MOLECULAR AND CELLULAR BIOLOGY, June 1997, p. 3137–3145 Vol. 17, No. 6
0270-7306/97/$04.00+0

Two Types of Zinc Fingers Are Required for Dimerization of the serendipity δ Transcriptional Activator

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Received 9 September 1996/Returned for modification 22 November 1996/Accepted 25 March 1997

The serendipity (sry) δ zinc finger protein controls *bicoid* gene expression during *Drosophila melanogaster* oogenesis. In addition, *sry* δ mutants display various zygotic phenotypes, ranging from abnormal embryogenesis to sex-biased adult lethality. We report here that sry δ is a sequence-specific transcriptional activator. A single sry δ consensus binding site (SDCS), in either orientation, is sufficient to promote transcription activation in cell culture, and multiple SDCSs mediate a strong synergistic activation, reflecting the cooperativity of sry δ binding to DNA. Further, several lines of evidence strongly suggest that sry δ binds to DNA as a dimer. While each of three point mutations located in the third zinc finger of sry δ drastically reduces its DNA binding affinity, a fourth mutation, located in the N-terminal region of the protein, specifically affects the cooperativity of DNA binding. This mutation reveals the functional importance of a putative Cys_2/Cys_2 zinc finger motif of a novel type, located outside the DNA binding domain. A systematic deletion analysis shows that interaction between this proposed Cys_2/Cys_2 motif and a classical Cys_2/His_2 zinc finger mediates homodimerization, which is required for DNA binding cooperativity.

Since the report of the structure of TFIIIA (20), much evidence has established that stabilization of peptide domains through the chelation of zinc atoms is widespread among nucleic acid-binding proteins, from prokaryotes to mammals (for reviews, see references 16 and 31). A growing number of different types of zinc binding domains, so-called zinc fingers, have been described elsewhere, and in several cases, crystallographic data are available (35). The common point between these domains resides in the key role of regularly spaced cysteine and histidine residues that bind the zinc atom, the valence of this interaction being itself variable between the different domains. The TFIIIA-type zinc finger, generally referred to as a Cys_2/His_2 zinc finger, has been shown to fold into an independent domain (18) capable of specifically interacting with DNA. Atomic resolution of DNA-zinc finger complexes (25) and comparison of the DNA recognition properties of closely related Cys_2/His_2 proteins (14, 22) led to the proposal of a zinc finger DNA recognition code. This code was based on the ability of 3 amino acids (aa), located in the alpha-helical C-terminal region of the zinc finger (positions −1, 2, and 6 [9, 26] or x, y, and z [15]), to make direct contact with the bases and thus determine the DNA binding specificity. While the proposed code has allowed the design of semisynthetic zinc finger modules with a predicted DNA binding specificity (8), it was mostly based on the analysis of a subclass of zinc finger proteins related to Sp1 (1, 9, 26). The crystal structure of the five-finger Gli protein-DNA complex showed that, in other subclasses, separate zinc fingers can contribute unequally to DNA recognition (26). Furthermore, some Cys_2/His_2 zinc fingers have been shown to mediate protein contacts (7, 21), subcellular localization (19), or a puzzling nuclease activity (12). Therefore, for a given protein, the role of individual Cys_2/His_2 fingers in both DNA binding and protein-protein contacts remains an open question.

We report here a functional analysis of serendipity δ (sry δ), a *Drosophila melanogaster* zinc finger protein, that reveals a previously unrecognized role for a Cys_2/His_2 zinc finger in DNA binding cooperativity. *sry* δ is a zygotic lethal gene, mutations of which are associated with a variety of phenotypes including embryonic head defects, gonadal defects, and sex-biased lethality (6). Analysis of female germ line clones further revealed a specific requirement for sry δ at several steps during oogenesis, including the transcription of the morphogen *bicoid* (5, 27). *sry* δ encodes a 50-kDa protein containing seven Cys_2/His_2 zinc fingers which binds in vitro to the consensus sequence 5′-YTAGAGATGGRAA-3′ (29) and in vivo to about 200 specific sites on polytene chromosomes (28). The domain responsible for both in vivo and in vitro binding specificity of sry δ comprises six adjacent Cys_2/His_2 zinc fingers (10, 23). This zinc finger cluster is also conserved in a close paralog of sry δ, sry β, which is nonetheless unable to substitute for sry δ and recognizes a binding site differing from that of sry δ at four nucleotide positions (29). Individual zinc fingers do not present the same extent of sequence conservation, and surprisingly, the replacement of the two more divergent (including at positions −1, 2, and 6) zinc fingers 5 and 6 of sry δ by those of sry β has no major effect on DNA recognition (27). In vivo, this sry δ/β chimeric protein was found to substitute for all recognizable sry δ functions except the positive control of *bicoid* transcription (27). This result indicated that the mode of sry δ DNA binding does not fit the proposed zinc finger-DNA recognition rules of the Sp1 subfamily and that individual zinc fingers of sry δ may be differentially involved in the control of its different target genes. To gain further insight into the mode of interaction of sry δ with DNA and its relation to transcriptional regulation, we studied the properties of wild-type and mutant sry δ proteins. Results from this analysis show that sry δ is a sequence-specific transcriptional activator which binds to DNA as a dimer and displays a strong synergism in transactivation upon multimerization of its binding sites. Two separate protein regions of sry δ are required for cooperative binding to DNA:

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the sixth Cys_2/His_2 zinc finger in the DNA binding domain and a novel type of Cys_2/Cys_2 motif, which act together to promote sry δ homodimerization.

MATERIALS AND METHODS

Plasmid constructs. Complementary oligonucleotide D524 (29) or D51 was phosphorylated, hybridized, and subsequently ligated with T4 DNA ligase. Concatemers consisting of one, two, three, or four copies were gel purified and cloned into the *Bam*HI site of the pTKCAT plasmid. Recombinant plasmids were screened by dideoxy sequencing, and constructs containing tandem copies of intact D524 oligonucleotide were kept for analysis. *Escherichia coli* vectors expressing the DSF1, DSF2, and D12 mutant proteins result from the exchange of a *Sac*I restriction fragment between the wild-type T7 expression vector pSDAI (30) and the mutant genes (6). The D14 mutation was introduced by site-directed mutagenesis. To produce truncated forms of the sry δ protein, appropriate restriction fragments were cloned in pET vectors (Novagen).

Cell lines and DNA transfection. *Drosophila* SL2 cells were grown in Schneider medium with 10% fetal calf serum and 1% penicillin-streptomycin (GIBCO). Cells were transfected by a standard procedure (4) with a mix of 3 μg of pADH-β-Gal, from 0 to 5 μg of reporter plasmids, and from 2 to 7 μg of pUC18. Cells were collected 48 h after and washed once in phosphate-buffered saline, and the cell pellet was resuspended in 250 mM Tris-HCl (pH 8.0)–5 mM dithiothreitol (DTT)–15% glycerol–2.5 mM phenylmethylsulfonyl fluoride. Cells were broken by freeze-thaw cycles, and after clearing, the supernatant was kept as whole-cell extract. β-Galactosidase activity was used to normalize cell transfection efficiency. Chloramphenicol acetyltransferase (CAT) activity measurement and quantification were done as described by Vesque and Charnay (37).

Protein extracts. Producer plasmids were introduced in *E. coli* BL21(DE3):: pLysS, and protein production was induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were collected by centrifugation and washed in 50 mM Tris-HCl (pH 8.0)–1 mM EDTA–20% D-glucose–0.1 mM phenylmethylsulfonyl fluoride, and inclusion bodies were prepared as previously described (29). Proteins were solubilized in buffer Z (6 M guanidine-HCl, 20 mM HEPES [pH 7.8], 100 mM KCl, 5 mM DTT, 50 μM $ZnSO_4$, 20% glycerol), and guanidine was removed with a PD10 desalting column (Pharmacia). The soluble fraction was then loaded on a Hitrap heparin column (Pharmacia). Bound proteins were eluted at 500 mM KCl, diluted to a conductivity corresponding to 200 mM KCl, and purified on a MonoQ column (Pharmacia). Embryonic nuclear extracts containing partially purified sry δ protein were prepared as previously described (heparin-Sepharose followed by MonoQ [29]).

DNA binding and electrophoretic mobility shift assay (EMSA). Oligonucleotides were 5′ labeled with T4 polynucleotide kinase, hybridized, and gel purified (specific activity, 1,500 Ci/mmol). sry δ consensus binding site (SDCS) fragments were generated by *Hin*dIII/*Eco*RI digestion of the pDnI-CAT plasmids, end labeling, and gel purification (specific activity, 1,000 Ci/mmol). Each DNA fragment contains 52 nucleotides from the vector in addition to one, two, three, or four tandem copies of D524 (25 nucleotides). A standard DNA binding assay contained 0.5 nM labeled DNA and 1.5 μg of yeast tRNA, calf thymus DNA, and poly(dI-dC) (and if specified, 10 or 100 ng of duplex cold competitor) in 10 μl, with final concentrations of 50 mM KCl, 20 mM HEPES (pH 7.8), 1 mM DTT, 50 μM $ZnSO_4$, and 10% glycerol. Five microliters of diluted protein was added to the DNA mixture, and the mixture was incubated for 20 min at 30°C. DNA-protein complexes were loaded onto an electrophoresis gel, either 1% SeaKem agarose (FMC) or 4.5% acrylamide in 20 mM Tris–200 mM glycine (pH 8.3)–1 mM EDTA at 4°C. Gels were transferred to DE81, and the dried paper was exposed for fluorography or quantification with a Fuji Bas1000 phosphorimager (PCBAS software). DNase I footprinting experiments were done as described by Payre and Vincent (29) with a 117-bp *Acc*I/*Pst*I restriction fragment of the wild-type or mutated *bcd* promoter region.

Protein interaction. After ultracentrifugation (100,000 × *g*, 1 h), the protein to be analyzed was loaded onto an HR10/30 S200 Superdex column (Pharmacia) and equilibrated in 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–1 mM DTT–50 μM $ZnSO_4$–0.1% Tween 20, at a flow rate of 0.4 ml/min. Protein elution was monitored by optical absorbance at 254 nm. Fractions of 500 μl were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Retention times were calculated with the fast protein liquid chromatography manager software from Pharmacia; molecular weight calibration was established by using a mixture of thyroglobulin, ferritin, β-amylase, albumin, ovalbumin, carbonic anhydrase, and cytochrome *c*.

RESULTS

sry δ binding sites mediate a strong transcriptional activation. The specific DNA binding properties of sry δ, in vitro and in vivo (28, 29), suggested that it is a gene-specific transcriptional regulator. We therefore tested the ability of sry δ to regulate transcription, by introducing SDCSs in the minimal promoter region of a reporter gene.

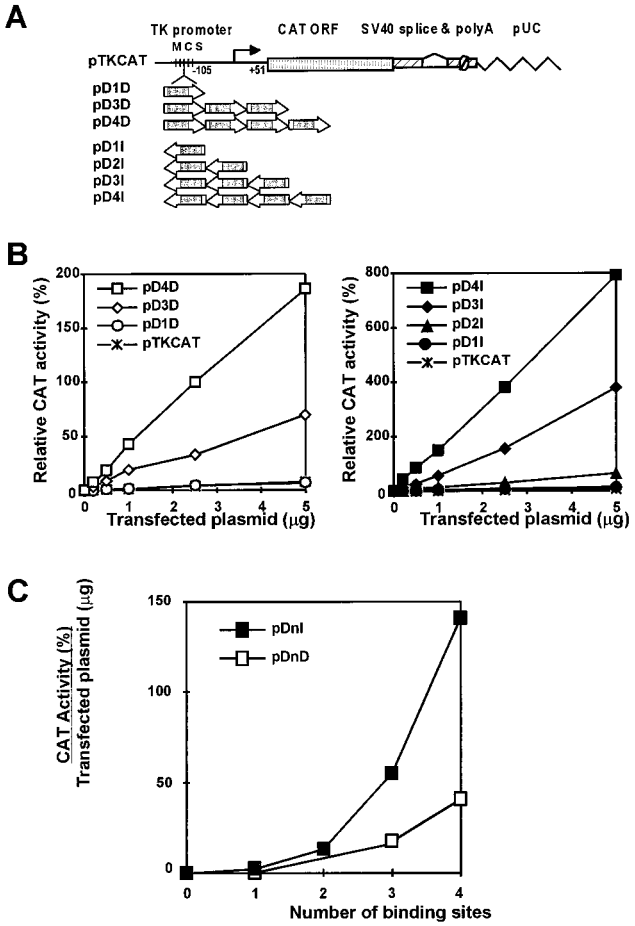

FIG. 1. sry δ binding sites mediate transcriptional activation in *Drosophila* tissue culture cells. (A) Schematic structure of reporter constructs containing from one to four tandem copies of the SDCS (gray boxes) in both orientations. TK, thymidine kinase; ORF, open reading frame; SV40, simian virus 40. (B) *Drosophila* Schneider cells were transfected with various amounts of each reporter plasmid. Control experiments with the pTKCAT reporter plasmid indicate that no significant activation occurs in the absence of SDCS. Each datum point represents the mean value of from three to five independent experiments. (C) Multimerization of sry δ binding sites strongly synergizes transcriptional activation. The slope of the variation of CAT activity in function of the amount of transfected reporter plasmid was plotted against the number of sry δ binding sites present, in either I (black squares) or D (white squares) orientation.

From one to four tandem copies of an oligonucleotide (D524) containing an SDCS were inserted in the pTKCAT reporter plasmid in one (D) or the other (I) orientation (Fig. 1A). Each construct, referred to as pD_n(D or I)CAT, was transiently transfected in *Drosophila* SL2 cultured cells, and promoter activity was assayed by CAT activity measurements. The presence of a single SDCS induced transcriptional activation of the CAT reporter gene. The level of activation was higher in the I than in the D orientation, but transactivation was effective in both cases. This indicates that SDCSs behave as bona fide enhancer elements when placed in a promoter-proximal configuration.

The level of activation was strongly increased with the copy number of SDCS (Fig. 1B). Going from one to two tandem SDCSs resulted in a sixfold increase in the transcriptional rate of the reporter gene, with a further 10-fold increase when going from two to four binding sites (Fig. 1C). In both instances, it is very different from a twofold increase characteristic of an ad-

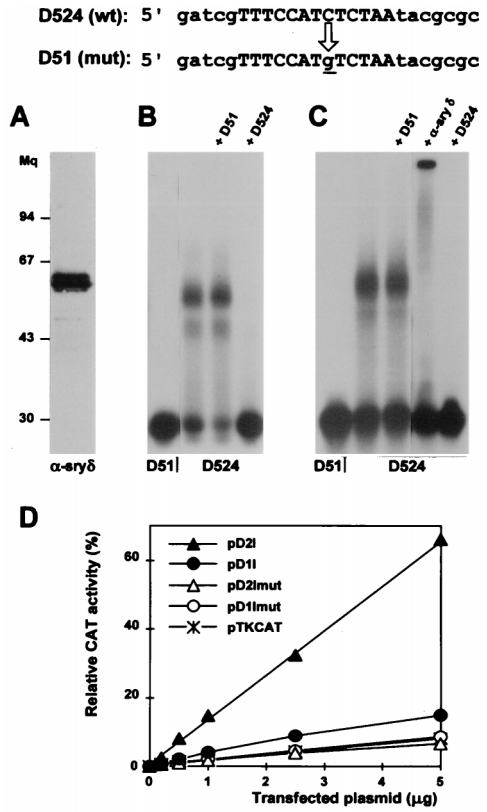

FIG. 2. Introduction of a single point mutation in SDCS abolishes sry δ binding. (Top) Sequence of wild-type (D524) or mutated (D51) oligonucleotide used, where positions matching the sry δ consensus site (SDCS) are uppercase. (A) Western blot showing the presence of a high level of endogenous sry δ protein in whole-cell extract from untransfected SL2 cells. Mq, molecular mass markers in kilodaltons. (B) D524 or D51 oligonucleotide was subjected to EMSA in the presence of recombinant sry δ protein. Where specified, a 100- or 1,000-fold molar excess of cold D524 or D51 (+D524 or D51), respectively, was added to the binding reaction. (C) EMSA with whole-cell extract from SL2 cells with D524 or D51 oligonucleotide. + α-sry δ, addition of affinity-purified anti-sry δ immunoglobulin G (the same batch as used for panel A). (D) Binding of sry δ is required for SDCS-mediated transcriptional activation. One or two tandem copies of the D51 mutant oligonucleotide were inserted, in the I orientation, in the pTKCAT plasmid (pD1Imut and pD1Imut and pD2Imut, respectively).

ditive comportment, showing that SDCS multimerization results in a strongly synergistic transcription activation.

Endogenous sry δ is present in SL2 cells, as shown by Western blotting (Fig. 2A). That this endogenous protein was responsible for the transactivation of the SDCS-CAT reporter genes was verified by EMSAs. A single G-to-C nucleotide replacement in the SDCS (at a position found to be invariant in all genomic sry δ binding sites; oligonucleotide D51 [Fig. 2, top]) drastically decreased binding of the recombinant sry δ protein (Fig. 2B). Neither binding of sry δ nor significant competition of sry δ binding to SDCS was detected with this mutated D51 oligonucleotide. When an SL2 cell extract was used, a DNA-protein complex with migration similar to that obtained with the recombinant sry δ protein formed with wild-type SDCS (D524), while no complex was detected with D51 (Fig. 2C). Addition of monospecific anti-sry δ antibodies resulted in a supershift of all the SDCS-protein complexes (Fig. 2C), further identifying sry δ as the major, if not only, protein binding to SDCS in SL2 cells. In transfection experiments, neither one nor two copies of D51, inserted in the promoter region of pTKCAT (I orientation), mediated detectable transcriptional activation (Fig. 2D). Taken together, transfection and EMSA results indicate that SDCS enhancer activity is dependent upon binding of the sry δ protein. Therefore, sry δ acts as a sequence-specific transcriptional activator, with a strong synergism in transcriptional activation resulting from multimerization of its binding sites.

Cooperative DNA binding of the sry δ protein. To determine the contribution of DNA binding to the synergistic transactivation observed upon multimerization of SDCS, we measured the binding affinity of sry δ for DNA fragments containing from one to four SDCSs. EMSA analysis revealed that sry δ binds more efficiently to multimerized SDCS than to a single SDCS (Fig. 3A). The apparent dissociation constant was estimated by titration curve experiments, with the protein concentration shifting 50% of the input DNA considered as the $K_{d\mathrm{app}}$. The strong increase in sry δ affinity ($K_{a\mathrm{app}} = 1/K_{d\mathrm{app}}$) observed upon SDCS multimerization (20-fold between one and two and 5-fold between two and four binding sites) did not fit the linear relationship expected for independent additive DNA binding. The plot of the apparent affinity of sry δ versus the number of SDCSs shows this strong cooperative binding of sry δ to multimerized SDCS (Fig. 3B). This suggests that the synergism in transcriptional activation observed in cell culture with multiple tandem SDCSs mostly reflects the cooperative DNA binding properties of sry δ.

Cooperative DNA binding generally reflects the existence of protein contacts. Supporting this hypothesis, the electrophoretic migration of the major DNA-sry δ complex (II) formed with one SDCS was identical to that with two SDCSs, indicating the same protein-DNA stoichiometry in both cases (Fig. 3A). With a DNA fragment containing either three or four tandem SDCSs, a first complex (complex II) formed at a low protein concentration and then a second complex of lower electrophoretic mobility (complex III) gradually appeared with increasing protein concentration (Fig. 3A). This strongly suggests that sry δ binds as a dimer, one dimer on either one or two binding sites, the presence of three or four sites thus allowing for the binding of two sry δ dimers.

Properties of sry δ mutant proteins in vitro. Each of the four *sry* δ mutant alleles isolated to date corresponds to a single amino acid replacement in the protein (6). The influence of these mutations on DNA binding was assayed with the four mutant proteins (DSF1, DSF2, D12, and D14) produced in *E. coli*. With a single SDCS, sry δ proteins mutated in the third zinc finger (DSF1, DSF2, and D12) each displayed a drastic reduction in DNA binding (Fig. 4A and Table 1). Surprisingly, the D14 mutation, which corresponds to an amino acid change outside the Cys_2/His_2 zinc finger DNA binding domain, also affected binding to DNA. Aside from a reduction of D14 affinity for one SDCS (Table 1), the apparent mobility of the D14-SDCS complex was markedly altered (Fig. 4A). Migration of this complex corresponded to that of a fast-migrating complex (complex I) observed as a very minor species with the wild-type protein (Fig. 3A), whereas no complex II (the major wild-type complex) formed.

As for wild-type sry δ, the use of an SDCS dimer resulted in an increased binding of DSF1, DSF2, and D12 (Fig. 4B). Moreover, the effect of *SF2* and δ*12* mutations was far weaker on two SDCSs than on one SDCS, the affinity of the D12 mutant protein approaching that of wild type (Table 1). At the opposite extreme, duplication of SDCS resulted in a drastic decrease of the D14 binding. Again, no complex II was visible, while a slowly migrating and smearing complex indicated formation of an abnormal protein-DNA complex (Fig. 4B). While a small increase of the ionic strength (from 60 to 85 mM KCl) further significantly reduced the binding of mutant proteins altered in

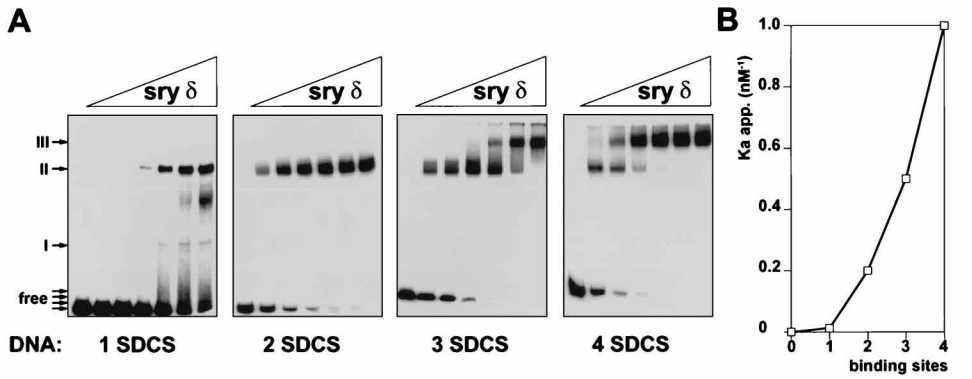

FIG. 3. Cooperative binding of sry δ protein on multimerized SDCS. (A) EMSA analysis of DNA fragments from pDnI-CAT plasmids containing one, two, three, or four tandemly repeated SDCSs, with increasing amounts of sry δ protein. From left to right, sry δ concentrations were 0, 5, 10, 20, 40, 80, and 160 nM for one and two SDCSs and 0, 1, 2, 4, 10, 20, and 40 nM for three and four SDCSs. Quantification of the bound/free ratio for SDCS-containing fragments (the concentration of which was held constant at 1 nM) was performed with a Fuji Bas1000 phosphorimager, in triplicate. Each experiment was repeated four times with two independent batches of protein. The concentration of protein found to shift half the DNA was estimated as the apparent dissociation constant. (B) Plot of the sry δ apparent affinity ($K_{aapp} = 1/Kd_{app}$) versus the number of SDCSs.

the DNA binding domain (DSF1, DSF2, and D12), it did not affect either wild-type or D14 protein (Table 1). These results suggested that, rather than specific DNA contact, the D14 mutation alters the structural conformation of the sry δ protein, consequently impairing its DNA binding cooperativity.

Protein regions required for DNA binding cooperativity of sry δ. The δ*14* mutation replaces a cysteine by a tyrosine residue at position +7 in sry δ. This cysteine is part of a C-X_2-C motif found twice in the N-terminal region, a region which is highly conserved between distantly related *Drosophila* species (10).

To test the role of this N-terminal region in DNA binding, a series of N-terminally truncated versions of sry δ were tested by EMSA on DNA fragments containing either one or two SDCSs (Fig. 5A). Removal of the N-terminal 30 aa (protein DN2) led to an altered migration of the protein-one SDCS complex. At a low protein concentration, a smearing complex with low electrophoretic migration formed. This complex only partly resolved in a complex similar to the wild-type complex II at a higher protein concentration. In the presence of two SDCSs, a type II complex formed, one whose smearing appearance, indicative of an abnormal DNA-protein complex, again diminished with increasing protein concentration. Further truncation of the N-terminal region (protein DN4, 67 aa; DN7, 182 aa; D2, 192 aa) led to a drastic change in the DNA binding mode. At any protein concentration, a single fast-migrating complex formed on one SDCS. This complex was also observed with two SDCSs at a low protein concentration while a second, more slowly migrating complex formed at a higher protein concentration. This behavior is typical of an additive rather than a cooperative binding. Deletion extending to the Cys_2/His_2 zinc finger domain (protein D10) abolished DNA binding. Further analysis of the mode of binding of D2 to DNA was performed on DNA fragments containing between one and four SDCSs (Fig. 5B). For each fragment, the number of complexes strictly paralleled the number of SDCSs. Measurement of binding constants indicated an affinity of D2 similar to that of wild-type sry δ for one SDCS (estimated K_d, 95 ± 10 and 118 ± 4 nM, respectively). However, and in contrast to wild type, the increase in affinity observed for multimerized SDCS is purely additive (Fig. 5C). Together, these data indicate that, in contrast to wild type, sry δ derivatives lacking the N-terminal region bind to DNA as monomers, with no sign of cooperativity.

To further extend these conclusions to a known physiological target of sry δ, we analyzed the binding of sry δ and D2 on the *bicoid* promoter. The wild-type sry δ protein protected a 44-bp region from DNase I digestion (Fig. 6A). This region contains two binding sites (SDBS A and SDBS B), with sequences matching the SDCS at 11 of 13 and 10 of 13 positions, respectively, and separated by 7 instead of 12 bp in the case of

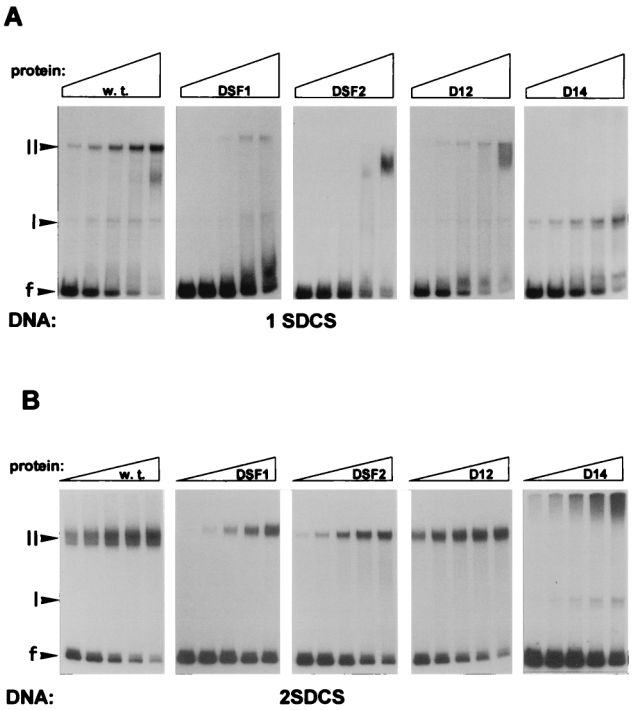

FIG. 4. sry δ mutations alter two distinct DNA binding properties. Analysis of DNA interaction of the mutant sry δ proteins was done by EMSA, with increasing amounts of protein. (A) Binding to a DNA fragment containing a single SDCS. A total of 0.5 nM labeled DNA fragment was incubated with, from left to right, 20, 40, 80, 160, and 320 nM each protein. (B) Binding to a DNA fragment containing a tandem SDCS. In this case, 0.5 nM labeled DNA fragment was incubated with, from left to right, 2.5, 5, 10, 20, and 40 nM each protein. Lanes corresponding to the D14 protein were overexposed. w.t., wild type; f, free DNA fragment.

TABLE 1. K_{dapp} for sry δ proteins

Protein	One SDCSs-DNA (60 mM)		Two SDCSs-DNA				60/85 mM ratio
			60 mM		85 mM		
	K_{dapp} (nM)	%	K_{dapp} (nM)	%	K_{dapp} (nM)	%	
Wild type	118 ± 4.0	100	5.1 ± 0.4	100	5.1 ± 0.2	100	1.00
SF1	850 ± 70.7	14	35.9 ± 2.6	14	111.5 ± 17.1	4	0.32
SF2	455 ± 24.7	26	16.0 ± 2.1	32	39.0 ± 1.0	13	0.41
D12	394 ± 24.3	30	6.4 ± 0.6	80	11.0 ± 0.4	46	0.58
D14	228 ± 8.1	52	38.8 ± 0.9	13	38.5 ± 0.5	13	1.00

two SDCSs (Fig. 6B). At all protein concentrations tested, sry δ protected equally both SDBSs, whereas D2 protected only SDBS B at a low protein concentration, while starting to protect SDBS A only at higher protein concentrations. The dramatic DNase I hypersensitivity, induced specifically by D2 binding, further confirmed the modification of sry δ conformational properties subsequent to N-terminal deletion. Disruption of SDBS A, mutation N1, impaired binding of both sry δ and D2 to SDBS A. However, while N1 had no effect on binding of D2 to SDBS B, it strongly reduced the binding of sry δ to this site, demonstrating the cooperative binding of sry δ on the *bicoid* promoter (Fig. 6A and C).

Since alteration of sry δ zinc fingers 5 and 6 was previously shown to impair *bicoid* transcription (27), we tested the influence of C-terminal truncations on DNA binding (Fig. 7). While removal of the seventh sry δ zinc finger has no apparent effect on binding to either one or two SDCSs, removal of zinc finger 6 led to loss of binding cooperativity to two SDCSs without a decrease in affinity for one SDCS. This result is similar to that observed for N-terminal deletions (Fig. 7). Further removal of the fifth zinc finger, protein D5, completely abolished DNA binding.

From the above results, we can conclude that two separate regions of sry δ, the N-terminal (+1/+67) region and the sixth Cys_2/His_2 zinc finger, are required for cooperative binding of sry δ to multiple binding sites, the sequence and spacing of which are not critical for cooperativity. Deletion of either one or the other of the two defined protein regions is sufficient to change the DNA binding mode of sry δ, leading the modified proteins to bind as monomers.

sry δ homodimers. To further test the ability of sry δ to interact with itself and determine whether this requires prior binding to DNA, we used a gel filtration assay with different forms of the sry δ protein.

The wild-type sry δ protein, either recombinant or partially purified from embryonic nuclear extracts, eluted from gel filtration as two distinct peaks (Fig. 8), indicating the existence of two molecular species composed of sry δ protein differing in their apparent masses. Comparison of the retention times showed that the species that eluted first was roughly twice as large as the other. When the protein present in either peak of elution was resubmitted to gel filtration in the same conditions, two peaks were again observed (not shown), pointing to the existence of an equilibrium between the two forms of sry δ. This indicates that sry δ is able to interact with itself in the absence of DNA and that homodimerization is an intrinsic property of the protein. The δ*14* mutation alters this property since the D14 protein eluted as a single peak, corresponding to a protein species apparently larger than the wild-type protein, indicative of aggregation (Fig. 8). This confirmed that the δ*14* mutation leads to a deregulation of sry δ homodimeric protein interaction, a conclusion already drawn from EMSA data (Fig. 5B). Progressive N-terminal truncations of sry δ resulted in a single peak of elution (proteins DN3 to D2), corresponding to the smaller size of the two forms of the protein observed with wild type (Fig. 8 and 9). Furthermore, specific deletion of the C-terminal Cys_2/His_2 zinc finger 6 (protein D9) gave the same result (Fig. 8). Together, these results established that the peak of lower molecular mass corresponds to a monomeric form of

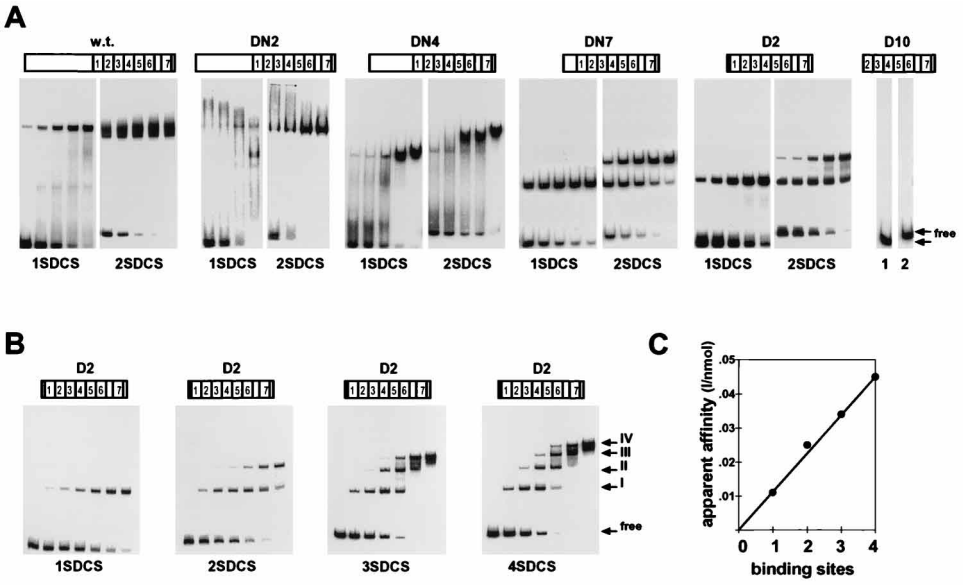

FIG. 5. The N-terminal region of sry δ is required for cooperative DNA binding. (A) Increasing amounts of either wild-type sry δ or N-terminally truncated proteins were incubated with DNA fragments containing one and two SDCSs. Boxes are drawn to scale; each zinc finger is represented by a numbered box. DN2, aa 30 to 434; DN4, aa 67 to 434; DN7, aa 182 to 434; D2, aa 192 to 434. (B) Increasing amounts of truncated D2 protein were incubated with DNA fragments containing one, two, three, or four SDCSs under the same conditions as above. From left to right, protein concentrations were 0, 10, 20, 40, 80, 160, and 320 nM. (C) Plot of the D2 protein affinity ($K_{aapp} = 1/K_{dapp}$) versus the number of SDCSs. The relationship follows a linear relation with a slope of 1.0.

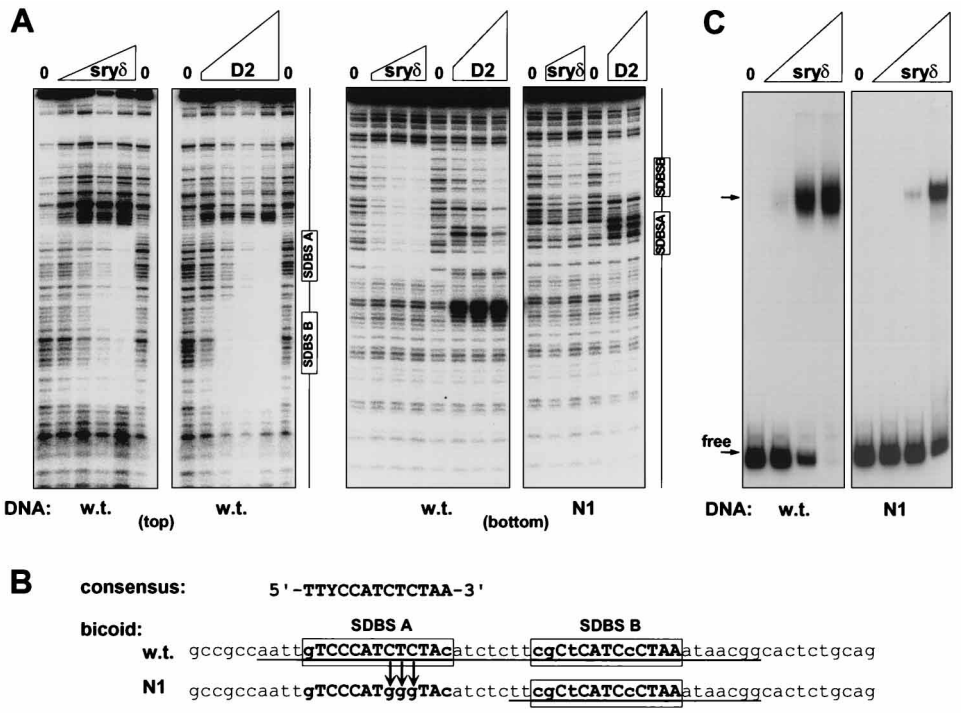

FIG. 6. Cooperative binding of sry δ to the *bicoid* promoter region. (A) DNase I footprinting analysis of binding of wild-type (sry δ) and D2 proteins to the *bicoid* promoter DNA. On one wild-type strand (top), we used 0, 1, 2, 5, and 10 nM sry δ and 0, 10, 20, 50, and 100 nM D2 protein. On the opposite strand (bottom), we used 0, 2, 5, and 10 nM sry δ and 0, 10, 20, 50, and 100 nM D2. Totals of 5 and 10 nM sry δ and 50 and 100 nM D2 were used with the N1 bottom strand. (B) Sequence of the DNA fragment from the proximal *bcd* promoter showing the position of the two sry δ binding sites SDBS A and SDBS B, identified by footprints in the wild-type (w.t.) gene. Positions protected from DNase I by wild-type sry δ are underlined, and nucleotides matching the consensus are uppercase. In N1, the first binding site (SDBS A) was modified by site-directed mutagenesis (27). (C) Wild-type sry δ protein (0, 1, 10, or 100 nM) was incubated with 1 nM either wild type (w.t.) or N1 *bcd* DNA, and formation of DNA-protein complex was analyzed by EMSA as for Fig. 3.

sry δ. In the cases in which two peaks were observed (proteins sry δ, DN2, and D7), the ratio of their apparent molecular weights ranged from 1.8 to 2.7 ± 0.4, strongly suggesting that the larger form is a dimer. Complementary to DNA binding (Fig. 5 to 7) and glutathione S-transferase fusion data (not shown), the gel filtration analysis confirmed that sry δ forms homodimers and that two separate regions of the protein (N terminus and zinc finger 6) are required for dimerization (Fig. 9).

DISCUSSION

Genetic analyses of development, especially in *Drosophila* and nematodes, have brought to light both the fundamental role and the functional diversity of Cys_2/His_2 zinc finger proteins. *sry* δ is an essential *Drosophila* gene coding for a Cys_2/His_2 zinc finger protein, with multiple functions in both the soma and the germ line. Here we show that sry δ is a sequence-specific transcriptional activator with a complex mode of DNA interaction. Whereas the block of the six contiguous Cys_2/His_2 zinc fingers is responsible for sequence-specific DNA binding, the sixth zinc finger is also strictly required for sry δ dimerization. Together, this Cys_2/His_2 zinc finger 6 and a putative Cys_2/Cys_2 zinc binding motif located outside the DNA binding domain, in the N-terminal region, form a dimerization domain. Proper dimerization is required for sry δ cooperative binding to multiple binding sites and synergistic transactivation of target genes.

The binding specificity of sry δ is very stringent with a consensus sequence of 13 nucleotides, and all identified genomic sry δ binding sites match this consensus at least eight positions

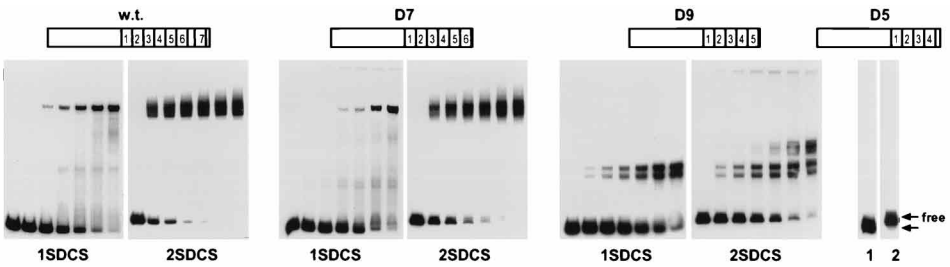

FIG. 7. The sixth Cys_2/His_2 zinc finger sry δ is required for cooperative DNA binding. Increasing amounts of either wild-type sry δ or C-terminally truncated proteins (D7, 1 to 338 aa; D9, 1 to 348 aa; D5, 1 to 310 aa) were incubated with DNA fragments containing one or two SDCSs. From left to right, protein concentrations were 0, 10, 20, 40, 80, 160, and 320 nM. In the case of D5 protein, only the lane corresponding to 320 nM is shown. In each case, the DNA concentration was held constant at 0.5 nM.

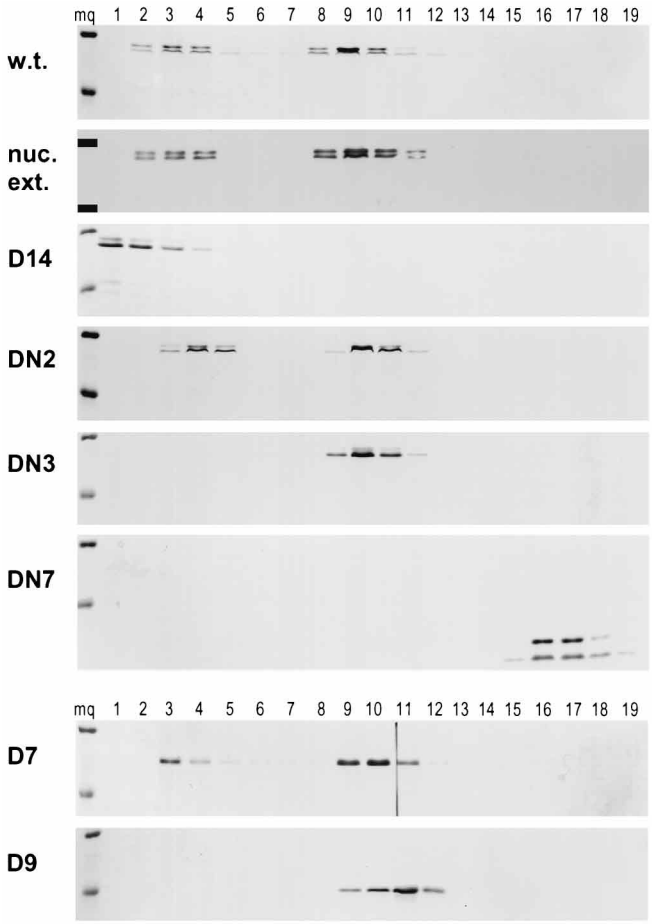

FIG. 8. Dimerization of the sry δ protein in solution. Wild-type sry δ and several derivatives were analyzed by gel filtration chromatography. w.t., recombinant wild-type sry δ protein; nuc. ext., *Drosophila* sry δ protein partially purified from embryonic nuclear extract; D14, sry δ14 mutant protein; DN2, DN3, and DN7, N-terminally truncated sry δ proteins lacking 30, 38, and 182 N-terminal aa, respectively; D7 and D9, C-terminal truncations of 46 and 86 aa, corresponding to removal of zinc fingers 7 and 6 and 7, respectively. After elution, fractions 1 to 19 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were revealed by Coomassie staining, except for nuclear extract, for which sry δ detection was done by Western blotting. mq, molecular mass marker; only bovine serum albumin (67 kDa) and ovalbumin (45 kDa) are visible in the figure.

(27, 29). This unusually long sry δ recognition site probably reflects the fact that several Cys_2/His_2 zinc fingers contribute to DNA binding and make direct contact with specific DNA. The high binding specificity of sry δ is also illustrated by its inability to bind the consensus binding site of its paralog sry β, although sry β and sry δ recognition sites differ at only 4 of 13 positions. Finally, we show here that a single G-to-C base substitution in the SDCS impairs both sry δ binding and sry δ-mediated transcriptional activation.

The DNA binding domain of sry δ can be restricted to the six adjacent Cys_2/His_2 zinc fingers, i.e., they by themselves recognize and bind to a sry δ binding site. Binding of this zinc finger domain results in the formation of as many different DNA-protein complexes as the number of SDCSs present in the bound DNA. With multiple binding sites, the binding is strictly additive, indicating that it occurs independently on each adjacent site on DNA. These data demonstrate that, by itself, the Cys_2/His_2 zinc finger domain of sry δ binds to DNA as a monomer.

Each of the *sry* δ point mutations isolated to date impairs both its DNA binding properties in vitro and its ability to mediate *bicoid* transcription, confirming that DNA binding is a primary determinant of sry δ function in vivo. The relative decrease in binding affinity of each sry δ mutant protein is not the same, whether there are one or two binding sites in the DNA fragment. The relative binding of mutant proteins appears also to be sensitive to the spacing and/or sequence of the adjacent binding sites. For example, DSF1 binds fivefold less efficiently than D12 to two SDCSs but twofold better to the *bcd* promoter (data not shown). These results explain the difficulty encountered in ordering *sry* δ mutations in a simple allelic series (6). Indeed, depending upon the phenotype analyzed (period of lethality, ratio of escapers, and male and female gonadal defects) the severity of each *sry* δ mutation is different, suggesting that expression of different *sry* δ target genes is differentially affected in relation to the structure of the sry δ binding site(s) in their promoters. Supporting this conclusion is the close parallel observed between the effect of different *sry* δ mutations on in vitro binding to the *bicoid* promoter and the level of *bicoid* transcription (reference 27 and data not shown).

Several studies of transcription factors containing multiple Cys_2/His_2 zinc fingers have suggested that not all zinc finger motifs present in a given protein may have equivalent roles (for

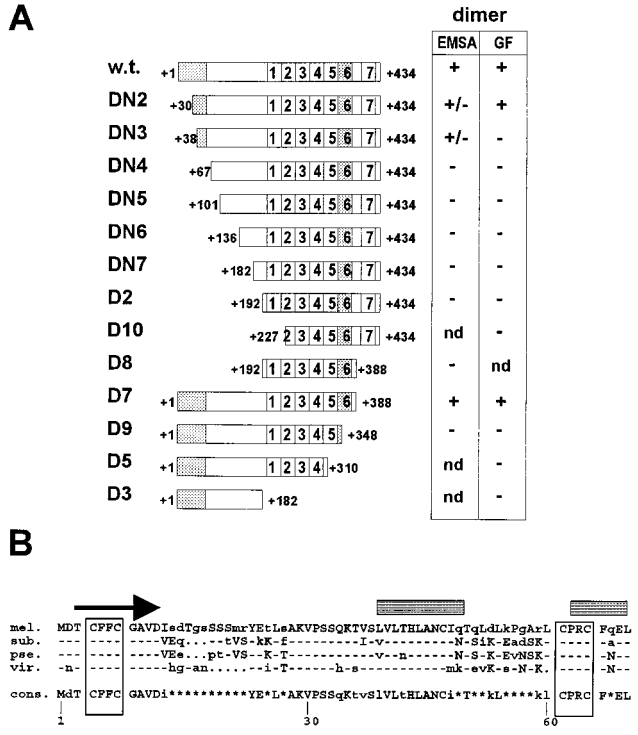

FIG. 9. Two separate protein regions, containing different types of zinc finger, are required for sry δ dimer formation. (A) Schematic representation of the sry δ N-terminally and C-terminally truncated proteins and summary of results obtained for dimer formation in DNA binding (EMSA) and gel filtration chromatography (GF) analyses. The two regions of the protein responsible for dimer formation are shaded. nd, not done. (B) Structure and evolutionary conservation of a putative zinc coordination motif in the N-terminal region of sry δ. Shown are sequence alignments (clustalV) of the first 70 aa of sry δ required for dimerization. Sequences are from *D. melanogaster* (mel.), *Drosophila subobscura* (sub.), *Drosophila pseudoobscura* (pse.), and *D. virilis* (vir.). Residues identical to *D. melanogaster* sry δ are represented by dashes, gaps are represented as dots, and residues retrieved in at least two sequences are shown in uppercase. The consensus sequence (cons.) is below. A predicted strand (SSCD, SSPRED, NNPREDICT, and PHD software programs gave similar results) is shown by an arrow, helices are shown as gray boxes, and the two Cys-X_2-Cys motifs are boxed. Cysteine 7 is replaced by a tyrosine in the *sry δ14* mutation.

an example, see reference 31). The isolation from genetic screens of three independent *sry* δ mutations located in the same (third) zinc finger revealed the critical importance of this specific finger for DNA binding (6). One mutation, δ*SF2*, changes a residue at a position thought to make nucleotide contacts (position −1). Yet, δ*SF2* is a weak mutation in terms of both DNA binding affinity and severity of the homozygous mutant phenotype. By contrast, the δ*SF1* mutation, which changes a residue (E to K) in the linker region between fingers 2 and 3, drastically affects DNA binding. The fact that a similar mutation has no effect when introduced in the first finger link of TFIIIA (3) suggests that the overall protein context determines the relative function of fingers and finger links. The δ*12* mutation, which substitutes M for I at the first position of the histidine helix (14), is of specific interest as no structural interpretation is so far available. First, no example of base or phosphate contact for a residue at this position has ever been reported (9, 25, 26). Second, it seems unlikely that an isoleucine would induce a structural change incompatible with the overall finger structure, as isoleucine is frequently found at this position (for example, in two of the three Zif268 fingers).

The δ*14* mutation is a very interesting case as it is located outside the DNA binding domain and is the strongest *sry* δ allele isolated to date. We have now shown that the strength of this mutation is linked to the key role of the binding cooperativity in sry δ function. While binding of sry δ to a single site results in transcriptional activation of a proximal promoter, the presence of multiple sites strongly synergizes both binding efficiency and transactivation. This cooperative binding on multiple sites explains why inactivation of only one of the two sry δ binding sites in the *bicoid* promoter is sufficient to impair *bicoid* transcription (27). The crucial role of the multimerization of binding sites in the control of tissue-specific gene expression by widely expressed transcription factors may be of a general nature. This is exemplified by the case of inherited hypercholesterolemia in humans, due to a single base substitution in one of the three functional Sp1 sites of the low-density lipoprotein receptor, leading also to a silent promoter (17).

The cooperativity of sry δ binding to multiple sites results from the ability of the purified sry δ protein to bind to itself, in vitro, in the absence of DNA. DNA binding and gel filtration analysis of wild-type and truncated proteins further shows that sry δ dimerizes in solution and binds to DNA as a preformed dimer. This property of sry δ raises the possibility that sry δ is capable of bringing together *cis* regulatory elements located at a distance from each other.

Two regions of sry δ contribute to the dimerization of the protein. First, whereas largely dispensable for DNA recognition and binding, the sixth Cys_2/His_2 zinc finger of sry δ is strictly required for protein contact. Second, an evolutionarily conserved N-terminal region, delimited by two pairs of cysteines (Fig. 9), is also required for dimerization. The functional importance of the cysteine residues of this motif is demonstrated by the strength of the δ*14* point mutation. Our current hypothesis is that this N-terminal region is able to fold into a Cys_2/Cys_2 zinc-finger-like motif that plays a key role in homodimerization. The evolutionary conservation of several hydrophobic residues located between the two cysteine pairs is consistent with a role for this motif in mediating protein contacts. A similar N-terminal Cys_2/Cys_2 motif is found in the sry β paralog, and despite sequence divergence (36% similarity), the N-terminal regions of sry β and sry δ can partially functionally substitute for each other (data not shown). However, the specific comportment of the D14 protein in both DNA binding and gel filtration assays was never mimicked by any tested N-terminal deletion, suggesting that the effect of this peculiar mutation is to strongly deregulate protein contacts rather than to abolish dimerization.

Several types of zinc coordination motifs involved in specific protein contacts have been described for transcription factors. This includes the Cys_2/Cys_2 motif from the nuclear hormone receptor superfamily (reviewed in reference 11) and the $His-Cys/Cys_2$ motif recently identified in EBF/OLF1 (13). These motifs are, however, not separable from the DNA binding domain, unlike in the case of sry δ. The sry δ Cys_2/Cys_2 motif appears to be more closely related to the LIM domain (composed of four cysteine, or cysteine/histidine, doublets), since the LIM motif is both functionally and evolutionarily separated from DNA binding domains (33). Nevertheless, a striking characteristic of the Cys_2/Cys_2 motif of sry δ differentiates its role from both that of the LIM motif (33) and those of other protein motifs involved in homodimerization of zinc finger proteins (2, 24, 34); while required, the sry δ Cys_2/Cys_2 region is not sufficient by itself for dimerization, as this requires also the presence of the Cys_2/His_2 zinc finger 6, from the DNA binding domain. We therefore postulate that interaction of these two different types of zinc fingers confers on sry δ its cooperative DNA binding properties and the resulting synergism in transcriptional activation. Experiments are currently under way to better define the structure-function relationship of this new type of Cys_2/Cys_2 motif and to get further insight into a novel type of dialogue between different types of zinc fingers.

ACKNOWLEDGMENTS

We thank P. Ferrer and D. Jullien for providing us with the sequence of sry δ from *Drosophila virilis*, P. Dumont for her precious help, and M. J. Guinaudy for excellent technical assistance. We thank David Cribbs, Claude Desplan, and Helene Richard-Foy for discussion and comments on the manuscript. We also thank P. Charnay, in whose laboratory cell transfection experiments were initiated, and C. Vesque for help and advice.

This work was supported by the Centre National de la Recherche Scientifique, l'Institut National de la Recherche Médicale (CRE 93-0112), and the Groupement de Recherches et d'Etudes sur les Génomes.

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