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Identification of the molecular lesions in four EMS-induced alleles of the *daughterless* gene of *Drosophila melanogaster*.

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The *Drosophila daughterless* (*da*) gene encodes a basic helix-loop-helix (bHLH) transcription factor, and although this protein regulates many aspects of development, the molecular mechanisms of its multifunctionality have not been fully elucidated. Using polymerase chain reaction (PCR) and DNA sequencing, we analyzed four ethyl methane sulfate (EMS)-induced *da* alleles (da^{s22} , da^5 , da^2 , and da^{f75}) to determine their molecular lesions and possibly identify functionally significant regions of the *da* protein

We chose these alleles because they have been extensively characterized genetically, or their genetic behavior suggested that their mutations appear to affect some of the *daughterless* functions more severely than others. da^2 and da^5 have been used in numerous developmental studies (e.g., Cronmiller and Cline, 1987; Caudy *et al.*, 1988; Cummings and Cronmiller, 1994; Brown *et al.*, 1996). Not only do these alleles behave like genetic nulls, but both also appear to be protein nulls, as determined by immunohistochemical staining (Cronmiller and Cummings, 1993). da^{s22} is a strong hypomorph, whose effects on oogenesis are somewhat more severe than those on sex determination or viability (Cummings and Cronmiller, 1994). Of all of the alleles analyzed, da^{f75} is the least well characterized genetically, but it was included in this analysis because it had been found previously to exhibit reduced viability with da^{lyh} (K. Curran and C. Cronmiller, unpublished observations), which otherwise appears to be an ovary-specific allele with no adverse effects on viability (Smith and Cronmiller, 2001).

Because each of the four *da* alleles chosen for sequence analysis is recessive lethal, we took advantage of their viability in combination with the da^{lyh} insertion allele to recover sequencing template DNA by PCR. DNA was prepared from each transheterozygous combination (e.g., da^2/da^{lyh}), and primers that flanked the springer insertion site of da^{lyh} (in the intron, upstream of the protein-coding exon: Smith and Cronmiller, 2001) were used to amplify *da* gene DNA. DNAs amplified from the two alleles would be distinguishable following electrophoretic separation of the PCR products, since the 8 kb transposon inserted in da^{lyh} would substantially increase the size of the DNA fragment amplified from that allele. In each case, however, PCR product was recovered only from the allele to be sequenced, indicating that the larger (>11 kb) product was not amplifiable by the PCR conditions used. As a precaution against contamination, each experimental band was excised, purified and re-amplified; either the original primers or nested primers were used for re-amplification.

Automated DNA sequencing was carried out at the University of Virginia Biomolecular Research Facility (Charlottesville, VA). Sequencing primers (Operon Technologies, Sigma) were spaced 500-700 nucleotides apart and spanned the entire *da* gene, from the 3' end of *mRpS7* (located upstream of the 5' end of *da*) to the 3' end of the *da* protein coding region. DNA sequence analysis was

accomplished with the MacVector, AssemblyLIGN (Sequence Analysis Software), and BLAST (NCBI) programs. The sequence of this entire region was determined for da^{s22} , while only the protein coding regions for da^5 , da^2 and da^{f75} were sequenced. Single point mutations were identified for all four da alleles analyzed (Figure 1).

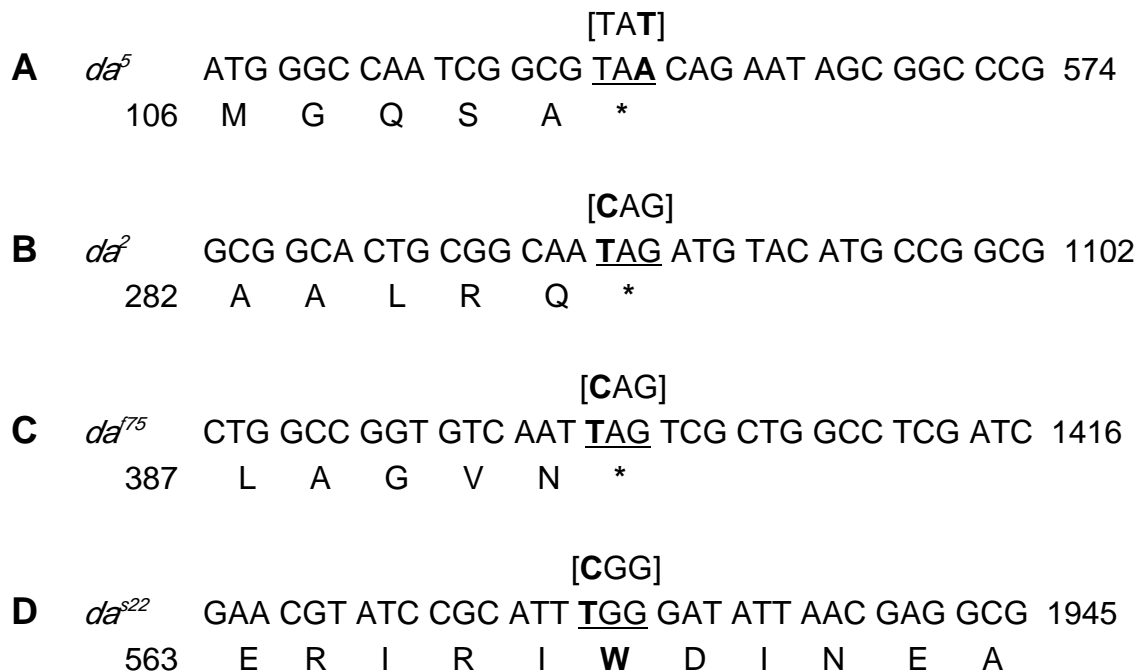


Figure 1. Mutational lesions in EMS-induced alleles of da . DNA sequences of (A) da^5 , (B) da^2 , (C) da^{f75} and (D) da^{s22} are shown with each single base change indicated in bold. The affected codons are underlined with their corresponding wild-type amino acids bracketed above. All sequence alterations were confirmed by numerous independent sequence analyses. Position numbers on the right refer to the nucleotide sequence of the 3.0-kb da cDNA, MN6 (Cronmiller *et al.*, 1988); numbers on the left refer to the amino acids of Da.

Nonsense mutations were found in da^{f75} and the null alleles, da^2 and da^5 . The most severe truncation of the da protein product would occur in da^5 , which had a T to A transversion at position 559 (at the beginning of exon 2). This converted a Tyr codon (TAT) to a stop (TAA), truncating the open reading frame to encode only the N-terminal 110 amino acids (~16%) of the normal protein. The nonsense mutation of da^2 resulted from a C to T transition at position 1085, which changed a Gln codon (CAG) to a stop (TAG) and reduced the protein coding potential of the mutant allele to 286 amino acids (~41% of the normal length). Finally, da^{f75} was found to result from a C to T transition at position 1399, replacing another Gln codon with a stop codon. This stop codon would truncate the protein to 391 amino acids, approximately 55% of normal Da. On western blots of extracts from heterozygous flies, we were able to detect only one of the predicted mutant proteins, that from da^2 ; however, the amount of truncated protein was drastically reduced, relative to that of the wild-type protein. Thus, if translated, the truncated proteins appear to be unstable. Nevertheless, since the essential bHLH domain has been deleted in all three nonsense mutations, any small/trace amounts of mutant protein that might be stable

would be nonfunctional.

A missense mutation was found in the strong hypomorph, *da*^{s22}. In this mutant a C to T transition at position 1928 changed an Arg codon (CGG) in the HLH domain into a Trp codon (TGG). The substituted amino acid is part of a stripe of basic residues that are located on the outer surface of helix 1 of the bHLH domain. The amphipathic helices of bHLH transcription factors mediate dimerization, a requirement for DNA binding (Murre *et al.*, 1989; Voronova and Baltimore, 1990), and stripes of acidic and basic residues of helix 1 may be important for heterodimer formation (Ellenberger *et al.*, 1994; Shirakata *et al.*, 1993). Although the Arg568>Trp substitution of *da*^{s22} probably does not change the protein's structure (because this residue sits on the surface of helix 1), the change in the helix 1 surface charge that resulted from replacing a positively charged amino acid with a nonpolar residue might disrupt normal protein interactions and thereby hamper dimerization with normal binding partners. Alternatively, since Arg568 is located only two residues away from the highly conserved Arg566 in the basic domain, which mediates DNA binding, perhaps the charge alteration that results from the *da*^{s22} missense mutation reduces the efficiency of target site recognition and/or binding.

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