain a region of considerable secondary structure (residues 4851–4946 of Figure 4), and the final sequence was only determined by using a combination of dITP, AMV reverse transcriptase (Promega) and Taq polymerase (Promega). A *Xho*I/*Sal*I genomic fragment that included the *Eco*RI site between DmH61 and DmH95 was also partially sequenced to confirm the continuity of sequence between DmH61 and p95Z7.

The *Xba*I/*Xho*I fragment (residues 2438–2875 Figure 4) from each of the p95Z clones was subcloned and sequenced to confirm the intercistronic sequence for all of the cDNA clones. The equivalent *Xba*I/*Xho*I fragment (1.4 kb) from DmH95 was also partially sequenced and located an intron 3′ to the intercistronic region.

The RT-PCR reactions were set up using head total RNA, MMV-reverse transcriptase and Taq polymerase as suggested by the supplier (Promega). Oligonucleotide primers that spanned the intercistronic region and the intron were used (residues 2477–2887 Figure 4).

Preparation of fusion proteins, antibodies and Western blots: The pMAL-C2 and pGEX-4T-1 vectors were obtained from New England Biolabs and Pharmacia, respectively, as were the amylose and glutathione affinity matrices.

The *Xho*I fragment from DmH61 (residues 115–1161) was subcloned into the *Sal*I site of Bluescript SK+. The orientation was determined, and the fragment was then cut out using *Eco*RI/*Xho*I and cloned into *Eco*RI/*Sal*I-cut pMal-C2 (pMAL-33X) and pGEX-4T-1 (pGEX-33X). The 3′ terminal fragment of p4.8Z3 (residues 5791–8122) was cut from p4.8Z3 using *Pst*I and subcloned into *Pst*I-cut Bluescript. The orientation was determined, and the fragment was cut out using the *Bam*HI and *Hin*dIII sites in the polylinker and cloned into *Bam*HI/*Hin*dIII-cut pMal-C2 (pMAL-EP1). For expression in pGEX, the *Bam*HI/*Eco*RI fragment from the pMAL construct was cut out and cloned into *Bam*HI/*Eco*RI-cut pGEX-4T-1 (pGEX-EP1). Fusion proteins were induced, and the proteins affinity purified as per suppliers instructions. With the exception of pGEX-EP1, which produced an insoluble protein product, milligram quantities of the fusion proteins were readily purified.

Antibodies were raised as previously described (KELLY 1990), using 200 μg of each of the maltose-binding protein (MBP) fusion proteins (MBP::STNA and MBP::STNB) for both the primary and booster immunizations. The anti-stoned antibody titer was monitored using the glutathione-S-trans-

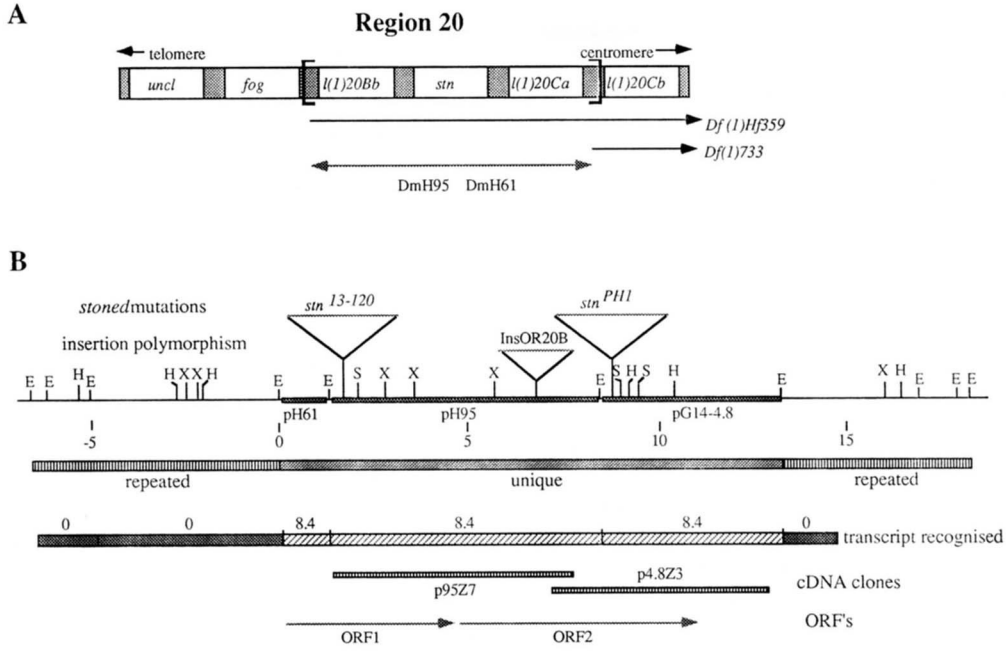

FIGURE 1.—Summary of cloning of the *stoned* gene. (A) The arrangement of lethal complementation groups in the proximal region of cytological division 20 are shown with the eight adjacent complementation groups in order, with the exception of the loci in brackets, *l(1)EA1, stn,* and *l(1)13E3.* The extent of the deficiencies used in this study are also shown. Clones from the microdissected mini library, DmH95 and DmH61, lie within *Df(1) HF359* but outside *Df(1)733.* (B) The restriction map of the genomic clones is diagrammed, however, the proximal-distal orientation of these clones with respect to the chromosome is unknown (E, IRI; S, *Sal*I; X, *Xba*I; H, *Hin*dIII). The position of the two insertion mutations stn^{PH1} and $stn^{13\text{-}120}$ are defined as being within the region bracketed by the closest restriction sites. The position of the insertional polymorphism InsOR20B, which is present only in the E and G genomic clones derived from Oregon-R wild-type flies, is also shown. The sizes of the insertions are not drawn to scale. The arrangement of unique and repetitive sequence are diagrammed as are the regions that recognize the 8.4-kb transcript. The relative positions, with respect to the genomic clones, of the two longest overlapping cDNA clones, p95Z7 and p4.8Z3, are indicated along with the positions of the two ORFs.

ferase (GST) fusion proteins. As insufficient GST::STNB (from pGEX-EP1) fusion protein could be purified for competition studies; MBP::STNB fusion protein was linked to a 1-ml NHS-activated High-Trap column (Pharmacia) according to the manufacturer's recommendations. This affinity matrix was used for the purification of anti-MBP::STNB antibodies. Five hundred microliters of serum was applied directly to the column and equilibrated for 2 hr at room temperature. The column was then washed in Tris-buffered saline (TBS) until the $O.D._{280}$ reached zero, at which point bound antibodies were eluted with 4 M $MgCl_2$. Bovine serum albumin was added to the eluant to a final concentration of 1 mg/ml, and the mixture was dialyzed against TBS overnight, freeze dried and stored at −20°.

Western blots were performed as previously described (KELLY 1990) with the exception that an horse radish peroxidase-linked secondary anti-rabbit antibody was used and was visualized using Enhanced Chemi-Luminescence (Amersham).

RESULTS

Cloning the *stoned* genomic region: A microdissected mini-library cut from *X* chromosome region 20 (MIKLOS *et al.* 1987) was used to identify unique clones that lie within the region excluded by *Df(1)733* but included in *Df(1)HF359* (Figure 1A). A total of 37 of the mini-library clones were screened using quantitative Southern blot analysis with genomic DNA from wild-type males and females, and heterozygous *Df(1)733* and *Df(1)HF359* female flies. Five of these clones, DmH61, DmH70, DmH79, DmH88 and DmH95, gave reduced cross hybridization signals with *Df(1)HF359* DNA but not with *Df(1)733* DNA, indicating their location within the *stoned* genetic region (Figure 2A). To determine if any of these DNA fragments originated from the *stoned* locus, they were used to probe Southern blots of genomic DNA from the various *stoned* mutant strains. Two clones, DmH95 and DmH61, hybridized to polymorphic DNA fragments in the stn^{PH1} and $stn^{13\text{-}120}$ mutants and were chosen for further analysis.

The screening of three genomic λ libraries, using DmH95 and DmH61 clones as probes, yielded six overlapping positive clones covering ~25 kb of the genome. The orginal clones from the microdissected library, DmH95 and DmH61, derive from adjacent genomic fragments (Figure 1B). A comparison of the separate genomic isolates indicated that the G and E genomic clones (prepared from Oregon-R wild-type flies, see MATERIALS AND METHODS) contained a polymorphic 2.4-kb insertion, named InsOR20B, that was absent both from the C clones (derived from Canton-S wild-type flies) and the DmH95 microdissected fragment (Figure 1B). A number of *stoned* mutant strains including stn^{ts1}, stn^{ts2}, stn^{C}, stn^{S64} and stn^{8P1}, along with the Oregon-R chromo-

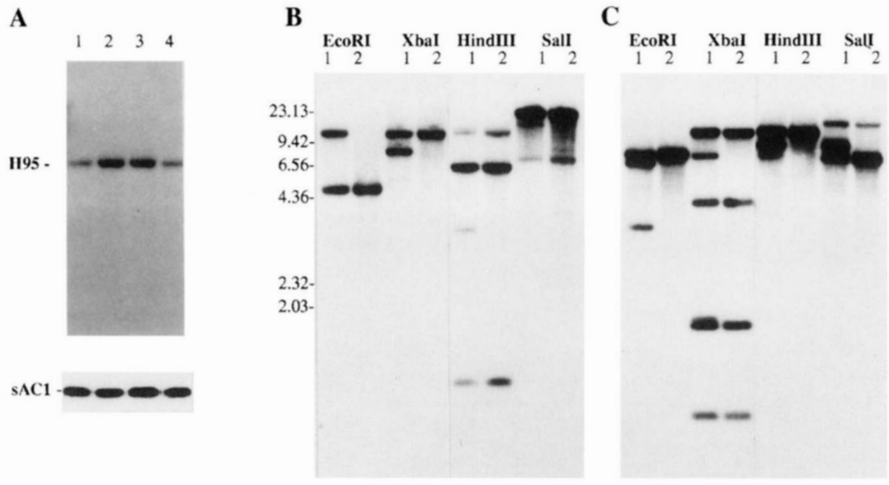

FIGURE 2.—Southern blot analysis of *stoned* clones. (A) A quantitative Southern blot of whole genomic DNA digested with *Eco*RI and probed with clone DmH95 from the microdissected library, and the same blot probed with an *Adh* probe (sAC1). Lane 1, *Df(1)HF359/FM7;* lane 2, *Df(1)733/FM6;* lane 3, Oregon-R wild-type females; lane 4, Oregon-R wild-type males. (B) Restricted whole genomic DNA probed with pG14-4.8. Lanes 1, DNA from stn^{PH1}/*FM6* heterozygous flies; lanes 2, DNA from $In(1)sc^{8}$ (stn^{+}) flies. (C) Restricted whole genomic DNA probed with DmH95. Lanes 1, DNA from $stn^{13\text{-}120}$/*Binsn* heterozygous flies; lanes 2, DNA from M56i (stn^{+}) flies. With the exception of the *Hin*dIII digest in B, all digests reveal different cross-hybridizing fragments in the mutant strain by comparison with the progenitor strain, and the aberrant fragments were shown not to arise from the *FM6* and *Binsn* chromosomes. These differences are consistent only with insertion events.

some, contained this insertion. Southern blots of genomic DNA probed with subclones of InsOR20B identified multiple copies in the fly genome (data not shown) suggesting that the insertion may be a remnant of a mobile genetic element.

Various cloned DNA fragments were used as probes on whole genomic Southern blots to determine if they represented unique sequences. Three contiguous *Eco*RI fragments, pG14-4.8, DmH95 and DmH61, exist as a 12.5-kb island of unique DNA surrounded by repetitive DNA (Figure 1B).

Using the pG14-4.8, DmH95 and DmH61 fragments as probes, novel insertions were apparent in the $stn^{13\text{-}120}$ and stn^{PH1} mutant chromosomes (Figures 1B and 2, B and C). In both cases these insertions are absent from the progenitor chromosomes. Figure 2 also indicates that the insertion in $stn^{13\text{-}120}$ is within DmH95 and the stn^{PH1} insertion is within pG14-4.8. The deduced restriction maps of the stn^{PH1} and $stn^{13\text{-}120}$ insertions are consistent with those of "I" elements and "doc" elements, respectively.

Identification of the *stoned* transcript: Northern blots of total RNA from both heads and bodies of adult flies were probed with DmH95 insert DNA (Figure 3A). The probe identifies an ~8.4-kb transcript that is largely expressed in the head of the fly with reduced expression of the same size transcript in thoraces and abdomens. To determine the extent of the genomic region that might encode this transcript, various genomic DNA fragments were used to probe Northern blots. The 8.4-kb transcript derived from a 12-kb region encompassed by clones DmH95, DmH61, and pG14-4.8 (Figure 1). No hybridization to any transcript was observed using any of the adjacent genomic regions as probes.

Northern blots were prepared using RNA derived from the heads of the various *stoned* mutant strains. When these blots were probed with the DmH95 clone, both larger and smaller cross-hybridizing transcripts were observed in $stn^{13\text{-}120}$ and stn^{PH1} heterozygotes, consistent with the presence of insertions in both of these mutants (Figure 3B). Presumably at least part of the insertion sequences were being included in the mature transcript to produce a population of larger mRNA molecules or were causing premature termination to produce truncated transcripts. None of the other *stoned* mutant strains showed alteration in the transcript size, including those that contained the polymorphic insertion (InsOR20B).

Sequence determination of the *stoned* transcript: A number of cDNAs were isolated from head cDNA libraries using the DmH95 and pG14-4.8 genomic clones as probes (see MATERIALS AND METHODS). However, no cDNAs that hybridized with DmH61 were found. Using the cDNAs as probes on genomic Southern blots indicated that the cDNAs did not extend beyond the region covered by clones DmH95 and pG14.48, and Northern blot analysis showed that all cDNAs recognized only the 8.4-kb transcript (data not shown). Five of the cDNAs that cross-hybridized with DmH95 all terminated at the same *Eco*RI site that, from sequence analysis of the respective cDNA and genomic clones, corresponds to the *Eco*RI site separating the DmH61 and DmH95 genomic fragments. The two largest and overlapping cDNAs (p95Z7 and p4.8Z3) that, on the basis of restriction maps, covered the greatest proportion of the genomic region were sequenced. In total 7.1 kb of cDNA sequence was obtained from these cDNAs (Figure 4). As no cDNAs corresponding to the DmH61 region of the

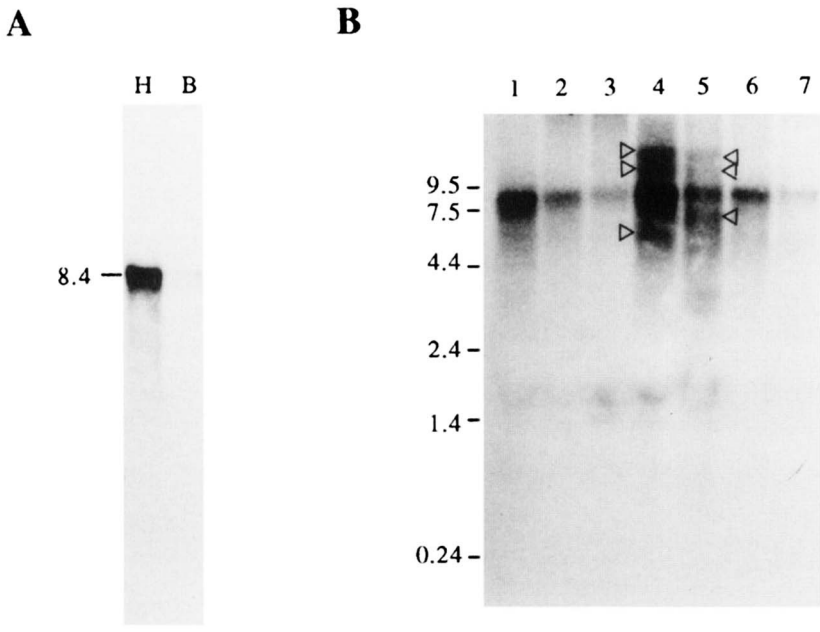

FIGURE 3.—Analysis of the *stoned* transcript. (A) Northern blot of total RNA from heads (H) or bodies (B) of Oregon-R adults probed with DmH95. A single-sized transcript of ~8.4 kb is evident in head RNA, and the same-sized transcript is present at much reduced levels in body RNA. The total amount of RNA loaded (20 μg) is equivalent in the two tracks. (B) Northern blot of total RNA from heads of lane 1, Oregon-R; lane 2, stn^{X3}/*FM6*; lane 3, $stn^{R\text{-}9\text{-}10}$/*FM6;* lane 4, stn^{PH1}/*FM6;* lane 5, $stn^{13\text{-}120}$/*Binsn;* lane 6, stn^{8P1}/*FM6;* lane 7, stn^{S64}/*FM6*. The open arrowheads indicate abnormal size transcripts present in the stn^{PH1}/*FM6* and $stn^{13\text{-}120}$/*Binsn* strains. Each track does not represent equivalent amounts of RNA.

genomic clone were found, this region of genomic DNA was sequenced to extend the 5′ end. This genomic fragment contained a single ORF with no indication of introns. The completed sequence is shown in Figure 4. The proposed transcript, including sequence determined from the DmH61 genomic fragment, totals 8.1 kb, which approximates the size of the mRNA seen on Northern blots.

The *stoned* transcript contains two tandemly arranged ORFs: Identification of ORFs in the completed sequence revealed no single ORF but rather two tandemly arranged ORFs, which we name cistron A and cistron B. The two cistrons are separated by a 55-bp intercistronic region containing termination codons in all three reading frames (Figure 4). To confirm that the presence of the termination codons was not a sequencing artifact, the sequencing was repeated using dITP and deaza-dGTP. All four cDNAs that cross-hybridized with DmH95 and could be considered unique isolates on the basis of variation in size and/or orientation within the λZAP vector were also sequenced. All four cDNAs spanned both ORFs and contained the same intercistronic region. This region of genomic clone DmH95 was also sequenced and confirmed the presence of the same intercistronic region. The presence of an intron immediately 3′ to the intercistronic region (nucleotide position 2835, Figure 4) in the genomic clone, confirmed that the cDNAs had been derived from mRNA that had been subjected to normal splicing. RT-PCR across the intercistronic region, using a primer that is 3′ to the intron and one that is 350 bp 5′ to the intercistronic region, gave only the fragment of the size expected from the cDNA sequences. Thus we have no evidence to show that the intercistronic region is spliced out of some mRNA species. All of the data we have obtained are consistent with the presence of two sequential ORFs in a single *stoned* transcript; however, we have not eliminated the possibility that other, less abundant forms of mRNA might derive from this locus. We propose that the protein product of the first cistron be called STNA and the second STNB.

Analysis of the proteins encoded by the two ORFs: The cDNA sequence of cistron A encodes a hydrophilic and presumably soluble protein of 93 kDa. The amino acid composition of STNA is unusual in that it is high in acidic amino acids and is free of cysteine and internal methionine residues. A comparison of this sequence to those in the databases using the FASTA program failed to reveal any significant homology, indicating that this is a novel protein. An internal homology search did, however, reveal the presence of four repeat sequences near the carboxy-terminal end of STNA (Figure 4). The first two repeat sequences are more extended than the second two, and there is a short eight-amino acid sequence starting at residue 481, which has homology to the carboxy-terminal end of the two longer repeats (Figure 5A). Using the repeat motif to screen the databases, no significant homology was found to other known proteins.

The protein encoded by cistron B, STNB, has a predicted molecular mass of 138 kDa, and analysis of the amino acid sequence indicates that it is also a soluble protein with no extended regions of hydrophobicity. The amino-terminal portion of this protein is proline rich. Comparison of the STNB amino acid sequence with those in the databases revealed limited homology between the carboxy-terminal domain of STNB and the rat AP50 subunit of the clathrin-associated protein AP2 complex (THURIEAU *et al.* 1988). This subunit is homologous to an equivalent protein (AP47) in the mouse AP1 complex (NAKAYAMA *et al.* 1991) and to a yeast

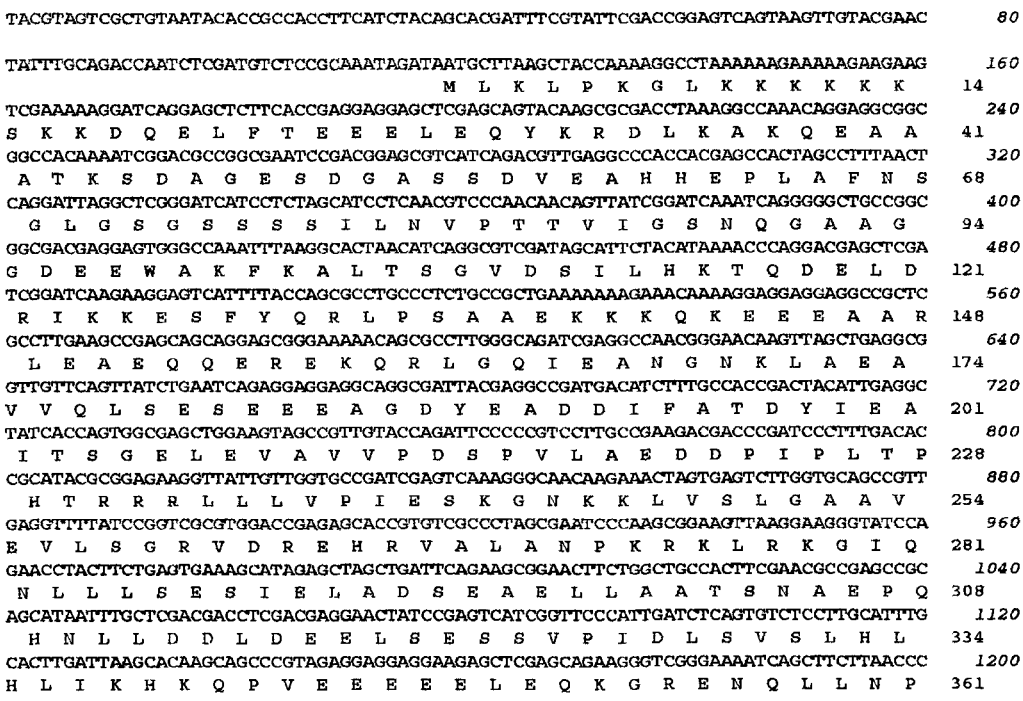

FIGURE 4.—The complete sequence of the *stoned* locus (Genbank accession no. U54982). The region of the sequence from the start to the arrow labeled pZ7 is from the DmH61 genomic fragment. The arrows labeled pZ7 indicate the beginning and end of the cDNA p95Z7, while the arrow labeled pZ3 indicates the start of the cDNA clone p4.8Z3. The intercistronic region is from bases numbered 2669 to 2723 and contains five termination codons, shaded, of which at least one is present in each of the three possible reading frames. The position of the intron immediately 3′ to the intercistronic region is also shown. The shaded regions in the first ORF are the repeated sequences, while the shaded region in the second ORF is the region that shows homology to the mammalian and yeast AP50 family of proteins.

protein (yAP57), thought to be functionally homologous to AP47 (NAKAYAMA *et al.* 1991). The homology of the STNB AP50 homology domain with this family of proteins is shown in Figure 5B. The amino acid identity between this domain of the STNB protein and each member of the AP50 family is ~23%, but the identity rises to 42% when comparison is made with the family as a whole. In contrast, the level of amino acid identity for yeast AP57-rat AP50 is 39% and for the yeast AP57-mouse AP47 comparisons is 56%. However, when the residues that are conserved in all of the AP50 family members are identified, 60% of these are also conserved in the STNB protein (Figure 5B).

Immunochemical identification of two *stoned* translation products: As the presence of two tandemly arranged ORFs in a single transcript is unique in eukaryotes, we sought to determine if both of these cistrons are translated as independent units *in vivo*. Antisera were raised against residues 27–350 of STNA (Figure 4) or residues 1024–1260 of STNB (Figure 4), fused to the *Escherichia coli* maltose-binding protein (MBP) (see MATERIALS AND METHODS). When the MBP::STNA fusion protein was produced, it exhibited a lower mobility than expected on SDS-PAGE. This was also the case when the same DNA fragment was subcloned into the pGEX vector to produce a glutathione-S-transferase-STNA (GST::STNA) fusion protein. In both cases the fusion proteins gave molecular weight estimates some 11 kDa greater than anticipated (Figure 6A). The high acidic amino acid content of this fragment of STNA may well be the cause of this anomalous behavior. The proteins produced from the fusion of the STNB fragment to either MBP in the pMAL vector or to GST in the pGEX vector gave products of the expected mobility on SDS-PAGE.

Both antisera were then used to identify proteins on Western blots of Oregon-R Drosophila head soluble protein extracts. The anti-STNA antiserum recognized a single protein species, and this cross-reactivity was effectively competed out by the preincubation of the antiserum with GST::STNA fusion protein (Figure 6B). The STNA protein had an apparent molecular mass of ~145 kDa on SDS-PAGE, in contrast to the 93 kDa predicted from the amino acid sequence. This is not unexpected given the reduced mobility of the fusion proteins containing a portion of the STNA protein. Western blots of samples prepared from thoraces and abdomens, and containing an equivalent amount of total protein, gave a low level of cross-reacting protein with identical molecular mass, using the anti-STNA antiserum (Figure 6B). The mobility of the anti-STNA cross-reacting protein was unaffected by the absence of reducing agent in SDS-PAGE.

Affinity-purified anti-STNB antibodies also recog-

FIGURE 4.—*Continued*

nized a single protein on Western blots of protein fractions from heads. This protein had a mobility on nonreducing SDS-PAGE consistent with a molecular mass of 138 kDa as predicted from the amino acid sequence of the STNB polypeptide. However, under reducing conditions, the anti-STNB cross-reacting protein migrated with a molecular mass of 70 kDa (Figure 6C). Presumably the STNB protein has been proteolytically cleaved, either *in vivo* or in preparation of the extracts, but the polypeptide fragments remain associated due to disulfide cross bridges that are removed on reduction. Low levels of cross reactivity with the anti-STNB antibodies were observed in fractions from thoraces and abdomens (data not shown).

DISCUSSION

We have identified a region of the Drosophila genome that encodes the *stoned* gene. The genetic data

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GACTTACTGCATGACGATCTGGACGAGACGATTGGGGAGGGTGAACCGCCGGAACAAGAGGAGCCGGACACGGAGCAGAG   3760
D  L  L  H  D  D  L  D  E  T  I  G  E  G  E  P  P  E  Q  E  E  P  D  T  E  Q  S   346
CAATGAAATTAGCTCTCGAGATGAACCAGTATTTACTTCCCTTCTTATACGACCAGATGAAAGCACCCATGATATAACAT   3840
 N  E  I  S  S  R  D  E  P  V  F  T  S  L  L  I  R  P  D  E  S  T  H  D  I  T  S  373
CCCAACCACAGGCGGCTACTGGATTGGAACGACAGGTGAATAACATGGCTGCACCACTGGGAACCGCCAGCACGCAGAGG   3920
  Q  P  Q  A  A  T  G  L  E  R  Q  V  N  N  M  A  A  P  L  G  T  A  S  T  Q  R   399
GCGACCACGCCCGATATTGAGATAACCACGGTAGAGGACCTGCCGCGATCTGATGACGAGGATGAGCCGGAGGCTATGCA   4000
A  T  T  P  D  I  E  I  T  T  V  E  D  L  P  R  S  D  D  E  D  E  P  E  A  M  Q   426
GGAGCCCGAAACAGAGACGAAACCACAAATCGAACCCGATACGGAGCCCGAGATCGTATCTGAACACAGCCCACCAACGG   4080
 E  P  E  T  E  T  K  P  Q  I  E  P  D  T  E  P  E  I  V  S  E  H  S  P  P  T  E  453
AACGCCTTGTGACTCAGGCAGCCCTCGTCGATGGTGAGCTAATAGCCGCCGAGCCTGAGCCGGAAGAAATGGACACCGGT   4160
  R  L  V  Y  Q  A  A  L  V  D  G  E  L  I  A  A  E  P  E  P  E  E  M  D  T  G   479
CTAGATTTTCCGCTGGCTTCCAGCGCCAGTTGTCCGCCAACCCCTTCGCCAGCCCCGACGAGGAGGAGCCGAACTTCGCG   4240
L  D  F  P  L  A  S  S  A  S  C  P  P  T  P  S  P  A  P  T  R  R  S  R  T  S  R   506
CCCATGCCAGCGAGTTGCCAATATATTTGCAGTGAACGATCCGGACTCGCAGATGGAGACCCCGAAAGCGCCTAGCCATA   4320
 P  C  Q  R  V  A  N  I  F  A  V  N  D  P  D  S  Q  M  E  T  P  K  A  P  S  H  T  533
CGGCCAATATCTTTGCTTCCGATCCAGACGAGTTCGACGCCTTCTCAGCCAAGTTTGACTCGGTGAAGAAGGACAACATC   4400
  A  N  I  F  A  S  D  P  D  E  F  D  A  F  S  A  K  F  D  S  V  K  K  D  N  I   559
AGCATAATGGACGGATTTGGCGGTTCCGGAGCGATAACGCCCACGTGCCGATGCTTGGGAGGTAGTGCTTTTGGATCAAC   4480
S  I  M  D  G  F  G  G  S  G  A  I  T  P  T  C  R  C  L  G  G  S  A  F  G  S  T   586
```

FIGURE 4.—*Continued*

indicates that the original microdissected library clones derive from the region of the *X* chromosome encompassing the *stoned* locus. This genomic region hybridizes with an abundant 8.4-kb transcript in adults, and the preferential expression of this transcript in the head is indicative of neural expression. This is consistent with the data obtained from *stoned* mosaic flies that indicated a neural location for the *stoned* gene product (PETROVICH *et al.* 1993). The presence of transposable element insertions in two of the *stoned* lethal mutants (stn^{PH1} and $stn^{13\text{-}120}$), along with the alteration in transcript size in these strains, confirms that the cloned region encodes the *stoned* locus. The increase in the the size of the transcript observed in $stn^{13\text{-}120}$ mimics the situation observed in the abo^{1} mutant allele of the *abnormal oocyte* gene. This mutant also results from the insertion of a doc element and produces an abnormally large transcript (TOMKIEL *et al.* 1995). The alteration in transcript size in stn^{PH1} and $stn^{13\text{-}120}$ is in contrast to the findings for Oregon-R, stn^{S64}, stn^{ts2} and stn^{C}, all of which carry the InsOR20B insertion polymorphism but fail to show any difference in transcript size. Presumably InsOR20B is inserted in intronic sequences and is spliced out to produce a normal sized mRNA.

The failure of any of the adjacent genomic regions to hybridize with the 8.4-kb transcript suggests that either the contiguous unique sequences in this genomic region represent the complete *stoned* transcription unit, or that 5′ untranslated sequences derive from more remote regions. The combination of the cDNA sequences and the ORF of H61 produces a total of 8.1 kb indicating that most of the *stoned* gene must be contained within the sequenced region. We had hoped to address the question of the extent of the *stoned* gene using rescue experiments, but none of the lambda genomic clones cover the entire *stoned* region, and we were unsuccessful in isolating cosmid clones. This latter finding may result from the repetitive nature of the DNA surrounding the *stoned* region and the propensity of such regions to undergo extensive recombination in bacteria.

The most unusual feature of the *stoned* locus is the apparent presence of a single spliced transcript with the potential to encode two distinct polypeptides. Apart from prokaryotes, the only previously described nonoverlapping dicistronic mRNAs are in the Epstein-Barr virus, where two nuclear proteins are translated from from a single mRNA (WANG *et al.* 1987), and in some cellular mRNAs where there is a small ORF ("minicistron") in the 5′ leader sequence (KOZAK 1989). This means that *stoned* appears to represent the first eukaryotic dicistronic gene where both cistrons appear to be part of the same mature mRNA. In support of this conclusion we note the following.

In the report detailing the complementation patterns of the lethal and behavioral *stoned* mutants, it was suggested that this locus encodes a product with two distinct functional domains (PETROVICH *et al.* 1993). The ERG complementation data placed the stn^{C} mutation in the same domain as the $stn^{13\text{-}120}$ mutation, but in a separate domain from the stn^{PH1} mutation, while the viability data placed stn^{ts} in the same domain as stn^{PH1}. This correlates with the observation that the $stn^{13\text{-}120}$ mutation is an insertion in the stn^{A} cistron, while the stn^{PH1} insertion lies in the stn^{B} cistron, and would explain the complete complementation observed between stn^{ts} and stn^{C} (PETROVICH *et al.* 1993). Presumably the failure of $stn^{13\text{-}120}$ to complement stn^{PH1} would be due to polarity effects of the insertion in cistron A on the translation of cistron B. In an earlier report it was noted that two lethal *stoned* alleles, known as *l(1)X-3* and *l(1)R-9-15*, also complemented each other (LIFSCHYTZ and FALK 1969), indicating that interallelic complementation may not be restricted to the viable behavioral alleles. Unfortunately the *l(1)R-9-15* allele is no longer available.

We have found no indication that the *stoned* mRNA undergoes alternative splicing that might fuse portions

FIGURE 4.—*Continued*

FIGURE 5.—Homology domains of STNA and STNB proteins. (A) Comparison of the sequence of the internal homology regions in STNA. Identical amino acids are shaded. The numbers on the left refer to the position in the complete sequence (Figure 4) of the first amino acid of the repeat. (B) Comparison of the carboxy-terminal region of STNB (residues 887–1133) with the AP50 family of proteins. Identical amino acids are shaded. The boxed sequences are those residues that are identical in STNB and/or all five AP50 family proteins. The alignment of *Saccharomyces cerevisiae* YAP54–1, rat AP50 and mouse AP47 is taken from NAKAYAMA *et al.* (1991), on which the STNB sequence has been superimposed. The YAP54-2 and the POMBE sequences derive from the *S. cerevisiae* chromosome *XV* sequencing project (Genbank accession no. X91067) and the *Schizosaccharomyces pombe* chromosome *I* sequencing project (Genbank acccession no. Z50113), respectively. These five were chosen as representing the greatest diversity of amino acid sequence among the AP50 family of proteins. The alignment at the extreme carboxy-terminus has been added even though there is a 34-amino acid addition in the STNB sequence.

of the first ORF in frame with the second ORF. Only low copy number transcripts would have remained undetected on Northern blots. Furthermore, all four of the independent cDNA clones that contain portions of the first ORF also include the intercistronic region and portions of the second ORF. In the genomic clones, the presence of an intron immediately 3′ to the intercistronic region, which is spliced out of the cDNAs, confirms that these cDNAs result from normally spliced mRNA. We have scanned the cDNA sequences 5′ to the intercistronic region for putative 5′ splice junction consensus sequences. No obvious sites exist.

Finally, the antibodies raised against the recombinant fusion proteins derived from each of the two ORFs cross-react with very different protein species in the fly's head. Anti-STNA antibodies do not cross-react with the same protein species that cross-reacts with anti-STNB antibodies and vice versa. Both proteins have similar mobilities on SDS-PAGE, but only the STNB protein dissociates into smaller subunits under reducing conditions.

All of the above data suggest that the *stoned* locus encodes a single dicistronic mRNA that is actively translated *in vivo* into two distinct polypeptides. However, we cannot exclude the possibility that a smaller, less abundant transcript exists that splices out the first ORF, leaving the second ORF to be translated in a normal manner.

The problem associated with dicistronic mRNAs in eukaryotes, in contrast to prokaryotes, derives from the observation that translation initiation is dependent on the recognition of the m7G cap at the 5′ end of mRNAs by the 40S ribosomal subunit. This led to the development of the scanning model of initiation of translation (KOZAK 1989). Hence for termination and reinitiation, the 60S ribosomal subunit would have to dissociate on contact with a "stop" codon and the 40S subunit would be left to scan the mRNA until it encounters another "start" codon, or falls off the mRNA. However scanning can also allow for reinitiation at the start of a second ORF, as demonstrated in synthetic dicistronic mRNAs (KOZAK 1987), even where the first ORF is relatively large and encoding the luciferase gene (LEVINE *et al.* 1991). In the latter work the nature of the intercistronic region was found to define the efficiency of such reinitiation. Thus, the absence of any AUG codons in the

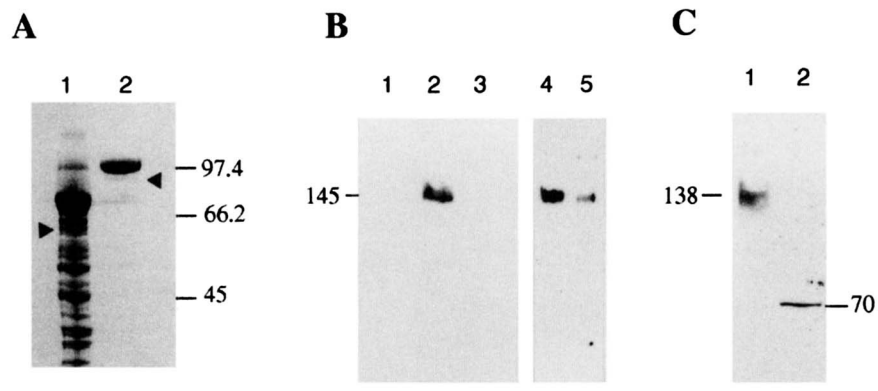

FIGURE 6.—The *stoned* locus encodes two polypeptides. (A) Coomassie blue-stained SDS-PAGE of *E. coli* lysate expressing the GST::STNA fusion protein (lane 1) and affinity-purified MBP::STNA fusion protein (lane 2). The arrowheads indicate the expected positions of the two fusion proteins, with the GST::STNA fusion protein having an expected molecular mass of 61.4 kDa (observed 73 kDa), and the MBP::STNA fusion protein having an expected molecular mass 86.3 kDa (observed 98 kDa). (B) Western blot of head fractions from adult Drosophila probed with anti-STNA preimmune serum (lane 1), anti-STNA immune serum (lane 2), and anti-STNA immune serum preincubated with 40 μg GST::STNA fusion protein (lane 3). The distribution of the STNA protein in heads (lane 4) and bodies (lane 5) is shown. Equal amounts of total protein were loaded in each lane. (C) Western blot of head fractions probed with affinity-purified anti-STNB antibodies where the SDS-PAGE was run under nonreducing conditions (lane 1) and reducing conditions (lane 2).

intercistronic region, the number of bases between the two ORFs, and the presence of an optimized consensus start AUG for the second ORF are all critical (LEVINE *et al.* 1991). The intercistronic region between *stnA* and *stnB* fits these criteria. The 55-bp *stoned* intercistronic sequence contains no AUG codons and is within the range (15–78 bp) for maximum levels of reinitiation (LEVINE *et al.* 1991). The start codon for the second *stoned* ORF is preceded by a purine (G) at the −3 position, and at the +4 position is the canonical G residue (KOZAK 1989). It would appear that significant levels of reinitiation might be expected following such an intercistronic sequence.

A second model for translation initiation, known as the internal entry model (PEABODY and BERG 1986), derives from the observation that certain picornaviral mRNAs and some cellular mRNAs, including the immunoglobulin heavy chain binding protein (BiP), are actively translated even in the absence of the m7G cap (PELLETIER and SONENBERG 1988; MACEJAK and SARNOW 1991). These data suggest the presence of internal ribosome binding sites in the 5′ leader sequences of these mRNAs. Dicistronic mRNAs in which these 5′ leader sequences are located between the two cistrons have been constructed and shown to produce both translation products *in vivo* (KAUFMAN *et al.* 1991; MACEJAK and SARNOW 1991). However, the intercistronic sequences necessary to produce the active translation of the second cistron are large (400–700 bp), and there is no homology between these sequences and those of the intercistronic or adjacent regions of the stn^A and stn^B cistrons. Although internal ribosome entry remains a possibility for the translation of both *stoned* cistrons, we suggest that the scanning model is more likely.

That such an unusual eukaryotic gene structure has evolved for the *stoned* locus implies a particular functional relationship between the two cistrons. Certainly the genetic evidence suggests that both products are involved in similar processes (PETROVICH *et al.* 1993) and implies a need for coordinate expression of the two proteins. Coordinate regulation can be achieved without producing a nonoverlapping dicistronic mRNA. The vesicular acetyl choline transporter (VAChT) and the choline acetyltransferase (ChAT) gene in the rat are encoded by a single primary transcript but the VAChT mRNA derives from the first intron of the ChAT gene (ERICKSON *et al.* 1994).

One possible reason for the presence of the *stoned* dicistronic mRNA is to produce a precise stoichiometric ratio between the two products. This might be most easily maintained by linking their transcription and translation. If such an arrangement of the cistrons is important for stoichiometric reasons, one might expect that it would be retained across species. It will be of interest, therefore, to determine the structure of the *stoned* gene in other species.

The structure of the STNA protein is unusual in a number of respects. The amino-terminal end of STNA has a high probability of forming extended α-helices, and in conjunction with the absence of cysteines, suggests that this represents an extended, nonglobular domain of the protein. The protein is highly acidic (20% acidic amino acids compared with 11% basic amino acids). This would explain its anomalous mobility on SDS-PAGE, as many previously identified acidic proteins, including neurofilament proteins (KAUFMANN *et al.* 1984), chromogranin-B (BENEDUM *et al.* 1987), GAP-43 (KARNS *et al.* 1987) and amphiphysin (LICHTE *et al.* 1992), all show the same anomalous mobility on SDS-PAGE. The presence of the four repeats in the carboxy-terminal domain suggests a specific function for this region of the protein. However, the lack of homology

between the repeats and any other known domain sequences of proteins in the databases means that their possible function remains to be determined. It seems likely, given the dicistronic nature of *stoned* and the *stoned* mutant phenotypes, that the STNA protein functions in the same cellular pathway as the STNB protein.

The observed homology between the carboxy-terminal region of the STNB protein and the AP50 family of clathrin assembly proteins does give some clues as to the possible function of the *stoned* locus. AP50 is a subunit of the AP2 adaptor complexes that are involved in directing the assembly of clathrin coats around invaginating plasma membrane vesicles in mammalian brain, and AP47 is the equivalent subunit of the Golgi-associated AP1 adaptor complexes (AHLE *et al.* 1988). Both the AP50 and AP47 (renamed μ_2 and μ_1, respectively) have recently been shown to specifically interact with the tyrosine-based signals of a number of integral membrane proteins, and it has been suggested that μ_1 and μ_2 are involved in membrane protein sorting (OHNO *et al.* 1995). The presence of the proline-rich extended amino-terminal region in STNB, and the reduced level of homology by comparison with the homology between yeast yAP57 and rat AP50, suggests that STNB is not the Drosophila cognate of either of the mammalian proteins. However, the homology of STNB protein to the AP50 protein family implies a role for STNB in neural membrane trafficking, perhaps as an alternative to the normal AP50 in a subset of AP complexes.

The complementation data (PETROVICH *et al.* 1993) indicates that the stn^{ts} mutation affects the same domain as stn^{PH1}, and stn^{C} the same domain as *stn13-120.* In molecular terms this means that stn^{C} is defective in STNA, while the stn^{ts} defect resides within STNB. Only the stn^{ts} allele interacts genetically with *shibire* and *dunce* mutant alleles, and hence this interaction must be via the STNB protein. As the *shibire* gene product, dynamin, has been shown to be intimately involved in the fission of nascent clathrin-coated endocytotic vesicles (HERSKOVITS *et al.* 1993; TAKEI *et al.* 1995), and the STNB protein contains an AP50 homology domain, the interaction seen between *shibire* and *stoned* mutations may reflect a common functionality. Both proteins may be involved in synaptic vesicle production/recycling or other cellular membrane trafficking. In this regard it is notable that reduction in the levels of the synaptic vesicle protein, synaptotagmin, in Drosophila mutants (LITTLETON *et al.* 1993) leads to an ERG phenotype identical to that of stn^{C} (DIANTONIO and SCHWARZ 1994), and synaptotagmin has been shown to act as a high affinity receptor for AP2 complexes (ZHANG *et al.* 1994). In fact, DIANTONIO and SCHWARZ (1994) suggest that such ERG defects could be used to identify mutations in genes that are involved in synaptic function. Perhaps we have done just that.

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