Single Amino Acid Exchanges in Separate Domains of the Drosophila serendipity \( \delta \) Zinc Finger Protein Cause Embryonic and Sex Biased Lethality

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ABSTRACT

The Drosophila serendipity (sry) delta (\( \delta \)) zinc finger protein is a sequence-specific DNA binding protein, maternally inherited by the embryo and present in nuclei of transcriptionally active cells throughout fly development. We report here the isolation and characterization of four ethyl methanesulfate-induced zygotic lethal mutations of different strengths in the sry \( \delta \) gene. For the stronger allele, all of the lethality occurs during late embryogenesis or the first larval instar. In the cases of the three weaker alleles, most of the lethality occurs during pupation; moreover, those adult escapers that emerge are sterile males lacking partially or completely in spermatozoa bundles. Genetic analysis of sry \( \delta \) thus indicates that it is an essential gene, whose continued expression throughout the life cycle, notably during embryogenesis and pupal stage, is required for viability. Phenotypic analysis of sry \( \delta \) hemizygote escaper males further suggests that sry \( \delta \) may be involved in regulation of two different sets of genes: genes required for viability and genes involved in gonadal development. All four sry \( \delta \) alleles are fully rescued by a wild-type copy of sry \( \delta \), but not by an additional copy of the sry \( \beta \) gene, reinforcing the view that, although structurally related, these two genes exert distinct functions. Molecular characterization of the four sry \( \delta \) mutations revealed that these mutations correspond to single amino acid replacements in the sry \( \delta \) protein. Three of these replacements map to the same (third out of seven) zinc finger in the carboxy-terminal DNA binding domain; interestingly, none affects the zinc finger consensus residues. The fourth mutation is located in the NH\(_2\)-proximal part of the protein, in a domain proposed to be involved in specific protein-protein interactions.

The Cys\(_2\)/His\(_2\) “zinc finger,” a DNA-binding motif, was initially identified by molecular analysis of TFIIIA, a Xenopus transcription factor specific for the 5S RNA genes (Miller, MacLachlan and Klug 1985; Brown, Sander and Argos 1985). This motif has now been described in a large number of eukaryotic transcriptional regulatory proteins (Berg 1990a). The 28–29-amino acid “finger” is postulated to exist in a folded conformation stabilized by the tetrahedral coordination of a zinc ion by two cysteine and two histidine residues located at invariant positions in each finger (Miller, MacLachlan and Klug 1985; Klug and Rhodes 1987). One proposed structure (Berg 1989), supported by two-dimensional NMR studies of a single “model” zinc finger consists of a two-stranded antiparallel \( \beta \) sheet containing the two cysteine residues and the histidine/cysteine (H/C) link, and an \( \alpha \)-helical domain containing the two histidine residues (Lee et al. 1989; Neuhaus et al. 1990). The \( \alpha \)-helical region is thought to bind to the major groove of the target DNA helix and to be responsible for the specificity of interaction of the finger with DNA [see Berg (1990b) for review]. The structure of the complex formed between a three finger fragment of the protein Zif268 and its consensus DNA-binding site recently provided an atomic level view of DNA recognition by Cys\(_2\)/His\(_2\) zinc fingers (Pavletich and Pabo 1991) and confirmed that the sequence-specific contacts have characteristic simplicity and modularity.

The identification of genes encoding Cys\(_2\)/His\(_2\) zinc finger proteins in yeast and Drosophila, organisms with powerful genetic tools, means that it is now possible to correlate the effect of alterations in individual residues of such proteins with cellular and developmental defects (Blumberg et al. 1987; Redemann, Gaul and Jäckle 1988; Gaul, Redemann and Jäckle 1989).

Within the Cys\(_2\)/His\(_2\) finger protein gene family, the Drosophila sry \( \beta \) and \( \delta \) genes present an interesting problem in the evolution and diversification of structure and function (Payre and Vincent 1988). The sry \( \beta \) and \( \delta \) genes map to a single chromosomal region and probably arose as a result of a duplication event. Comparison of their respective protein products shows that an extensively conserved region is the

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DNA-binding domain which includes six contiguous fingers (Vincent, Colot and Rosbash 1985; Payne et al. 1990). The sry β and δ proteins display partly overlapping, 13 bp long, in vitro DNA recognition sites and distinct patterns of in vivo binding sites on polytene chromosomes (Payne et al. 1990; Payne and Vincent 1991; Noselli, Payne and Vincent 1992). Both proteins are maternally inherited by the embryo but are also zygotically expressed at different levels throughout the rest of the fly’s life cycle (Payre, Yanischostas and Vincent 1989; Payne et al. 1990).

The presence of these proteins in embryonic nuclei before the onset of zygotic transcription and in nuclei of transcriptionally active cells throughout development suggested that sry β and δ are transcription factors involved in zygotic activation and maintenance of expression of general cellular functions (Payre, Yanischostas and Vincent 1989; Payne et al. 1990). We report here the characterization of four sry δ zygotic lethal alleles, showing that expression of the sry δ gene is essential for normal development. The rescue of viability and fertility of these mutants by sry δ DNA indicates that, despite their homology, the function of the sry δ and β genes has diverged to the extent that β cannot substitute for sry δ. Sequencing of four of the alleles shows that the mutations cause single amino acid changes in two separate domains of the sry δ protein, which both have previously been shown to contribute to the specificity of in vivo interactions of sry δ with chromatin (Noselli, Payne and Vincent 1992).

MATERIALS AND METHODS

Drosophila stocks: The y506 strain used in the transformation experiments was obtained from W. Bender, the balanced stock CyO;TM3, Sb, Ser, ry+/ap" from J. Deutsch, the MKRS, Sb/TM2, Ubx stock (Hilliker et al. 1980) from J. O'Tusa and the lethal alleles SF1 and SF2 and the Canton S parental strain (Warmke, Kreuz and Falkenthal 1989) from S. Falkenthal. Third chromosome balancers used were In(3R)Ubx'Mcp"8, which carries a duplication for the 99D region (Kongsuwan, Dellavalle and Meriam 1986); In(3L)P+;3LR]HR33+; 3R]H+ C+ (3)M6, Hw, Tb; In(3LR)TM3, ri Phyap Su(H)W2 Sb bx"u; and In(3LR)TM2, Ubx bx"u (Lindsley and Grell 1968); these chromosomes are referred to in the text as DpUbx'Mcp, TM6B, TM3 and TM2, respectively. The synthetic deficiency DfB81Dp67A is a combination of the chromosomes Df(3R)BS81 and Dp(3;1)67A; the proximal and distal breakpoints of the deficiency are in 99D1.2 and 99D9.1, respectively (Kongsuwan, Dellavalle and Meriam 1986). The p[rp49, ry+] transformant chromosomes carry the cp20.1- RP49 plasmid; this plasmid contains one copy of the ribosomal protein gene 49, and rescues the Minute phenotype caused by hemizygosity of the 99D region (Kongsuwan et al. 1985).

Isolation of recessive lethal mutations: Two mutagenesis schemes were used to identify lethal mutations in the chromosomal region around the sry gene. In both schemes, mutagenized chromosomes were tested for lethality over the synthetic deficiency DfB81Dp67A. In scheme I (Figure 1a), the Minute phenotype caused by the hemizygosity of the

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**FIGURE 1.**—Mutagenesis screens to induce and recover recessive lethal mutations in the 99D region. For screen 1, the A3 transformant, in which the p[rp49, ry+] insert maps to 84F (Kongsuwan et al. 1985), was crossed onto a ry, e chromosome; for screen 2, this same insert was crossed onto the DfB81Dp67A chromosome. * indicates the mutagenized chromosome. Each possible F2 genotype is given. If a lethal mutation was induced that was uncovered by the DfB81Dp67A deficiency, no F2 progeny of the F1 cross would be viable.

99D region was rescued by the p[rp49, ry+] A3 insert at 84F (Kongsuwan et al. 1985) on the mutagenized chromosome, while in scheme II (Figure 1b), the p[rp49, ry+] F1 transformant insert, mapping to 80A (Kongsuwan et al. 1985) was crossed onto the DfB81 chromosome. The scheme described in Figure 1a was carried out with both ethyl methanesulfate (EMS) and with X-ray mutagenesis.

For EMS mutagenesis, 3-5 day-old males were treated with 0.025 M EMS (Sigma) according to the procedure of Lewis and Bachner (1968). For X-ray mutagenesis, males aged 3-5 days were treated with 3000 rad. Following mutagenesis, males were mated en masse to virgin females in half-pint bottles and transferred every 2 days; after 6 days the adults were discarded. The F1 progeny from this cross were individually mated to the DfB81Dp67A flies according
to the schemes outlined in Figure 1. The resulting F$_2$ progeny were scored for the absence of the class carrying the mutagenized chromosome over the deficiency, as indicated in Figure 1. If this class was lethal, a stock was established from siblings bearing the mutagenized chromosome/balancer (TM3 or TM6B). Using scheme I, 4926 EMS-treated chromosomes were screened and 20 lethals were identified. Of these lethals, three were rescued when in trans to the DJB81Dp67A chromosome onto which the p[CHs, ry+] insert (see below) had been introduced by recombination and were therefore designated sry", sry" and sry". Using scheme II, 2560 EMS-treated chromosomes were screened and 17 lethals were identified. Of these lethals, one was rescued by p[CHs, ry+] and was therefore designated sry".

An X-ray mutagenesis was carried out using scheme I; 1465 chromosomes were screened and four lethal mutations obtained. None of the X-ray-induced mutations was rescued by p[CHs, ry+]. One of the X-ray-induced lethal mutations was found to uncover the 99D region, with breakpoints at 99D1.2 and 99E1.2; this mutation was designated Df(3R)X3F (referred to as DjX3F), and was used in P element rescue experiments described below. Note that the Df(3R)X3F chromosome provides a simple deficiency which uncovers the entire X chromosome and a hemizygous mutant copy on the third chromosome.

RESULTS

Isolation of sry δ mutants: The sry gene cluster maps to 99D4-δ (Roark et al. 1985). Because the rp49 gene, which encodes a ribosomal protein, maps very close to sry, flies carrying deficiencies of this region have a strong Minute phenotype and low fertility (Kongsuwun et al. 1985). In order to screen for lethal mutations over a deficiency uncovering the sry genes, it was therefore necessary to use a synthetic deficiency of the region and to provide a second copy of the rp49 gene. A deficiency of the region 99D3 to 99D9, E1 was provided by the synthetic deficiency Dj-B81Dp67A (described by Kongsuwun, DellaValle and Merriam 1986; see materials and methods). A Minute-rescuing copy of the rp49 gene was provided by using the P element construct p[rp49, ry+] (Kongsuwun et al. 1985), which was present either on the mutagenized chromosome or crossed onto the synthetic deficiency (see materials and methods). Two different protocols were used to screen for lethal mutations in the 99D region. In screen I, a (rp49, ry+), ry, e chromosome was mutagenized (with EMS in one experiment and X-rays in another experiment); the mutagenized chromosomes were then tested over the Df(3R)X3F synthetic deficiency chromosome (Figure 1a).

DNA sequencing: Double-stranded DNA preparations were used as templates for sequencing by the chain termination method of Sanger, Nicklen and Coulson 1977. Ten different 16-base oligonucleotides, scattered throughout the sry δ gene sequence, were used as sequencing primers. For each allele, the sequence of the entire mutant sry δ gene was determined on one isolated clone. The parental genes were cloned and the DNA sequence determined in the region where differences between mutant and wild-type DNA (Vincent, Colot and Rosbash 1985) were identified. In each case two or three independent clones of parental DNA were sequenced. Differences between the parental and the mutant genes were then verified by sequencing the appropriate regions from three to four additional independent clones of mutant DNA.
a p(rp49, ry+) transformant insert (Figure 1b). In this screen, 2560 chromosomes were tested and 17 lethal alleles obtained.

To determine which of the 41 lethal alleles obtained mapped to the sry region, we tested which alleles, in hemizygote combination, could be rescued by complementation with a chromosome carrying the construct p(CHs, ry+), which contains the sry α, β and δ genes and the closely linked Jan A and B genes (VINCENT, COLOT and ROSBASH 1985; YANICOSTAS, VINCENT and LEPESANT 1989; see Figure 2). Three lethal alleles (on the e-bearing chromosome) from the EMS-treated chromosomes of screen I and one lethal allele (on the ca-bearing chromosome) from the EMS-treated chromosomes of screen II were rescued. To more precisely define the transcription unit to which these lethal alleles could belong, they were tested for rescue by complementation with a chromosome carrying the p(CHs-2, ry+) insert which contains, in addition to an internally deleted and presumably nonfunctional sry α gene, a functional sry δ gene (VINCENT, COLOT and ROSBASH 1986; see Figure 2). All four alleles that were rescued by the p(CHs, ry+) insert were also rescued by the p(CHs-2, ry+) insert, suggesting that all were in the sry δ gene (further confirmation of this is presented in experiments described below). These four EMS-induced alleles were designated sry δ11, sry δ12, sry δ14, sry δ17, respectively (LINDSLEY and ZIMM 1990). From the X-ray mutagenized chromosomes in screen I (see MATERIALS AND METHODS) a simple deficiency, DfX3F, which deletes 99D1.2-99E1, was recovered; DfX3F uncovers all of the sry δ alleles. Crosses inter se revealed that the sry δ14 allele fails to complement the sry δ11, sry δ12 and sry δ13 alleles, consistent with the notion that all four alleles are in the same gene.

An independent screen of 3,000 EMS-mutagenized chromosomes produced 44 recessive lethal alleles defining 12 complementation groups in the 99D3-99E2,3 interval (WARMKE, KREUZ and FALKENTHAL 1989). Complementation tests between the sry δ14 allele and lethal alleles identified in the WARMKE et al. screen, generously carried out by SCOTT FALKENTHAL, revealed a failure to complement the mutants SF1 and SF2. These latter alleles are therefore additional sry δ alleles which we refer to as sry δ14 and δ17. Thus three screens, in which a total of 16,500 EMS-mutagenized chromosomes were examined, yielded 6 lethal alleles of sry δ.

Mutant phenotype: Because they displayed a range of phenotypic severities and complementation behaviors (see below), we concentrated on the analysis of the four alleles sry δ12, sry δ14, sry δ17 and sry δ19, which all displayed partial embryonic lethality when in trans to DfX3F. The level of adult lethality was assessed in crosses between the mutant and the DfX3F stocks balanced with TM3. For each allele the percentage of the viable hemizygotes (sry δ/DfX3F) was determined. The TM3/TM3 homozygotes are 100% embryonic lethal (data not shown). Thus, while close to 100% adult female lethality is observed for each of the four sry δ alleles, approximately 30% of δ17/DfX3F and 80% of δ19/DfX3F males survive to adulthood (Table 1).

To analyze more precisely the lethality period associated with the sry δ mutations we determined the
as embryos die as first instar larvae, while in the case to determine the relative numbers of sry chromosomes balanced over TM6B were counted of the sry pupae (Tb+) compared to the two classes of balanced shown). All izygotes, all the hatched larvae develop to give pupae (E).

Summed results of reciprocal crosses between sry mutants and DfX3F (deficiency) strains balanced with TM3. Among the offspring, 50% of the embryos are sry/TM3 or DfX3F/TM3, 25% sry/TM3 or sry and 25% are TM3/TM3. TM3/TM3 is 100% embryonic lethal; sry/TM3 and DfX3F/TM3 shows normal viability (data not shown). Among the offspring, phenotypically Stubble (noted Sb) and non-Stubble (non-Sb) flies (if 100% viable) were expected to segregate in a 2:1 ratio. The total number of adults scored (Sb and non-Sb) for each combination is indicated (Σ). Assuming a sex ratio of 1:1 as was the case for Stubble flies, the viability indices for each allele (denoted V, and Vm, respectively) were calculated from the following equations: Vm = non-Sb males 

\[
\text{V}_m = \frac{\text{total embryos}}{\text{non-Sb males}} \times 100, \quad \text{V}_m = \frac{\text{total embryos}}{\text{non-Sb females}} \times 100.
\]

TABLE 1
Percent viability of sry δ heterallelic combinations

<table>
<thead>
<tr>
<th>Strains</th>
<th>f</th>
<th>m</th>
<th>Sb</th>
<th>Sb/4</th>
<th>Vm %</th>
<th>Vm %</th>
</tr>
</thead>
<tbody>
<tr>
<td>DfX3F δ′</td>
<td>0</td>
<td>5</td>
<td>2720</td>
<td>3</td>
<td>2717.679</td>
<td>0</td>
</tr>
<tr>
<td>DfX3F δ′′</td>
<td>0</td>
<td>3</td>
<td>2477</td>
<td>3</td>
<td>2474.618</td>
<td>0</td>
</tr>
<tr>
<td>DfX3F δ′′′</td>
<td>2</td>
<td>158</td>
<td>2239</td>
<td>160</td>
<td>2079.520</td>
<td>0.4</td>
</tr>
<tr>
<td>DfX3F δ′′′′</td>
<td>13</td>
<td>693</td>
<td>4190</td>
<td>706</td>
<td>3484.871</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The lethality level associated to DfX3F/TM3 (viable), 25% are TM3/TM3 (embryonic lethal), 25% of adults scored (Sb and non-Sb) for each combination is indicated (Σ). Assuming a sex ratio of 1:1 as was the case for Stubble flies, the viability indices for each allele (denoted V, and Vm, respectively) were calculated from the following equations: Vm = non-Sb males 

\[
\text{V}_m = \frac{\text{total embryos}}{\text{non-Sb males}} \times 100, \quad \text{V}_m = \frac{\text{total embryos}}{\text{non-Sb females}} \times 100.
\]

TABLE 2
Percent viability of sry δ/DfX3F hemizygous embryos

<table>
<thead>
<tr>
<th>Strains</th>
<th>Hatched</th>
<th>Total</th>
<th>Hatched</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DfX3F δ′</td>
<td>681</td>
<td>1128</td>
<td>282</td>
<td>117</td>
</tr>
<tr>
<td>DfX3F δ′′</td>
<td>2306</td>
<td>3489</td>
<td>872</td>
<td>562</td>
</tr>
<tr>
<td>DfX3F δ′′′</td>
<td>1863</td>
<td>2813</td>
<td>703</td>
<td>457</td>
</tr>
<tr>
<td>DfX3F δ′′′′</td>
<td>1150</td>
<td>1732</td>
<td>433</td>
<td>284</td>
</tr>
</tbody>
</table>

heterozygous pupae (Tb). This affords a measure of the lethality of each sry δ allele prior and during the pupal stage (approximately 1000 pupae were counted for each allele, data not shown). About 0%, 26% and 65% of the sry δ, δSF1 and δSF2 hemizygous pupae, respectively, eclosed to give adults which are nearly exclusively males (data not shown).

In all the allelic combinations in which they were obtained, hemizygous "escapers" were significantly smaller and took (at 25 °C) about 25% longer than their wild-type siblings to progress from the embryonic to pupal stage. Some of the escaper adult males showed rough eyes, extra humeral bristles (a mild humeral phenotype); and some escaper males lacked thoracic macrochaetes, especially the presutural bristles (data not shown). While the penetrance observed for each of these subtle defects was incomplete, 100% of the male escapers were sterile in crosses to wild-type females.

Gonadal defects: Examination of adult dissected testes from δSF2/DfX3F showed that the gonads have a much reduced size and contain very few spermatozoa bundles, compared to wild type, while the associated tissues, i.e., the seminal vesicles and ejaculatory bulb, appear normal (Figure 4). Compared to δSF1/DfX3F males, δSF2/DfX3F escaper males show a more pronounced atrophy of the gonads; the associated tissues, however, are still normal if one takes into account the small size of these flies. Observations on squashes of dissected testes showed the complete absence of spermatozoa in the δSF2/DfX3F hemizygous males (Figure 4). The δSF1/DfX3F female escapers (two out of an expected 520) that emerged had seemingly normal ovaries (dissected postmortem), but these females were not tested for fertility because of their very short life span, a trait not observed for males of the same genotype. The few δSF2/DfX3F female escapers (13 out of an expected 871) had a normal life span and dis-
played normal appearing ovaries at the light microscopic level, but did not lay any eggs.

**Complementation results:** In complementation assays involving pairwise combinations of the four sry alleles, essentially no complementation between sry \( \delta^{14} \) and the other sry \( \delta \) alleles was found. These results were very similar to those obtained for hemizygotes suggesting that the sry \( \delta^{14} \) allele is amorphic or nearly so (Table 1). Low levels of complementation (escaper adults) were found in crosses between \( \delta^{12} \) and the \( \delta^{SF1} \) and \( \delta^{SF2} \) alleles (Table 1). As was seen for the same alleles in hemizygous combination with \( DfX3F \), the escaper double mutant flies \( \delta^{SF1}/\delta^{12} \) or \( \delta^{SF2}/\delta^{12} \) were largely males (Table 1). Complementation between sry \( \delta^{SF1} \) and \( \delta^{SF2} \) was relatively strong: more than 60% of double mutant males and females eclosed; this was the only case in which equal numbers of males and females escaper were obtained (Table 1). In all cases, however, escapers took longer to emerge than wild type flies and all, both females and males were sterile. Observation on squashes of dissected testes of transheterozygote escaper males showed a phenotype comparable to that of \( \delta^{SF1}/DfX3F \) hemizygotes (Figure 4 and data not shown). The complementation results, together with the different levels of embryonic lethality, allow the four sry \( \delta \) alleles to be arranged in allelic series: the sry \( \delta^{14} \) allele is the strongest, as it behaves like an amorph in complementation tests. The sry \( \delta^{12} \) allele is weaker than sry \( \delta^{14} \); the sry \( SF1 \) and \( SF2 \) alleles are weaker, with \( SF2 \) the weakest by a small margin, at least in terms of penetrance for lethality (see discussion).

**Rescue of the adult viability and fertility of mutant alleles by sry \( \delta \), but not sry \( \beta \) DNA:** Since there was a small possibility that the truncated sry \( \alpha \) gene in the \( p[CHs-2,\ \beta^{\gamma}] \) insert might be providing some sry \( \alpha \) function and thus rescuing some of the lethal alleles obtained in the screen, we carried out rescue experiments with a chromosome carrying an insert with only the sry \( \delta \) gene. That the four alleles described above are in the sry \( \delta \) gene was shown unambiguously by full rescue of sry \( \delta/DfX3F \) hemizygotes by the transformed insert \( p[sry \ \delta, \ \gamma^{\gamma}] \), which contains only the sry \( \delta \) gene (see Figure 2). This \( p[sry \ \delta, \ \gamma^{\gamma}] \) insert on the X chromosome was able to suppress the sry \( \delta \) mutant lethality and sterility: for each sry \( \delta \) allele, the number of non-Sb (sry \( \delta/DfX3F \)) females (in this cross, only the hemizygote females carry the transformed DNA insert) was that expected for 100% rescue by the transposed DNA (Table 3). Mating of the rescued females \( (p[sry \ \delta, \ \gamma^{\gamma}]/X; \ sry \ \delta/DfX3F) \) to wild-type males showed that these females were fully fertile. Similar results were obtained using a \( p[sry \ \delta, \ \gamma^{\gamma}] \) insert on the second chromosome and tested with the strongest sry \( \delta \) allele, \( \delta^{14} \) (data not shown).

Transgenic flies carrying an extra copy of the sry \( \beta \) gene (construct \( p[sry \ \beta, \ \gamma^{\gamma}] \)), see Figure 2) on the X chromosome were also constructed. This \( p[sry \ \beta, \ \gamma^{\gamma}] \) -bearing X chromosome was introduced by crosses into sry \( \delta/DfX3F \) flies. The non-Sb (sry \( \delta/DfX3F \)) females carried the \( p[sry \ \beta, \ \gamma^{\gamma}] \) insert while males did not. For all four sry \( \delta \) alleles, there were very few progeny of either type (Table 3). The small number
TABLE 3
Rescue of sry δ alleles in presence of transformed sry δ or sry β DNA

<table>
<thead>
<tr>
<th>Strains</th>
<th>Strains</th>
<th>f</th>
<th>m</th>
<th>Σ</th>
<th>Non-Sb</th>
<th>Sb</th>
<th>Sb/4</th>
<th>V_d %</th>
<th>V_m %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sry δ*</td>
<td>DFX3f</td>
<td>δ*</td>
<td>257</td>
<td>1</td>
<td>1288</td>
<td>258</td>
<td>1030</td>
<td>257</td>
<td>100</td>
</tr>
<tr>
<td>DFX3f</td>
<td>δ12</td>
<td>290</td>
<td>1</td>
<td>1353</td>
<td>291</td>
<td>1062</td>
<td>265</td>
<td>109</td>
<td>0.4</td>
</tr>
<tr>
<td>DFX3f</td>
<td>δ18</td>
<td>218</td>
<td>71</td>
<td>1125</td>
<td>289</td>
<td>856</td>
<td>299</td>
<td>164</td>
<td>34</td>
</tr>
<tr>
<td>DFX3f</td>
<td>δ22</td>
<td>600</td>
<td>160</td>
<td>3332</td>
<td>1060</td>
<td>2272</td>
<td>588</td>
<td>106</td>
<td>81</td>
</tr>
</tbody>
</table>

To introduce a p transposon p[sry, ry+] containing either sry δ or sry β DNA into a sry δ hemizygous background, we used the following cross:

$$X_{TM3, Sb} \times p[sry, ry+] \times TM3, Sb \times p[sry, ry+]$$

In this cross, males do not receive the p[sry, ry+]. Therefore the non-Sb males (designated as m non-Sb) are escapers, (see text and Table 1). All females receive one copy of either the sry δ or sry β transposon. Non-Sb females (noted as f non-Sb) (sry δ/DFX3F) that emerge are rescued adults and escapers (see Table 1). Viability indexes (noted V_d and V_m for males and females, respectively) were calculated, using the same equation given in Table 1.

*The transposon used was p[sry δ, ry+].

**The transposon is p[sry β, ry+]; both transposons are described in Figure 2.

of non-Sb flies observed for the SF1 and SF2 alleles was that expected from the occurrence of "escaper" males among these hemizygotes (see Table 1). Consistent with this interpretation, the non-Sb flies obtained from this cross were sterile, in contrast to the fertility of the progeny recovered by the sry δ DNA. Failure of an extra copy of the sry β gene to rescue sry δ mutations was confirmed using the p[sry β, ry+] insert on the second chromosome and the δ* allele (data not shown). We conclude that sry β DNA does not seem to rescue adult viability and fertility of sry δ/DFX3F hemizygotes and therefore that an extra copy of sry β gene does not seem to compensate for the loss of sry δ function.

Cloning and sequence analysis of four sry δ alleles:
We took advantage of the fact that the p[sry δ, ry+] transposon and the genomic sry gene yield HindII1 and EcoRI restriction fragments of different sizes to isolate and clone the mutant sry δ DNA from p[sry δ, ry*/X; sry δ/DFX3F lines (see MATERIALS AND METHODS). The DNA fragments containing the sry δ*, sry δ12, sry δ18, and sry δ22 mutant alleles were cloned into the pTZ18R plasmid, and the entire sry δ transcribed region sequenced and compared to wild type [Figure 5; for the full nucleotide and protein sequence of the wild type sry δ gene, see VINCENT, COLOT and ROSBASH (1985) and PAYRE et al. (1990)]. The four sry δ alleles sequenced are point mutations due to G to A (C to T) transitions, consistent with the mode of action of the EMS mutagen (WILLIAMS and SHAW 1987). At the amino acid level, sry δ* results in a cysteine (residue position 7) to tyrosine replacement in the NH2-terminal part of the protein. The three other mutations are localized in the third zinc finger. Sry δ12 causes a methionine (residue position 271) to isoleucine replacement in the His-His a-helical domain of the finger. sry δ22 results in an arginine (residue position 263) to cysteine replacement and sry δ* causes a glutamic acid (residue position 251) to lysine replacement (Figure 5). DNA cloned from the sry δ* mutation also has an additional A to G transition which results in an isoleucine to valine replacement at residue position 144. This conservative replacement is located within a segment of the sry δ protein showing high primary sequence divergence during evolution as opposed to the third finger (P. FERRER, N. CROZATIER and A. VINCENT, manuscript in preparation). Differences between the parental and the mutant genes were verified by cloning the sry δ genes from the parental strains used to isolate the mutations, and sequencing the appropriate regions from two or three independent clones. None of the base exchanges identified in the mutants has been found in the corresponding parental gene indicating that the modification found in each clone is the relevant mutation.

DISCUSSION
Generation of lethal alleles in the sry δ gene:
By providing a transformed copy of the rp49 gene to rescue the 99D hemizygous Minute phenotype, we were able to screen over a deficiency for lethal alleles of the sry genes, and identified six alleles of the sry δ gene. From screens of 7500 chromosomes (Figure 1), in which four sry δ alleles were identified, no lethal alleles in the other genes carried by the p[CH1, ry+] insert (which contains sry β, sry α, Jan A and Jan B in addition to sry δ; see VINCENT, COLOT and ROSBASH 1986) were recovered. These other genes might be less mutable, have homologs elsewhere in the genome able to replace their function, and/or are not required for viability. An additional possibility is that the screen was not saturating (we did not test for frequency of lethal hits for other loci in the 99D region). Although the sry α gene has been shown to be required for normal cellularization at the blastoderm stage, it is not known whether lack of this gene results in lethality (SCHWEISGUTH, LEPESANT and VINCENT 1990). From the screen of 9000 mutagenized chromosomes obtained in S. FALKENTHAL'S laboratory, 26 lethal mutations were recovered and mapped into 12 complementation groups (WARMKE, KREUZ and FALKENTHAL 1989). One allele of each complementation group (kindly provided by A. KREUZ) was tested for rescue
by the p(sryβ, ry*) containing the sry β gene, but none seems to correspond to a sry β mutant.

An explanation for the failure to obtain sry β mutations specifically (as opposed to sry δ mutants) might be the difference in expression pattern between the two genes. Zygotic expression contributes only a minor fraction of the total sry β embryonic protein (PAYRE et al. 1990) and, further, sry β expression during later development is quite low (VINCENT, COLOT and ROSBASH 1985). Maternal expression of sry β might thus provide all of the sry β expression necessary for survival to adulthood, making it impossible to identify sry β alleles in a screen for zygotic lethals. Alternatively, it remains possible that sry δ may at least partly complement for sry β function, even though the reciprocal is not true.

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**Figure 5.** Sequence comparison of the sry δ wild-type (WT) and the four sry δ alleles. For orientation, the complete structure and the putative DNA-binding domain of the sry δ wild-type protein are schematically drawn on the top line: the position of each finger (numbered from 1 to 7) is represented by an open box, with the shaded area corresponding to the predicted His-His α-helical domain (see BERG 1988). Horizontal bars above correspond to regions whose nucleotide sequence was determined on both the parental and mutant chromosomes. The corresponding nucleotide and predicted protein sequences are given, with the third finger being bracketed. Mutations were found at nucleotide positions 20 (cysteine position 7 replaced by tyrosine) in sry δF'; position 751 (glutamic acid position 251 replaced by lysine) in sry δSF1; position 787 (arginine position 263 replaced by cysteine) in sry δSF2 and position 813 (methionine position 271 replaced by isoleucine) in sry δF2. In sry δF2 DNA, an additional nucleotide substitution is found at position 430 (isoleucine position 144 replaced by a valine). In both the diagram at the top and in the sequence, the sry δ mutations are indicated by upward pointing arrows. In the sequence, the cysteine and histidine residues of fingers are boxed, while cysteine residues in the Cys-X-Cys motifs present in the NH2-terminal domain are indicated by downward pointing arrows.
The sry δ allelic series: The four sry δ alleles characterized in greater detail displayed varying penetrance for lethality. Hemizygotes for the strongest allele, sry δ14, showed almost 100% lethality at either the embryonic or first instar larval stage. This phenotype shows that zygotic expression of sry δ is necessary for embryonic viability (Payre et al. 1990).

Large scale genetic screens have revealed that about 25% of all lethal mutations are embryonic lethal, while only about 3% affect severely the morphology of the mature embryonic cuticle (Wieschaus, Nüsslein-Volhard and Jürgens 1984; Nüsslein-Volhard, Wieschaus and Kluding 1984; Jürgens et al. 1984). The head involution defect of the sry δ hemizygotes places the sry δ gene in small subclass of all embryonic lethal producing "subtle" phenotypes, including head defects (Jürgens et al. 1984). Two mutants with more seriously defective cuticles were identified in the 99 region, *kayak* and *shroud* (Jürgens et al. 1984); complementation tests with DFX3F, however, indicate that neither gene could be sry (data not shown). Since the sry δ gene is expressed in virtually all transcriptionally active nuclei of the embryo (Payre et al. 1990), it is probable that the head involution defect of the sry δ mutant embryos is not due to a specific role of sry δ in head involution, but to the cumulative effect of inadequate levels of expression of diverse genes involved in many different processes. Because of the large maternal contribution of sry δ RNA and protein, it is plausible that a more severe embryonic phenotype of the sry δ alleles might be observed if the maternal component were removed; this possibility is currently under investigation.

For the three other alleles δ12, δ SF1 and δ SF2, we observed survival of a substantial portion of hemizygotes to the pupal stage. As there is a relatively high level of maternal sry δ RNA (Vincent, Colot and Rosbash 1985), it would not be surprising for embryos carrying weak alleles of sry δ to be able to survive embryogenesis and hatch. sry δ does not seem to be strictly required during larval stages, although larval development is considerably slowed, since all the δ12, δ SF1 and δ SF2 hemizygous hatched embryos develop to pupae (data not shown). sry δ function is required during pupation, however, since 0%, 26% and 65% of δ12, δ SF1 and δ SF2 hemizygous pupae, respectively, eclosed as adults (data not shown). Altogether, these data indicate that the continued expression of sry δ at distinct stages of the life cycle, notably during embryogenesis and pupal stage, is required for viability.

Pairwise combinations of the four sry δ alleles gave varying degrees of viability (Table 1). Only δ14 failed to complement the other sry δ mutants, suggesting that it is close to an amorph. The numbers of escapers obtained for the alleles in trans-heterozygous combination are greater to the numbers obtained for the alleles as hemizygotes; this partial complementation suggests that the alleles sry δ12, SF1 and SF2, are hypomorphs, and is consistent with each of the mutations mapping to the same functional domain of the protein (see below).

Specificity of sry δ rescue: That a single copy of the sry δ gene restores both full viability and fertility indicates that both lethality and sterility displayed by the sry δ12, δ14, δ SF1 and δ SF2 hemizygotes are due to loss of sry δ function. Even the weakest sry δ alleles, which can be fully rescued by sry δ DNA (Table 3), cannot be rescued by the sry β DNA with its complete upstream control region. This result shows that two doses of the sry β gene cannot compensate for (an even partial) loss of sry δ function, and therefore that the sry δ and β genes exert separate functions. Other evidence that supports a distinct function of sry δ and sry β is that the encoded proteins differ in both their expression during embryogenesis and in their DNA binding specificities in vitro and in vivo (Payre et al. 1990; Payre and Vincent 1991; Noselli, Payre and Vincent 1992). Our results do not distinguish whether this failure to rescue viability and fertility of the hemizygotes is due to a functional difference between the sry δ and β proteins or whether it is due to a difference in the pattern of expression driven by the two promoters.

Adult somatic and germ-line defects are associated with sry δ mutation: Subtle but reproducible somatic phenotypes are shown by the sry δ hemizygote adult escapers (small body size, slow growth rate, rough eyes and short bristles). These defects are reminiscent of the phenotypes of strong Minute mutations. About 80 loci corresponding to Minute mutations have been cytologically mapped on chromosomes (review by Ashburner 1989). From molecular cloning studies, it is clear that at least some Minute mutations code for ribosomal proteins. The cytological positions of three ribosomal proteins, at 5D3, 58F and 62E-63A, could possibly coincide both with Minute mutations and chromosomal binding sites of the sry δ protein (Ashburner 1989; Noselli, Payre and Vincent 1992). Whether the promoter region of any of these genes contains a sry δ binding site remains to be determined. Some specific defects of the sry δ mutant escapers, such as small rough eyes, are also reminiscent of the roughened eye phenotype that is associated with a dominant mutation of the *rap* 1 gene, a gene expressed throughout development and a putative antagonist of ras action (Harirhan, Carthew and Rubin 1991). A strong binding site of the sry δ protein at 67B7-12 coincides with the position of *rap* 1, raising the possibility of an interaction between sry δ and *rap* 1. Genetic experiments will be conducted to test for such an interaction.

In dissected testes of hemizygote "escaper" males,
which were all sterile, no (sry\(^{EF2}\)) or very few (sry\(^{SF2}\)) bundles of spermatozoa were seen (Figure 4). The few (sry\(^{EF2}\)) female escapers seemed to have anatomically normal ovaries but they did not lay any eggs. These fertility defects can be correlated with the expression of the sry \(\delta\) gene in premeiotic spermatocytes as well as in both follicle cells and nurse cells during oogenesis (PAYRE, YANNICOSTAS and VINCENT 1989). The reason for the presence of many fewer females than males among the escapers is not immediately obvious, but might be due to, an at least indirect, requirement for the sry \(\delta\) protein in expression of genes involved in sex determination, or \(X\) chromosome dosage compensation. Both processes are regulated through a key \(X\)-linked control gene, Sex-lethal (Sxl), whose activity must be on in females and off in males (for review see CLINE 1985). Results from genetic crosses, introducing the Sxl dominant alleles Sxl\(^{EF1}\) and Sxl\(^{ME4}\) (MAINE et al. 1985) into the sry \(\delta^{SF1}\) or sry \(\delta^{SF2}/DfX3F\) hemizygous genomes failed to reveal any interaction between sry \(\delta\) and Sxl (data not shown). The sex bias in sry \(\delta\) mutant lethality is therefore probably due to interactions between sry \(\delta\) and gene(s) downstream of Sxl in the sex differentiation regulatory pathway.

**Implications of amino acid replacements in sry \(\delta\) alleles:** The sry \(\delta^{12}\), \(\delta^{14}\), \(\delta^{SF1}\) and \(\delta^{SF2}\) mutations are all due to single amino acid replacements in the sry \(\delta\) protein product (Figure 5). The strongest allele sry \(\delta^{14}\) causes substitution of the cysteine at position 7 by a tyrosine; this substituted cysteine is part of a Cys-X\(_2\)-Cys doublet that is repeated twice in the NH\(_2\)-terminal domain of both the sry \(\delta\) and \(\beta\) proteins. Recent experiments making use of modified sry \(\delta\) and \(\beta\) genes fused to lacZ and expressed in Drosophila transgenic lines suggest that the NH\(_2\)-terminal domain of the sry \(\delta\) and \(\beta\) proteins may be involved in selective protein-protein contacts and may determine the specificity of in vivo interaction with chromatin (NOSELLI, PAYRE and VINCENT 1992).

The three other sequenced sry \(\delta\) mutations are located within the DNA-binding domain, in the same (the third) zinc finger, but none affects the zinc finger consensus residues. In Drosophila, Krüppel is the only gene where similar mutations have already been reported (Figure 6). Sry \(\delta^{12}\), a somewhat weaker allele than \(\delta^{14}\), causes a methionine to isoleucine replacement in the His-His \(\alpha\)-helical region; this region is thought to make sequence-specific contacts with DNA (BERG 1990b; PAVLETICH and PABO 1991). While methionine is found at this position in the second and third fingers of the sry \(\delta\) and \(\beta\) proteins (and the second and fourth fingers in the Krüppel protein) (Figure 6), isoleucine is found at this same position in numerous other fingers (GIBSON et al. 1988), including the first finger in sry \(\delta\) and \(\beta\) (Figure 6). Therefore, breaking of the \(\alpha\)-helix would not seem to be the reason for the lethal effect of this mutation. It seems more likely that the methionine replacement in the third finger modifies specific contacts between DNA and the protein.

sry \(\delta^{SF1}\) has two amino acid exchanges compared to the parental copy. The conservative replacement of an isoleucine (residue position 144) to valine is in a region displaying high primary sequence divergence during evolution (PAYRE et al. 1990; P. FERRER, M. CROZATIER and A. VINCENT, manuscript in preparation), suggests that this replacement might correspond to a silent mutation. The other modification found in sry \(\delta^{SF1}\) is a glutamic acid to lysine replacement in the "finger link" separating the second and the third sry \(\delta\) fingers. This exchange of negatively charged by a positively charged residue is likely to modify contacts between the sry \(\delta\) protein and the phosphate DNA backbone. The Kr\(^{I}\) mutation, a glycine to glutamic acid substitution, also maps in a "finger link" (GAUL, REDEMANN and JACKLE 1989; see Figure 6). The Kr TGEKP "finger links" (SCHUH et al. 1986) are all similar to each other, in contrast to those in sry \(\delta\) which differ from each other and from those found in the Kr protein (ROSENBERG et al. 1986; VINCENT, COLOT and ROSBASH 1985). The lethality of both the sry \(\delta^{SF1}\) and the Kr\(^{I}\) alleles demonstrates that specific sequences of the "finger links" are critically important for the function of Cys\(_2\)/His\(_2\) finger proteins.

sry \(\delta^{SF2}\) causes an arginine to cysteine replacement (position 263) (Figure 6). This replacement occurs at the residue position "X" in the three-dimensional structure of the Zf268 DNA complex proposed by PAVLETICH and PABO (1991), with X being one of the three residues involved in a direct base contact of each
finger with DNA. Each of the three Zif268 fingers also contains an arginine at the X position that contacts a guanine of the DNA. It is therefore plausible that the $\delta^{SF2}$ mutation results in loss of one base contact between the sry $\delta$ protein and its recognition site (5'-YTAGAGATGGRAA 3'; Payre and Vincent 1991), resulting in a weakened binding affinity. In vitro experiments to determine the consequences of the $\delta^{SF1}$, $\delta^{SF2}$ and $\delta^{SF4}$ mutations on the DNA recognition and binding properties of the sry $\delta$ protein are in progress.

That all three point mutations localized in the DNA-binding domain map to the third zinc finger indicates the crucial functional importance of this finger. Interestingly, it correlates with the fact that, out of the six contiguous sry $\delta$ fingers, only the third one could possibly make three direct contacts with bases (GAG) according to the pattern of recognition of DNA by Cys$_2$His$_2$ zinc fingers proposed by Klevecz (1991), based on amino acid positions X, Y and Z (Pavletich and Kaback 1991). Even though these different amino acid replacements are in the same finger, they do not appear to be equivalent. As already pointed out, $\delta^{SF1}$ results in greater lethality than does $\delta^{SF2}$, while $\delta^{SF2}$ results in a phenotype characterized by greater gonadal atrophy. Taken together with the strong complementation observed for $\delta^{SF1}/\delta^{SF2}$ trans-heterozygotes, these results suggest that the $\delta^{SF1}$ and $\delta^{SF2}$ mutations differentially affect the relative expression of two different sets of genes: genes coding for general cellular functions, i.e., required for viability, whose expression is more disturbed in $\delta^{SF2}$, and genes involved in gonadal development, whose expression is more disturbed in $\delta^{SF2}$ compared to $\delta^{SF1}$. Characterization of sry $\delta$ target genes, currently in progress, should aid a detailed understanding of the role of sry $\delta$ in development.

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