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## To cite this version:

Stéphane Noselli, Francois Payre, Alain Vincent. Zinc Fingers and Other Domains Cooperate in Binding of Drosophila sry 1 and 8 Proteins at Specific Chromosomal Sites. Molecular and Cellular Biology, 1992, 12 (2), pp.724-733. 10.1128/mcb.12.2.724-733.1992 . hal-03064569

**HAL Id: hal-03064569**

**https://hal.science/hal-03064569v1**

Submitted on 28 Oct 2022

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MOLECULAR AND CELLULAR BIOLOGY, Feb. 1992, p. 724–733
0270-7306/92/020724-10$02.00/0
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Vol. 12, No. 2

# Zinc Fingers and Other Domains Cooperate in Binding of *Drosophila sry* β and δ Proteins at Specific Chromosomal Sites

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Received 9 July 1991/Accepted 13 November 1991

**The closely related *Drosophila* serendipity (*sry*) β and δ zinc finger proteins display consensus in vitro DNA recognition sequences differing by 4 of 13 nucleotide positions and bind in vivo to distinct sets of sites on polytene chromosomes. We compared the pattern of in vivo chromosomal binding of deleted forms of the *sry* δ protein fused to β-galactosidase and expressed in *Drosophila* transgenic lines. Results show that the carboxy-terminal DNA-binding finger domain is required and sufficient for binding at specific chromosomal sites but that this binding does not nearly reproduce the wild-type pattern. An $NH_2$-terminal domain of the *sry* δ protein is essential to its specificity of in vivo interaction with chromatin. In vitro and in vivo experiments using reciprocal finger swap between the *sry* β and δ proteins suggest that the in vivo specificity is dependent on selective protein-protein contacts at defined chromosomal sites, in addition to DNA specific recognition.**

A major focus of eucaryotic molecular biology is the identification of the *cis*-acting DNA sequences and *trans*-acting proteins involved in regulation of gene expression. One principal challenge is to understand how specific protein-protein interactions modulate transcriptional activation at specific DNA sites and how these interactions are integrated into the overall network of gene regulation during development. The $Cys_2/His_2$ zinc finger (15, 22) is one of the major structural motifs involved in protein-nucleic acid interactions. Specific to this motif is its modular nature, since it occurs a variable number of times per finger protein (2, 8, 29). Identified or putative transcription activation domains associated with zinc fingers (and other DNA-binding motifs as well) include regions enriched in specific amino acids, such as proline, glutamine, and serine, or acidic residues (see references 10 and 23 for reviews). More specific modules defining subfamilies of $Cys_2/His_2$ finger proteins have been described in *Xenopus* and human cells, but the function of these conserved modules remains to be elucidated (1, 16, 25).

The *Drosophila* serendipity (*sry*) β and δ gene products provide an interesting model for studies of functional aspects of the modular organization of $Cys_2/His_2$ finger proteins. The *sry* β and δ genes map to a single chromosomal locus and probably result from the duplication of a common ancestral gene (38). The *sry* β and δ proteins show interspersed blocks of conserved and divergent amino acid sequences, the most conserved region being six contiguous zinc fingers (28). Their maternal inheritance by embryonic nuclei and presence in nuclei of transcriptionally active cells throughout development suggest that they are transcription factors involved in zygotic activation and maintenance of expression of genes coding for general cellular functions (28, 31). *sry* δ embryonic lethal mutants (21) can be rescued by transformation with a DNA fragment containing the wild-type *sry* δ gene but not the *sry* β gene, demonstrating that the two genes are functionally distinct (7).

We have recently shown that the respective consensus DNA recognition sequences of the *sry* β and δ proteins are closely related, differing at 4 of 13 nucleotide positions (30). At the chromosomal level, the two proteins display different, although possibly overlapping, patterns of binding sites. A detailed analysis of several *sry* δ mutant lethal alleles (7) has found that at least two distinct domains of the protein are required for the function of *sry* δ: the $NH_2$-proximal part and the zinc finger domain. To assess in vivo the respective contributions of these two domains of the *sry* β and δ proteins to binding at specific sites on chromosomes, we used an experimental approach in which *sry* β or δ/β-galactosidase fusion proteins were expressed in transgenic *Drosophila* lines. Fusing the *Escherichia coli* β-galactosidase to the almost full-length *sry* β and δ proteins does not change the specificity of this chromosomal binding (28; this report). We show here that while the zinc finger domain carries the in vitro DNA recognition specificity and is required and sufficient for binding to chromosomes, an $NH_2$-terminal domain of the *sry* β and δ proteins is essential to the specificity of this in vivo binding. Finger swap experiments suggest that the observed specificity is dependent on specific protein-protein interactions at defined chromosomal sites.

## MATERIALS AND METHODS

**Plasmid constructions.** Unless otherwise noted, nucleic acids were handled according to standard protocols (36). Plasmids pSDL1, pSBL1, pSDA1, and pSBA1 have been described elsewhere (28, 31). Nucleotide and amino acid numbers refer to the sequences published previously (28, 38).

**pSDAL construct.** A 1,510-bp *Bam*HI (3210)–*Apa*LI (4829) genomic fragment containing the *sry* δ promoter and coding region for amino acids 1 to 195 was made blunt by filling in with Klenow enzyme and ligated into the *Sma*I site of pTZ18R (Pharmacia) to create plasmid pBApa2. The same fragment was recovered from pBApA2 as an *Eco*RI-*Sal*I fragment and cloned into the P-element β-galactosidase vector pSDL1, cut with *Xba*I and *Sal*I (31). This results in an in-frame fusion of the N-terminal part of *sry* δ to β-galactosidase (plasmid pSDAL). All other *sry*/β-galactosidase fusion constructs described below in the pDm23 rosy-containing

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P-element vector were made by using pSDL1 cut with *Xba*I and *Sal*I.

**pSDFL construct.** An *Sph*I site was created in *sry* δ by site-directed mutagenesis at nucleotide position 4783, substituting Met-Pro with Lys-Ser (residues 176 and 177, 15 residues $NH_2$ to the first finger). A 620-bp *sry* δ fragment, bordered by the created *Sph*I site and a unique *Sal*I site (5401), was ligated into pTZ19R, recovered as a *Hin*dIII (blunted)-*Sal*I fragment, and inserted into pTZ18R *Bam*HI-(blunted)-*Sal*I, creating plasmid pDFN. A genomic fragment from *Eco*RI (1040) to *Sau*3AI (4170), including the coding region for the 10 *sry* δ $NH_2$-terminal residues, was inserted in two steps into pDFN cut with *Eco*RI and *Sma*I, creating plasmid pRDFN. The *Xba*I (2486)-*Sal*I (5401) fragment of pRDFN, which contains the *sry* δ promoter region and coding region for *sry* δ residues 1 to 10 and 178 to 384 (i.e., the six contiguous fingers), was cloned into pSDL1 to create pSDFL.

**pSDF4-6L construct.** The *Bam*HI, *Xba*I, and *Sal*I restriction sites of the residual polylinker sequence in plasmid pBApa2 were eliminated by filling in *Bam*HI and *Sal*I sites with Klenow enzyme before religation to create plasmid pBApA3. The *sry Eco*RI (1040)-*Nco*I (4008) fragment was substituted for the *Eco*RI-*Nco*I fragment of pBApA3 to create pRApa. The *sry* δ *Sph*I (5059)-*Hin*dIII (6070) fragment was cloned into pRApa cut with *Sph*I and *Hin*dIII to create plasmid pRDF4-6. The *Xba*I-*Sal*I fragment of pRDF4-6 that includes the coding region for *sry* δ amino acids 1 to 195 and 261 to 384 (i.e., carrying a deletion of fingers 1, 2, and 3) was cloned into pSDL1 to create pSDF4-6L.

**pSDBL and pSBDL constructs.** A 547-bp *Sph*I (1105)-*Sal*I (1652) fragment containing the six *sry* β fingers was substituted with the homologous *sry* δ region into pRApa to create plasmid pRDB. The *Xba*I-*Sal*I fragment of pRDB was inserted into pSDL1 to create pSDBL. Conversely, the *sry* δ *Sph*I (4783)-*Sal*I (5401) fragment was inserted into plasmid pSBL1 (28) to create plasmid pSBDL. This results in replacement of the six *sry* β fingers by their *sry* δ counterparts.

DNA of each P[$ry^+$] plasmid was used to transform $ry^{506}$ flies according to standard protocols (35) except that some injections were done into nondechorionated embryos (34). Transformant lines (as well as the corresponding fusion proteins) are referred to as SDAL, SDFL, SDF4-6L, SBDL, and SDBL. They were made homozygous, and chromosomes carrying the different P constructs were determined by chromosomal linkage, using balancer stocks. Two independent lines for each construct were used for analyses.

**pSDB and pSBD constructs for expression of finger swap proteins in *E. coli*.** A *Sac*I-*Sac*I fragment from plasmid pRDB containing the coding region for the six *sry* β fingers was substituted with the homologous *sry* δ region in plasmid pSDA1 (31) to create pSDB. The *Sph*I (4783)-*Sal*I (5411) fragment containing the six contiguous *sry* δ fingers was substituted with the corresponding domain of *sry* β in plasmid pSBA1 (28) to create plasmid pSBD. The proteins (not fused to β-galactosidase) encoded by plasmids pSBA1, pSDA1, pSDB, and pSBD, respectively, are schematically represented in Fig. 6A.

**Expression in *E. coli*, purification of the SBD and SDB proteins, and gel retardation assay.** Overexpression and purification of the chimeric SBD and SDB proteins was achieved by using plasmids pSBD and pSDB, respectively, as described previously (28). The gel retardation assay was done as described elsewhere (30).

**Histological and immunological detection of β-galactosidase fusion proteins.** Whole-mount 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining of third-instar larvae was performed as described previously (11). Extracts from female ovaries were prepared as described previously (26). Western immunoblotting and immunostaining of polytene chromosomes were done as described elsewhere (28). Views were taken at varied magnification, using bright-field Nikon optics. We used Agfa Ortho 25 film for black-and-white prints.

## RESULTS

**Cytogenetic mapping of *sry* δ binding sites on polytene chromosomes.** We previously reported that the *sry* β and δ zinc finger proteins bind to distinct sets of sites on salivary gland polytene chromosomes of third-instar larvae. Cytological location of 54 *sry* β binding sites was also reported. The *sry* δ protein binds to a much larger number of sites, of which only a few were previously mapped (28). We report here the cytological location of the whole set of *sry* δ chromosomal binding sites (Fig. 1). We have now mapped a total of 30 *sry* β- and 196 *sry* δ-specific sites as well as 23 sites which, at the cytological level, appear common for the two proteins (Fig. 1; see Discussion).

**Expression of *sry* β or δ/β-galactosidase fusion proteins in transgenic lines.** Carboxy-terminal fusion of the *E. coli* β-galactosidase to either the *sry* β (SBL) or *sry* δ (SDL) protein does not modify their respective patterns of chromosomal binding sites, except for a slightly decreased staining intensity of the fusion proteins at a small number of sites. Binding sites for the endogenous *sry* δ and fusion SDL proteins precisely colocalize even though the fusion protein does not contain the seventh carboxy-terminal *sry* δ finger (28; data not shown). To assay the relative contributions of different parts of the *sry* β and δ proteins to this specific binding, we constructed a series of deletions as shown in Fig. 2. These deletions were made by using restriction sites, either in the genomic DNA or created in vitro by mutagenesis, and were fused back together by using linkers that recreate the required reading frame and, in some cases, insert a few additional residues. Each of the constructs includes DNA upstream of the *sry* β or *sry* δ transcription start site sufficient to reproduce the developmental pattern of expression of the corresponding wild-type gene in transgenic lines (31, 39). Of particular interest here, the two promoters are active in salivary gland cells of third-instar larvae (26, 28). Most constructs made were deleted forms of the *sry* δ protein, in relation with genetic and molecular analysis of several *sry* δ mutant alleles revealing an embryonic lethality associated with missense mutations in separate domains of this protein (7; see the introduction). All of the constructs were fused to the β-galactosidase gene coding region subcloned into a P-element transformation vector (see Materials and Methods). Synthesis of the different fusion proteins was verified by Western blot analysis of protein extracts made from dissected ovaries, probed with either a monoclonal anti-β-galactosidase antibody (Fig. 3) or an anti-*sry* β or anti-*sry* δ antibody (not shown). For a given construct, each line expresses a β-galactosidase fusion protein migrating in the gel at a position correlating with its calculated molecular weight.

Expression in salivary glands of third-instar larvae, as monitored by whole-mount X-Gal staining, gave similar results except that the SDFL protein accumulates at lower levels (data not shown). The X-Gal staining assay further allowed us to determine the subcellular localization of each chimeric protein. All of the fusion proteins constructed are

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| X | 2L | 2R | 3L | 3R | 4 |
|---|---|---|---|---|---|
| 1A | 21E3-F3 | 43B1,2 | 61A3,4 | 82AB | 102B |
| 1B11,14 | 22A6,8 | 44AB | 61B | *82F | 102D |
| 1E | 23B1,3 | 44F3,5 | 61F | 83C | 102E |
| *2B13,18 | 23E | 45A7,8 | 62A4,7 | 83E | |
| 2C5,8 | *23F1,4 | 46A1,2 | 62B7,11 | 84B3,6 | |
| 2E | 24D | *46F | 62D4,6 | 84C1,2 | |
| 3A1,4 | 25A6,8 | 47C | 62E6,9 | 84E | |
| 3B1,2 | 25C1,2 | 48D | 63B | 84F13,16 | |
| 4C | 25D3,7 | 49D | 63C1,4 | 85A7,11 | |
| *4F9,10 | 25F3,5 | 50A9,10 | 63D3 | *85C | |
| 5A8,14 | 26A5,9 | 50B1,2 | 63E7,9 | 85D | |
| 5D3,4 | 26B5,8 | 50E3,5 | 64A | 85E | |
| 5E1,2 | 26D8,11 | 51B | 64B | *85F14,16 | |
| 5F1,2 | 26F7-27A | 51C | *65A | 86C5,10 | |
| *6C | 27C | 51D | 65B4,5 | *86E | |
| 6D1,6 | 27D1,2 | 51E | 65C3,5 | 87A6,10 | |
| 6E4-F1,2 | 28F | 51F4,7 | 65E5 | 87D5,7 | |
| 7C1,2 | 29A | 52E | 65F | 88D | |
| 7C4,5 | 29D1,2 | *52F | *66A3,4 | 88E | |
| 7F-8A | 30E1,2 | 53E1,5 | 66A10,13 | 89AB | |
| 8B | *30F6 | 53F | 66B1,6 | 89D | |
| 8C | *31B | 54C1,2 | *66C | 89F | |
| 9F12,13 | 31C | 54DE | 66D | 90A4,7 | |
| 10A3,4 | 31F | 55C5,13 | 66E5 | 90F | |
| 10B2,3 | 32A3,5 | 55F-56A | 66F1,2 | 91D1,3 | |
| 10F7,11 | 33E1,2 | 56B3-C7 | 67A9-B3 | 92C3,6 | |
| 11A7,12 | 34A3,4 | 56D3,8 | *67B6,10 | 92E | |
| *11B7,11 | 34B10,12 | 56E | 67C3,5 | 93B4,8 | |
| 11D4-E13 | 34D6,8 | 56F | 67D3,7 | 94C | |
| 12B1,2 | 35B | 57A | 68C15-D2 | 95C | |
| 13A | 35C4,5 | 57B | 68D4,5 | *95DE | |
| 13D3,4 | 36B | 57C | 68F5,8 | 96A | |
| *13E8,9 | 36CD | 58F | 69A5-B2 | *96B | |
| 13E14,15 | 37C | 59B | 69C1,2 | *96C | |
| 13F1,2 | 37EF | 59C | 69E1,2 | 97A | |
| 13F14,18 | | *59F-60A | 69F4,7 | 98E | |
| 14B7,10 | | 60B | 70B | 98F | |
| 14B13,16 | | | 70C | 99B | |
| 14C4,8 | | | 70F4,7 | 99C | |
| 14F1,2 | | | 71A3,4 | *99D | |
| 15A | | | 72D4,5 | 99F-100A | |
| 16D3,4 | | | 72F | 100B6,9 | |
| 17E | | | 73A5,11 | | |
| 18C1,4 | | | 73D1,4 | | |
| 18D | | | 73E | | |
| | | | 73F | | |
| | | | 74C | | |
| | | | 74F | | |
| | | | 75D6,8 | | |
| | | | 76A5,7 | | |
| | | | 77B | | |
| | | | 77C | | |
| | | | 77E6-F5 | | |
| | | | 78A3,4 | | |
| | | | 78B1,2 | | |
| | | | 78D1,2 | | |
| | | | 78EF | | |

FIG. 1. Locations of *sry* δ binding sites on salivary gland polytene chromosomes of Oregon R larvae. The cytological positions of the binding sites were determined by comparison with the map of Bridges (cited in reference 20). *, *sry* δ sites that cytologically overlap with *sry* β sites (28).

nuclear, consistent with the fact that they were designed to contain a nuclear localization signal present in the *sry* δ protein between residues 180 and 197. This 18-amino-acid-long fragment was previously shown, when fused to a cytoplasmic β-galactosidase, to drive autonomously the fusion protein into nuclei of transgenic flies (SDCN construct [26]).

**The *sry* δ finger domain is required for specific binding of the protein on polytene chromosomes.** The SDAL fusion protein is a carboxy-terminal deletion that removes all six fingers present in the SDL protein. This protein does not bind to chromosomes, showing that the DNA binding domain is required for such chromosomal association. The SDF4-6L fusion is a shorter deletion of the DNA binding domain that removes only the three N-terminal fingers, with fingers 4, 5, and 6 being conserved in their entirety (Fig. 2). Similar to the SDAL protein, the SDF4-6L protein does not show any binding to polytene chromosomes. Therefore, the three carboxy-terminal *sry* δ fingers appear to be insufficient for binding of the SDF4-6L fusion protein to even a limited number of sites.

Conversely, we made a reciprocal deletion of the amino-terminal domain of the *sry* δ protein. The SDFL protein contains the 10 $NH_2$-terminal *sry* δ residues linked to the six contiguous fingers (Fig. 2). This protein binds to specific sites on chromosomes (Fig. 4 and 5A). However, the number

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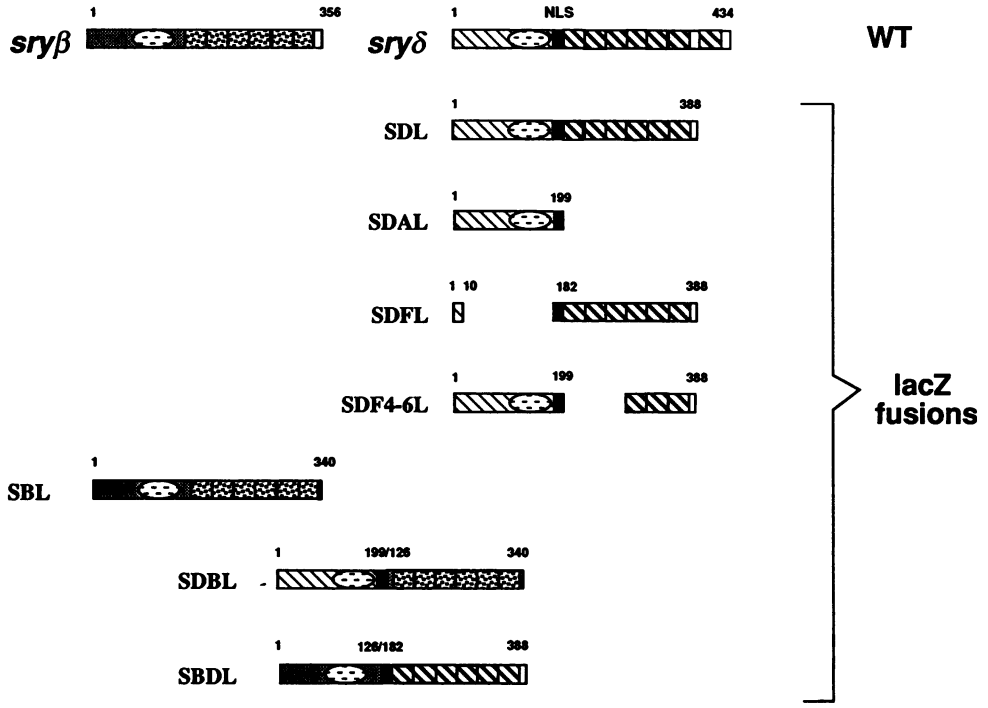


FIG. 2. Diagrammatic representation of the *sry-lacZ* fusion proteins. The different *sry-lacZ* protein fusions are schematically drawn below the wild-type (WT) *sry* β and *sry* δ proteins. Only the *sry* protein coding sequences are drawn, the β-galactosidase-fused carboxy end being omitted for clarity. Numbers above each protein domains refer to amino acid numbers of the wild-type proteins. Symbols: ▒, *sry* β $NH_2$-terminal part; ▨, *sry* β zinc fingers; ▧, *sry* δ $NH_2$-terminal part; ▧, *sry* δ zinc fingers; ■, *sry* δ nuclear localization signal; ⊖, acidic domain.

of detected sites, five (Fig. 4), is dramatically lower than the number of sites displayed by the native *sry* δ protein or the SDL fusion (Fig. 1). This finding shows that while zinc fingers are required and sufficient for association of the *sry* δ protein with chromatin, this association is severely reduced or unstable when the protein lacks sequences outside the finger domain. The five SDFL binding sites were mapped, and their cytological positions were found to correspond to binding sites of the endogenous *sry* δ protein (Fig. 4 and 5A). They therefore represent a specific subset of the native sites. Two correspond to the strong *sry* δ sites at 65A and 84C1,2 (Fig. 5A), while the three others correspond to weaker sites. Conversely, it is interesting to note that some of the strongest binding sites of the endogenous *sry* δ protein are not bound by the SDFL protein, reinforcing the importance of sequences amino to the finger domain in the specificity of interaction of the *sry* δ protein with chromatin at the majority of its chromosomal binding sites.

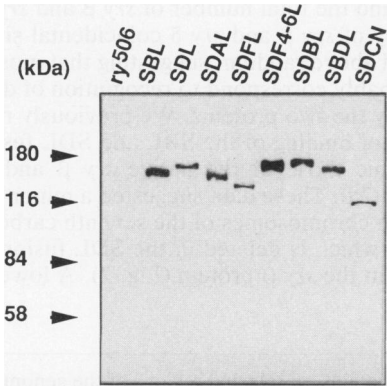


FIG. 3. Western blot analysis of protein extracts from control and *lacZ* fusion-expressing lines. In each lane, 100 μg of ovarian protein extract from control ($ry^{506}$ and SDCN [26]), SBL, SDL, SDAL, SDFL, SDF4-6L, SDBL, and SBDL lines was separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose filter, and probed with a monoclonal anti-β-galactosidase antibody (Promega). On the left is given the mobility of known $M_r$ protein standards.

**Chimeric SBDL (*sry* β-δ) and SDBL (*sry* δ-β) proteins display novel patterns of binding sites on polytene chromosomes.** Deletion of the N-terminal domain of the *sry* δ protein drastically reduces the number of its binding sites on chromosomes (see above). To gain further insight into how specific this effect is, we constructed tripartite fusion proteins by reciprocal exchange (swap) of the *sry* β and *sry* δ finger domains. The SDBL fusion is made of the N-terminal domain of *sry* δ linked to the finger region of *sry* β, fused to β-galactosidase. The reciprocal SBDL fusion protein comprises the N-terminal part of *sry* β fused to the fingers of *sry* δ, fused to β-galactosidase (Fig. 2). Both the SDBL and SBDL proteins bind to specific chromosomal sites (Fig. 5B and C), consistent with the fact that both include an unmodified block of six contiguous fingers. Cytological mapping of these sites further shows that they bind to nonoverlapping sets of chromosomal sites (Fig. 4). The SBDL protein binds to about 40 sites. These sites correspond to a mixed pattern; i.e., 4 sites map to *sry* β-specific sites, while 25 correspond to *sry* δ-specific sites (Fig. 4 and 5C). In contrast, the SDBL fusion protein, which contains the *sry* δ N-terminal domain linked to the *sry* β fingers, binds to only six sites, all of which

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A

| X | SDBL | SBDL | SDFL |
|---|---|---|---|
| 1B | | ◊ | |
| 2B13,18 | | * | |
| 3B3,4 | | ■ | |
| 4F9,10 | | * | |
| 5A8,14 | | ◊ | |
| 6EF | | ◊ | |
| 7F | ◊ | | |
| 8A | | ◊ | |
| 8B | | ◊ | |
| 11C1,2 | | ■ | |
| 11C3,4 | | ■ | |
| 13A | | ◊ | |
| 13E | | * | |

| 2 | SDBL | SBDL | SDFL |
|---|---|---|---|
| 26F-27A | | ◊ | |
| 30F | | * | |
| 34AB | | ◊ | |
| 37EF | | ◊ | |
| 47C | | ◊ | |
| 48D | | ◊ | |
| 49F | | ■ | |
| 51C | | ◊ | |
| 51E | | ◊ | |
| 51F4,7 | ◊ | | |
| 55F-56A | | ◊ | |
| 56B | ◊ | | |
| 56E | | ◊ | ◊ |
| 56F | | ◊ | |
| 57BC | | | ◊ |
| 59F-60A | | ◊ | |
| 60B | ◊ | | |

| 3 | SDBL | SBDL | SDFL |
|---|---|---|---|
| 61F4,8 | | ◊ | |
| 63B | | ◊ | |
| 63D3 | | * | |
| 63E7,9 | | ◊ | |
| 65A | | * | * |
| 69A | | ◊ | |
| 70C | | ◊ | |
| 70F | | | ◊ |
| 84C1,2 | | ◊ | ◊ |
| 85C | | * | |
| 85D | | * | |
| 86C | | ◊ | |
| 87A6,10 | ◊ | | |
| 91C | | ◊ | |
| 99C | | ◊ | |
| 99D | | * | |

B

| | SDBL | SBDL | SDFL |
|---|---|---|---|
| Total | 6 | 38 | 5 |
| Sites common with : | | | |
| SDBL | | 0 | 0 |
| SBDL | 0 | | 3 |
| SDFL | 0 | 3 | |
| sryβ | 0 | 4 | 0 |
| sryδ | 5 | 26 | 4 |
| sryβ/sryδ | 1 | 8 | 1 |

FIG. 4. Binding sites of the SDBL, SBDL, and SDFL fusion proteins compared with those of native proteins. (A) Cytogenetic locations of the binding sites of the SDBL, SBDL, and SDFL *sry*/β-galactosidase fusion proteins. Sites on the X, second, and third chromosomes, respectively, are given in separate columns. Symbols indicate cytological colocalization with a *sry* β-specific site (■), with a *sry* δ-specific site (◊), and with a coincidental *sry* β/*sry* δ site (*). (B) Summary of coincidental binding sites of the SDBL, SBDL, and SDFL fusion proteins.

map to *sry* δ sites. Only one of these sites, at 99D (Fig. 5B), cytologically colocalizes with a *sry* β site. Strikingly, no signal is found at cytological positions of strong *sry* β sites (compare Fig. 4 with Table 1 in reference 28).

**Specificity of *sry* β and *sry* δ binding to DNA in vitro is not influenced by reciprocal exchange of their $NH_2$-terminal domains.** The specific binding of the SDBL protein to *sry* δ sites (and SBDL protein to some *sry* β sites) visualized on polytene chromosomes raised the possibility that the N-terminal part of the *sry* β and *sry* δ proteins could influence their DNA recognition properties. To test this possibility, the *sry* δ-β (SDB) and *sry* β-δ (SBD) finger swap proteins (Fig. 6A) were produced in *E. coli*, purified, and assayed for their DNA binding specificity in vitro compared with that of the native *sry* β and δ proteins.

The DNA recognition sites of the *sry* β and *sry* δ proteins were recently determined by using a newly developed immunoselection procedure for the isolation of genomic targets of DNA-binding proteins (30). The *sry* β and δ consensus recognition sites, both 13 bp long, differ at only 4 of 13 positions. Synthetic oligonucleotides B22I and D524 containing the consensus *sry* β and *sry* δ recognition sites, respectively, and bound exclusively by one or the other protein (30) (Fig. 6B) were used for gel retardation experiments with each of the four *sry* β, *sry* δ, SDB, and SBD proteins purified from *E. coli* (Fig. 6A). The results show that the SDB and SBD proteins have retained the in vitro DNA binding specificity of *sry* β and δ, respectively (Fig. 6B). This specificity was shown in conditions of high concentrations of competitor DNA ($10^5$-fold molar excess). These data demonstrate that specific in vitro DNA recognition properties of the chimeric SBD or SDB proteins are carried out by the six finger domains, irrespective of the origin (*sry* β or *sry* δ) of the $NH_2$-terminal part of the protein and of the presence or absence of the carboxy-terminal seventh *sry* δ finger.

## DISCUSSION

We show here that specific DNA recognition is only one of several requirements for selective in vivo binding of two related zinc finger proteins, *sry* β and *sry* δ, at specific sites on chromosomes. Our experimental approach is based on the expression, in *Drosophila* transgenic lines, of modified forms of these two proteins fused to β-galactosidase, taking advantage of the fact that polytene chromosomes of third-instar larvae permit the visualization of protein-protein and DNA-protein interactions, in situ, in a normal chromatin context (13, 37, 42, 43). This provides an efficient assay for visualizing the entire set of in vivo chromosomal binding sites of a given DNA-binding protein.

**Comparison of the cytological positions of binding sites of the *sry* β and δ proteins.** By mapping the chromosomal *sry* δ binding sites, we have confirmed here that at least 60% of the *sry* β and 90% of the *sry* δ binding sites appear specific for one or the other protein. The resolution obtained in cytological mapping does not allow us to distinguish whether coincidental *sry* β/*sry* δ binding sites (23 sites) correspond to recognition of the same DNA sequence by the two proteins or binding to nearby separate sequences. With respect to the number of chromosomal bands (5,162) (20), the precision (5 bands on the average) of cytological mapping of the *sry* β and *sry* δ sites, and the total number of *sry* β and *sry* δ sites, the hit frequency of *sry* β and *sry* δ coincidental sites (1/2.5) is only twofold above random, suggesting that most coincidental sites probably correspond to recognition of distinct DNA sequences by the two proteins. We previously reported that the patterns of binding of the SBL and SDL fusion proteins (Fig. 2) mimic those of the native *sry* β and δ proteins, respectively (28). These data suggested a minor contribution to binding on chromosomes of the seventh carboxy-terminal *sry* δ finger, which is deleted in the SDL fusion and has no counterpart in the *sry* β protein (Fig. 2). A lowered staining

FIG. 5. Comparison of binding sites of the *sry* β, *sry* δ, SDBL, SBDL, and SDFL proteins on selected regions of the genome. On the left, chromosomes from Oregon R larvae were immunostained with either an anti-*sry* β or an anti-*sry* δ antibody, and SDBL, SBDL, SDFL, and control $ry^{506}$ lines were immunostained with an anti-β-galactosidase antibody (Promega). Protein binding sites were visualized by the peroxidase substrate DAB. On the right, the same chromosome squashes were counterstained with Giemsa for visualization of Bridges' bands. (A) Right arm of the third chromosome, from centromere (left) to section 84. The site at position 84C1,2 is specifically bound by the *sry* δ, SBDL, and SDFL proteins (black arrowhead) but not the *sry* β protein (open arrowhead). (B) Tip of the right arm of the third chromosome. The arrowhead points to the site at position 99D bound by the SDBL protein, which is a coincidental *sry* β/*sry* δ site. (C) Distal part of the X chromosome (sections 1 to 6). The pattern of binding of the SBDL protein includes both *sry* δ-specific sites (5A8,14), *sry* β-specific sites (3B3,4), and *sry* β/*sry* δ coincidental sites (4F9,10).

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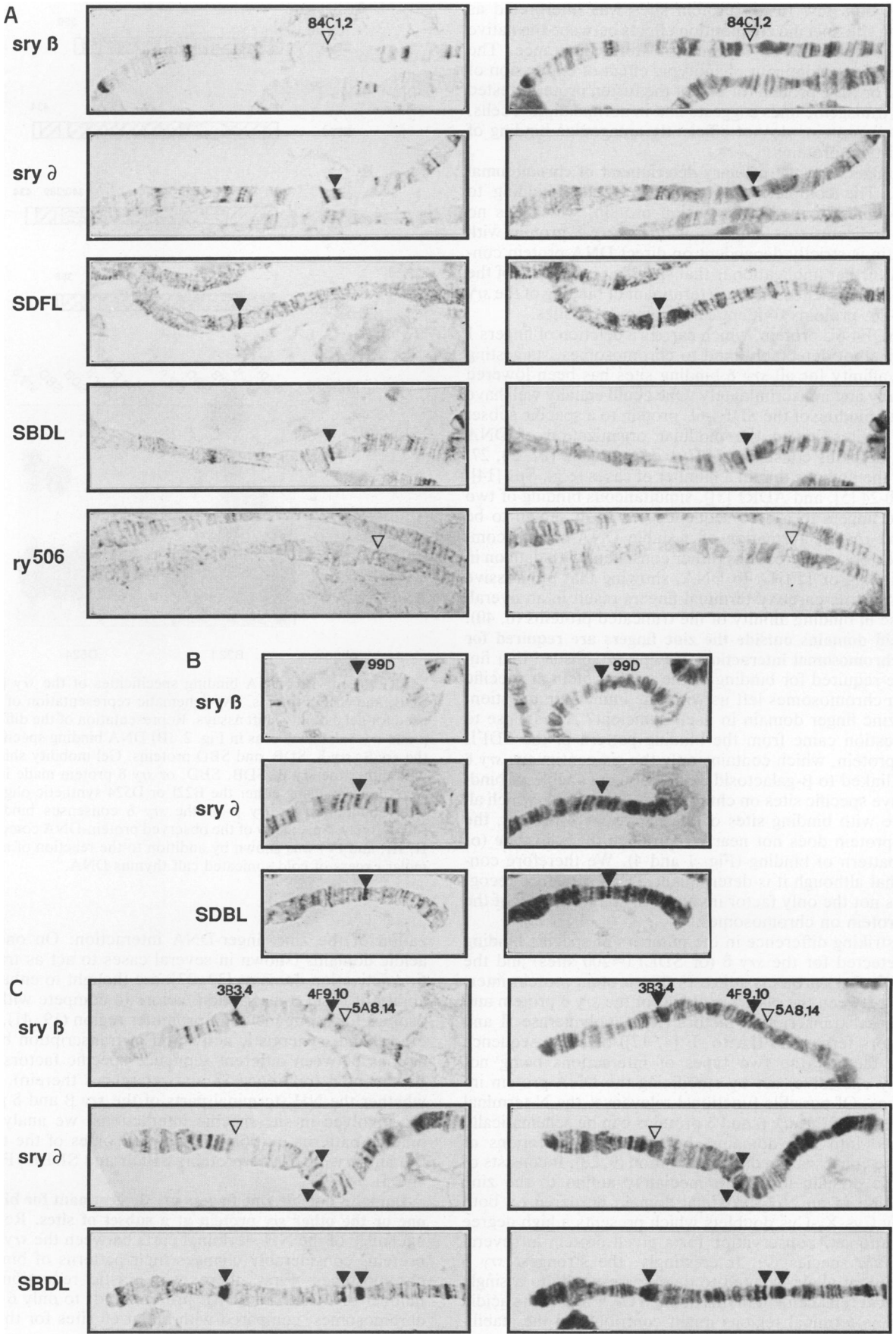


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intensity of a few fusion protein sites was interpreted as reflecting site-specific competition effects between the native and fusion proteins as a result of steric hindrance. The absence of any dominant phenotypic effect of expression of the SDL or SBL protein (or any of the fusion proteins tested here) in transgenic lines suggests that in normal diploid cells, the fusion proteins do not efficiently antagonize binding of the wild-type proteins.

**Zinc fingers are the primary determinant of chromosomal binding.** The complete absence of detectable binding to chromosomes of the SDAL fusion protein, which has no fingers, indicates that interaction of the *sry* δ protein with chromatin is strictly dependent on direct DNA-protein contacts. A further implication is that specific recognition of the same DNA sequences is the determinant of binding of the *sry* δ and SDL proteins to identical chromosomal sites.

The SDF4-6L protein, which carries a deletion of fingers 1 to 3, does not detectably bind to chromosomes, suggesting that its affinity for all *sry* δ binding sites has been lowered drastically and indiscriminately. One could equally well have expected binding of the SDF4-6L protein to a specific subset of *sry* δ sites, given the modular organization of DNA binding domains consisting of $Cys_2/His_2$ fingers (22, 24, 27, 29) and the findings that in a number of cases (e.g., Sp1 [14], Krox 20-24 [5], and ADR1 [3]), simultaneous binding of two or three fingers to a core sequence has been shown to be sufficient for the formation of a stable DNA-protein complex. The observed result is rather consistent with data on in vitro binding of TFIIIA to DNA, showing that progressive deletions of its carboxy-terminal fingers result in an overall decrease in binding affinity of the truncated proteins (6, 40).

**Protein domains outside the zinc fingers are required for stable chromosomal interaction.** Having established that fingers are required for binding of the *sry* δ protein at specific sites on chromosomes left us with one immediate question: Is the zinc finger domain in itself sufficient? A response to this question came from the binding pattern of the SDFL fusion protein, which contains only the six contiguous *sry* δ fingers linked to β-galactosidase. Although capable of binding to five specific sites on chromosomes (Fig. 5A), which all coincide with binding sites of the native *sry* δ protein, the SDFL protein does not nearly reproduce the wild-type (or SDL) pattern of binding (Fig. 1 and 4). We therefore conclude that although it is determinant, DNA sequence recognition is not the only factor involved in stable binding of the *sry* δ protein on chromosomes.

The striking difference in the numbers of specific binding sites detected for the *sry* δ (or SDL) (≈200 sites) and the SDFL (5 sites) proteins suggests that protein-protein interactions between the N-terminal half of the *sry* δ protein and either basal transcription factors (RNA polymerase II and the factors termed TFIIA to -F [4, 17]) or other sequence specific factors (the two types of interactions being not mutually exclusive) act by stabilizing the DNA-protein interactions. Of possible functional relevance, the N-terminal half of each of the *sry* β and δ proteins can be schematically separated into two domains, based on considerations of sequence conservation during evolution (9, 28). It consists of an acidic domain located immediately amino to the zinc fingers and of an $NH_2$-proximal domain bordered on both sides by Cys-$X_2$-Cys doublets which presents a high degree of evolutionary conservation for a given protein in several *Drosophila* species (9). Interestingly, the strongest *sry* δ point mutant allele isolated to date corresponds to a single amino acid replacement in this domain (7). Each of the acidic and amino-terminal regions might contribute to the stabilization of the zinc finger-DNA interaction. On one hand, acidic domains (shown in several cases to act as transcription activation domains [32, 33]) are thought to enhance the ability of basal transcription factors to compete with nucleosomes for occupancy of a promoter region (19, 41). On the other hand, synergistic activation of transcription by interactions between different sequence specific factors is well documented (reference 18 and references therein). To test whether the $NH_2$-terminal parts of the *sry* β and δ proteins are involved in site-specific interactions, we analyzed the binding patterns on polytene chromosomes of the two (*sry* β-δ and *sry* δ-β) swap proteins SBDL and SDBL (Fig. 2, 4, and 5).

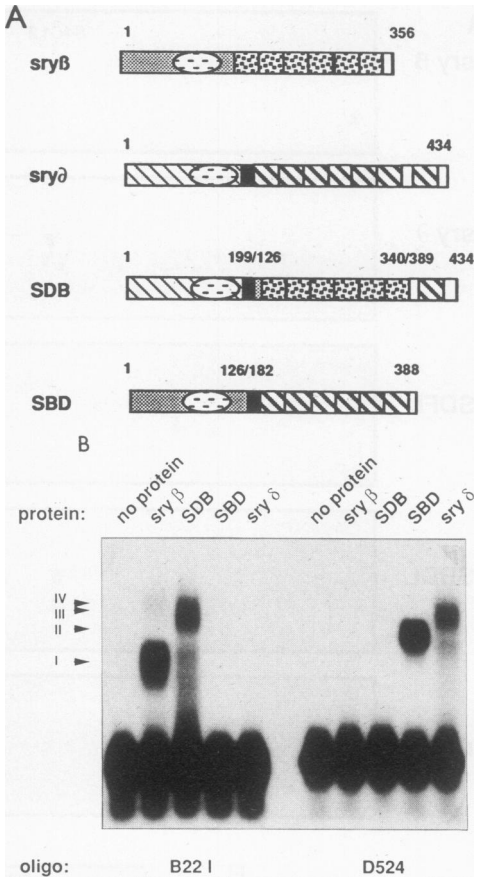


FIG. 6. In vitro DNA binding specificities of the *sry* β, *sry* δ, SDB, and SBD proteins. (A) Schematic representation of proteins used for gel mobility shift assays. Representation of the different *sry* β and *sry* δ domains is as in Fig. 2. (B) DNA binding specificities of the *sry* β, *sry* δ, SDB, and SBD proteins. Gel mobility shift assays with either the *sry* β, SDB, SBD, or *sry* δ protein made in *E. coli* were done by using either the B22I or D524 synthetic oligonucleotide, containing the *sry* β or the *sry* δ consensus binding site, respectively. Specificity of the observed protein-DNA complexes (I, II, III, and IV) was shown by addition to the reaction of a $10^5$-fold molar excess of cold sonicated calf thymus DNA.

**Domains outside zinc fingers are determinant for binding of one or the other *sry* protein at a subset of sites.** Reciprocal exchange of the $NH_2$-terminal parts between the *sry* β and δ proteins considerably changes their patterns of binding on chromosomes. First, there is a drastic reduction in the number of sites. The SDBL protein binds to only 6 sites on chromosomes, compared with about 60 sites for the native

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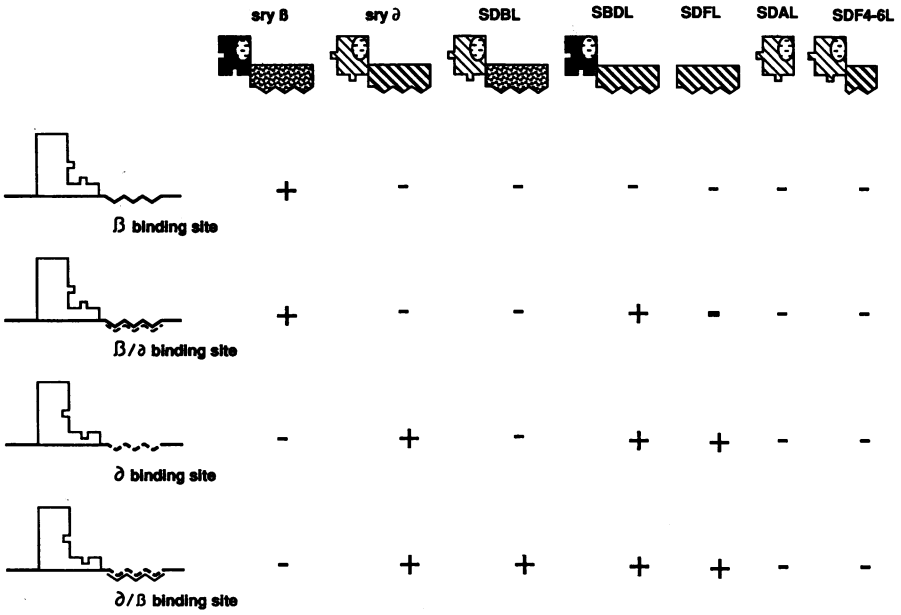


FIG. 7. Model for chromosomal binding of the *sry* β, *sry* δ, and β-galactosidase fusion proteins. Only sites specific to one or the other protein at the cytological level are considered in this model. The critical event is binding of the finger domain to its DNA recognition sequence. Three types of DNA binding sites can be distinguished: *sry* β-specific sites (〰), *sry* δ-specific sites (-~-~-), and sites possibly bound by both proteins (〰 and 〰). This primary recognition event is converted to stable binding of the proteins through interactions between their $NH_2$-terminal domains and other factor(s). The restricted pattern of binding sites of the SDBL protein compared with the SBDL protein suggests that factors that interact with the amino half of *sry* β are unable to interact properly with the *sry* δ counterpart. No implication is made that factors interacting with the *sry* β and *sry* δ proteins are specific DNA-binding proteins. Representation of the different domains of the *sry* β and δ proteins is as in Fig. 2. For clarity, the β-galactosidase sequences are not represented.

*sry* β (or SBL) protein. Likewise, the SBDL protein binds to 38 sites, compared with about 200 in the case of the native *sry* δ (or SDL) protein. Second, both swap proteins bind to subsets of the native *sry* β or δ chromosomal sites but not to an additional (novel) site (that does not correspond to either a *sry* β or a *sry* δ site), arguing against acquisition of new DNA binding specificities (Fig. 4). These data indicate that the $NH_2$-terminal part of the *sry* β and δ proteins, even though not sufficient in itself for positioning the β-galactosidase to specific chromosomal sites (SDAL fusion), is determinant in the selection of *sry* β and *sry* δ sites in the wild-type chromatin context.

That the SBDL and SDBL fusion proteins bind to many fewer sites than the native proteins further indicates that the N-terminal parts of the *sry* β and *sry* δ proteins cannot substitute for each other. It indicates interactions of these domains with other site-specific factors rather than (or in addition to) general transcription factors. Binding of the SDBL protein, which contains the *sry* β fingers, to (a small subset of) *sry* δ-specific sites and of the SBDL protein, which contains the *sry* δ fingers, to some *sry* β-specific sites (Fig. 4) suggests that protein-protein interactions could be epistatic over DNA-specific recognition at these particular sites. We propose that these sites contain DNA sequences possibly recognized by both proteins (Fig. 7). In vitro gel retardation experiments conducted with the SDB and SBD finger swap proteins confirm that the six contiguous fingers are sufficient for sequence-specific binding of the *sry* δ and *sry* β proteins and allow these two proteins to fully discriminate between their respective consensus binding sites. It is conceivable, however, that genomic sequences intermediate between the *sry* β and δ consensus recognition sequences are capable of binding both proteins, as observed in preliminary experiments using λ phage DNA restriction fragments (28, 30). Binding of one or the other protein to such a sequence in chromatin would be dependent on specific protein-protein interactions (Fig. 7). A parallel can be made with the analysis of gene-specific regulatory properties of different members of the homeodomain protein family which, in vitro, recognize the same DNA sequence. Models involving specific protein-protein contacts has been proposed to contribute to the in vivo specificity of action of each individual homeodomain protein (for a review, see reference 12).

**The DNA and protein-protein interactions specifying chromosome binding patterns segregate concertedly during evolution.** By looking at the entire set of chromosomal binding sites of modified *sry* β and δ proteins, we have shown here that specific DNA recognition together with other protein-specific interactions synergistically determine the in vivo interaction of such DNA-binding proteins with chromatin. That very few of the sites bound by the native *sry* δ protein are bound by a β-galactosidase fusion protein which contains only its DNA binding domain correlates with the fact that only a fraction of genomic sequences recognized in vitro by *sry* proteins corresponds to in vivo chromatin sites (30; unpublished data). Finally, the much lower number of binding sites for the SDFL, SDBL, and SBDL proteins than for the wild-type *sry* β and *sry* δ proteins indicates that the two elements determining binding of the proteins at specific sites, that is, presence of a DNA target and interaction with some third component(s), have segregated in a concerted fashion during evolution. This assumption is well supported by data from immunostaining chromosomal squashes from the sibling species *Drosophila simulans* and interspecific *D. melanogaster-D. simulans* hybrids. In the two cases, patterns identical to those of *D. melanogaster* are obtained, for either

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*sry* β or *sry* δ, except for one additional *sry* β site on chromosome 2L (not shown). Biochemical identification of the presumptive proteins interacting specifically with the *sry* β or *sry* δ proteins and functional characterization of some of their chromosomal targets have been undertaken.

Functional studies on numerous transcription factors in vitro has proven extremely valuable for the identification of different, usually separable, domains making up such factors (10). The chimeric protein approach developed in this study shows that an in vivo dissection should be of wide interest to identify determinants of specificity and potential target genes of regulatory factors.

## ACKNOWLEDGMENTS

We are grateful to David Cribbs and Michèle Crozatier for critical reading of the manuscript. We wish to thank Edmond Barbey for photography and Joëlle Maurel for editorial assistance.

This work was supported by CNRS and the Association pour la Recherche sur le Cancer (ARC) grant 6457 and by INSERM grant 881019. S.N. was supported by fellowships from Ministère de la Recherche et de la Technologie and ARC, and F.P. was supported by a fellowship from Ligue Nationale Contre le Cancer.

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