Equilibrium Binding Assays Reveal the Elevated Stoichiometry and Salt Dependence of the Interaction between Full-length Human Sex-determining Region on the Y Chromosome (SRY) and DNA*

Stephanie Baud‡, Emmanuel Margeat‡‡, Serge Lumbroso¶, Françoise Paris¶¶, Charles Sultan¶¶¶, Catherine Royer‡, and Nicolas Poujol‡**

From the ‡Centre de Biochimie Structurale, UMR INSERM 554, CNRS 5048, Université Montpellier I, 29 rue de Narabes, 34090 Montpellier, France, ¶INSERM U439, Pathologie Moléculaire des Récepteurs Nucléaires, 70 rue de Narabes et Laboratoire d'Hormonologie, CHU Montpellier, 34295 Cedex France, and ¶¶Unité Endocrinologie Pédiatrique, Hôpital A. de Villeneuve, CHU Montpellier, 34295 Cedex France

In an effort to better define the molecular mechanism of the functional specificity of human sex-determining region on the Y chromosome (SRY), we have carried out equilibrium binding assays to study the interaction of the full-length bacterial-expressed protein with a DNA response element derived from the CD13 gene enhancer. These assays are based on the observation of the fluorescence anisotropy of a fluorescein moiety covalently bound to the target oligonucleotide. The low anisotropy value due to the fast tumbling of the free oligonucleotide bound to the target oligonucleotide in solution increases substantially upon binding the protein to the labeled target DNA. Our results indicate that the full-length human wild-type SRY (SRYwt) forms a complex of high stoichiometry with its target DNA. Moreover, we have demonstrated a strong salt dependence of both the affinity and specificity of the interaction. We have also addressed the DNA bending properties of full-length human SRYwt in solution by fluorescence resonance energy transfer and revealed that maximal bending is achieved with a protein to DNA ratio significantly higher than the classical 1:1. Oligomerization thus appears, at least in vitro, to be tightly coupled to SRY-DNA interactions. Alteration of protein-protein interactions observed for the mutant protein SRYY122NN, identified in a patient presenting with 46,XY sex reversal, suggests that oligomerization may play an important role in vivo as well.

Sex-determining region on the Y chromosome (SRY)† is the master genetic switch that triggers development of the bipotential gonad into testes in mammalian embryos (1, 2). The protein it encodes is a member of a large family of nuclear proteins harboring a 79-amino acid motif known as a high mobility group (HMG) box (3). HMG box-containing proteins can be classified into two major groups based on the degree of sequence specificity in DNA binding and the number of HMG boxes within a protein. One group includes UBF, HMG-1 and MT-TF1, which have multiple HMG boxes and recognize DNA with low or no specificity. The other group includes transcriptional regulators such as LEF-1 and Sox (SRY-box related) proteins, including SRY, that possess a single HMG box and show sequence-specific DNA binding. Thus, the SRY protein is a DNA-binding protein that recognizes certain AT-rich sequences (4–6) including the consensus binding sequence A/TTAACCT/TA obtained by random site selection (7). Upon binding, human SRYwt induces a 60–83° bend in the DNA helix as demonstrated by circular permutation assays (6, 8, 9), NMR structure (10), and fluorescence resonance energy transfer (11). Both DNA binding and bending capacities were demonstrated as essential for testis development on the basis of the study of the biochemical consequences of SRY mutations (4, 9, 12). To date, 36 SRY mutations have been reported (13) in patients with gonadal dysgenesis/XY sex reversal, and the large majority (33 of 36) of the patients were phenotypically normal 46,XY females with complete gonadal dysgenesis. The strong bending of DNA together with the lack of a potential trans-regulation domain in human SRY has led to the suggestion that the protein may modulate transcription by acting architecturally in the assembly of a nucleoprotein complex (9). However, despite the critical role of SRY in the cascade of gene regulation leading to maleness, the direct targets of SRY remain to be positively identified.

Because the first step in such a cascade is DNA recognition, a thorough, quantitative understanding of the structure-energetic function relations in this system is essential. A number of studies of the interactions between SRY and duplex DNA, all using mobility shift assays, have been published (8, 9, 14, 15). For example, Ferrari et al. (8) examined SRY-DNA interactions, but their study was restricted to a construct containing only the HMG box. Similar studies using wild-type and mutant SRY isolated from complete gonadal dysgenesis have been performed but were still restricted to the SRY HMG box (9). However, domains of SRY distinct from the HMG box have been implicated in the modulation of DNA binding properties (16). Trimmer et al. (15) have reported studies of full-length human SRY and human SRY-HMG box domain interactions with a 20-bp DNA oligonucleotide. Their results suggested that the affinities of both constructs were in the nanomolar range but also pointed out sharp binding transitions and higher order

* This work was supported in part by the CNRS, INSERM, La Fondation pour la Recherche Médicale, L'Association pour la Recherche sur le Cancer, and the Région Languedoc-Roussillon. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a doctoral grant from the French Ministère de l’Education, de la Recherche et de la Technologie and a grant from the Fondation pour la Recherche Médicale, L’Association pour la Recherche sur le Cancer, and the Région Languedoc-Roussillon. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a doctoral grant from the French Ministère de l’Education, de la Recherche et de la Technologie and a grant from the Fondation pour la Recherche Médicale.

** A postdoctoral fellow (INSERM poste d’accueil recherche clinique).

§ Supported by a doctoral grant from the French Ministère de l’Education, de la Recherche et de la Technologie and a grant from the Fondation pour la Recherche Médicale.

¶ Supported by a doctoral grant from the French Ministère de l’Education, de la Recherche et de la Technologie and a grant from the Fondation pour la Recherche Médicale.

¶¶ Supported by a doctoral grant from the French Ministère de l’Education, de la Recherche et de la Technologie and a grant from the Fondation pour la Recherche Médicale.

¶¶¶ Supported by a doctoral grant from the French Ministère de l’Education, de la Recherche et de la Technologie and a grant from the Fondation pour la Recherche Médicale.

† The abbreviations used are: SRY, sex-determining region on the Y chromosome; FRET, fluorescence resonance energy transfer; HMG, high mobility group; HPLC, high pressure liquid chromatography.
complexes with multiples sites on the probes that precluded of a thermodynamic analysis with confidence. The gel mobility shift method provides interesting information concerning the number of stoichiometric complexes formed but suffers from its nonequilibrium nature and the relatively large signal to noise ratio inherent in the titration curves derived from quantification of the bands. The quality of such data is usually insufficient for determination of the presence and degree of cooperativity in binding.

In the present work, we have used a fluorescence-based binding assay to quantitatively characterize the interaction between full-length bacterial-expressed human SRY and its target DNA. Our equilibrium assays are based on the observations of changes in the fluorescence anisotropy of a fluorescein-labeled DNA target upon binding by the protein. Because rotational diffusion of the free oligonucleotide is quite rapid, the anisotropy of the fluorescent dye covalently bound to the oligonucleotide is quite low, i.e., little orientation of the polarized exciting light is retained in the emission. However, because binding by the protein significantly slows the rotational diffusion of the oligonucleotide, much more of the exciting light polarization is retained in the emission. These experiments can be performed with very low concentrations of target DNA and provide data of very high precision and reproducibility (17–19). Thus, they can be used to quantitatively characterize the affinity, cooperativity and, eventually, the kinetics of biomolecular interactions. To gain further insight into the molecular basis for SRY function, we performed binding experiments with a rare SRY mutant, identified in partial rather than complete gonadal dygenesis, that may present more subtle biochemical consequences and thus be more difficult to reveal. Only three SRY mutations have been reported to date with this partial clinical presentation, and all of them were located outside the HMG box (20–22). In contrast, the fourth such mutation (Y129N) examined here is located at the C-terminal end of the HMG box.

We have used this anisotropy-based assay to evaluate the affinity, specificity, and cooperativity of the interaction between the full-length wild-type and mutant human SRY with the consensus target DNA at various salt concentrations. We also addressed the DNA binding properties of both full-length human SRYs in solution by fluorescence resonance energy transfer (FRET).

**EXPERIMENTAL PROCEDURES**

**Construction of the Plasmid pQE30-SRY**—His-tagged proteins were overexpressed in *Escherichia coli* and purified by nickel-nitrilotriacetic acid-garos beads (Qiagen, Courtaboeuf, France) under denaturing conditions. DNA encoding for the full-length SRY protein was amplified from genomic DNA extracted from a fertile subject and from the patient presenting with the Y129N substitution. For this purpose, the oligonucleotides SRYs (5′-TACGAGCTCAGTCACTGATGCTTCTG-3′) and SRYas (5′-CTATATAGATTCAGTTGCTGCGG-3′) were purchased from Genosys (Montigny-le-Bretonneux, France). Reaction products and expression plasmid pQE30 (Qiagen) were cleaved with BamHI and HindIII, purified, ligated, and cloned into *E. coli* XL1. The resulting plasmids pQE30-SRYWT and pQE30-SRYY129N were checked by sequencing.

**Protein Expression and Purification**—The plasmids were introduced into *E. coli* S13909-repQ (Qiagen). The cells were grown in LB medium with 100 μg/ml ampicillin and 25 μg/ml neomycin at 37 °C and induced at an A600 of 0.7 with 0.5 mM isopropyl β-D-thiogalactopyranoside. After 2h, the cells were harvested by centrifugation and lysed under denaturing conditions (6 M guanidine hydrochloride, 20 mM Tris-HCl, pH 8.0, 5 mM imidazole, 500 mM NaCl, and 5 mM β-mercaptoethanol). The cell lysates were passed over a nickel-nitrilotriacetic acid-garos column (Qiagen), and the SRY proteins were eluted according to the manufacturer’s recommendations (6 M urea, 20 mM bis-Tris-HCl, pH 5.0, 200 mM imidazole, 500 mM NaCl, and 5 mM β-mercaptoethanol). The unfolded protein was subject to a concentration under nitrogen (Amicon, YM10) and then added dropwise to the renaturing buffer (10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, and 10% glycerol) at 0 °C on ice. The folded proteins were then loaded on a fast protein liquid chromatography Superdex 75 size exclusion column (AKTA prime; Amersham Biosciences). The proteins eluted at the expected monomer molecular mass were homogeneous by Coomasie-Blue staining of a 7% SDS-polyacrylamide gel, and their respective concentrations were calculated by using the extinction coefficient (34640 M⁻¹ cm⁻¹ at 280 nm).

**Oligonucleotides**—Oligonucleotides were purchased in HPLC-purified form from Genent S.A. (Paris, France). The fluorescein and rhodamine X labels were incorporated by the supplier using phosphoramidite chemistry, and all the free probe was thus eliminated in the synthesizer and subsequent HPLC purification. The labeling ratios for the oligonucleotides were 60% and 99% for fluorescein- and rhodamine X-labeled oligonucleotides, respectively. The sense and antisense strands were annealed by heating at 1:1 molar ratio of unlabelled antisense with fluorescein-labeled sense strands to 85 °C for 5 min and slowly cooling them in a thermocycler (Gen-amp 2400; PerkinElmer Life Sciences), resulting in a duplex probe used for the anisotropy assay (FSRBE). A double-labeled duplex probe (FSRBE-R) intended for FRET was similarly prepared, except that the antisense strand was rhodamine X-labeled. The 23-bp probe referred to here as SRSBE has the sequence given below for the sense strand: 5′-CCCTGCAAGTAAACATCGCTGGCT-3′.

**Anisotropy Assays**—Binding assays were performed using a Beacon 2000 polarization instrument (Panvera Corp., Madison, WI) regulated at 4 °C. Each point in the titration curves was obtained by starting with 200 μl of a concentrated solution of SRY and 5 mM F-SRBE. Aliquots of 40 μl were successively removed from the starter solution and replaced by 40 μl containing 5 mM F-SRBE. The buffer solution was 10 mM Tris-HCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, pH 7.5 (TEGD buffer) and contained the indicated concentration of KCl. Tubes were equilibrated at 4 °C for 5 min before measurement, and the anisotropy was measured successively until stabilized. The reporting values are the average of five to seven measurements after stabilization. Anisotropy is calculated as the ratio of the difference between vertical and horizontal emission intensities (I⊥ and I∥) normalized to the total intensity: A = (I∥ − I⊥)/(I∥ + 2I⊥).

**DNA Bending by FRET**—Time-resolved fluorescence experiments were performed in the frequency domain using ISS frequency-domain acquisition electronics (ISS Inc., Champaign, IL). The excitation light was at 450 nm from the frequency doubled mode-locked output of a Spectra Physics Tauanna Titanium-Saphir laser excited with the light of a Milenna X diode-pumped laser. Pulse width was 2 ps at 4 MHz, and the frequency response was measured at harmonic frequencies from 4 to 200 MHz. Emission was measured at 530 nm with a bandpass filter. For each point, we used a DNA probe containing a fluorescent donor (fluorescein) at the 5′ end of one strand and an acceptor (rhodamine X) at the 5′ end of the other strand. Labeled DNA concentration was 50 nM. The labeling ratio for acceptor was 99%. The labeling ratio for donor was lower (near 60%), but these unlabeled molecules were invisible when monitoring donor quenching. Bending is detected as enhanced FRET efficiency due to a decrease in end-to-end distance. FRET efficiency was calculated from donor intensity in the absence and presence of acceptor (I0D and IAD) as E = (I0D − IAD)/I0D and A-D distances (R) were calculated from E using a Ro value for this D-A pair of 55 Å, E = Ro(5Ro + R6) where Ro is the characteristic transfer distance. Fits of the frequency response curves in terms of an energy transfer model with a Gaussian distance distribution were carried out using the GlobalS Unlimited Program (Laboratory for Fluorescence Dynamics, Urbana, IL).

**RESULTS**

**Purification of Full-length Human SRY**—The His-tagged full-length human SRYWT and SRYY129N proteins were overexpressed in *E. coli* and purified by nickel chelate affinity chromatography under denaturing conditions, followed by renaturation and size exclusion chromatography as described under “Experimental Procedures.” In comparison with molecular mass standards and other proteins prepared in the laboratory, SRYWT eluted as a peak centered at a molecular mass of 22–25 kDa. The molecular mass calculated from the sequence of the gene expressed in *E. coli* is 25.7 kDa. It therefore appears that full-length SRY is a monomer in solution in the 1–10 μM concentration range under our purification conditions. The resulting protein is homogeneous by Coomasie Blue staining of a
SRY<sub>WT</sub> and SRY<sub>Y129N</sub> DNA Interactions

Unlabeled SRBE at a concentration of 45 nM was added to 5 nM labeled F-SRBE (total concentration, 50 nM) and titrated by SRY<sub>WT</sub> in TEGD buffer and 50 mM KCl at 4 °C. The anisotropy value is observed to continue to increase well beyond a 1:1 SRY<sub>WT</sub>/SRBE ratio.

Full-length SRY<sub>WT</sub> Binds Specifically to a Fluorescent Oligonucleotide—The target oligonucleotide used was 23 bp in length and derived from the CD<sub>3</sub> gene enhancer, except that it bears the sequence TAACAATG, which allows for 2-fold better binding of SRY HMG box (9). Fig. 1a shows a representative (one of four) anisotropy-based binding isotherm of the 5'-fluorescein-labeled SRY-responsive binding element (F-SRBE) at 2 nM with purified full-length SRY<sub>WT</sub> in TEGD buffer (●). a, stoichiometric titration of SRBE by SRY<sub>WT</sub>. The target oligonucleotide used was 23 bp in length and derived from the CD<sub>3</sub> gene enhancer—specifically, the CD3<sub>b</sub> receptor heterodimer and is devoid of any specific binding site for SRY. As observed in Fig. 2a, an 8- and a 40-fold excess of nonspecific DNA oligonucleotide concentration is 5-fold greater than the apparent binding midpoint in Fig. 1a, thus assuring stoichiometric binding conditions.

Stoichiometric Titration—Unlabeled SRBE at a concentration of 45 nM was added to 5 nM labeled F-SRBE (total SRBE, 50 nM) and titrated by SRY<sub>WT</sub>. At 50 nM SRBE, the oligonucleotide concentration is 5-fold greater than the apparent binding midpoint in Fig. 1a, thus assuring stoichiometric binding conditions. The latter is a 37-bp double-stranded DNA used in the laboratory that bears recognition sequences for the retinoid X receptor-retinoic acid receptor heterodimer and is devoid of any specific binding site for SRY. As observed in Fig. 2a, an 8- and a 40-fold excess of unlabeled SRBE induced shifts in the titration curves to higher concentrations for the C<sub>50</sub> (70 and 200 nM, respectively) in the presence of 50 mM KCl. These results (7- and 40-fold displacement of the C<sub>50</sub> by an 8- and a 40-fold excess of unlabeled
specific DNA, respectively) clearly demonstrate the equilibrium status of the interaction between SRY<sub>WT</sub> and its target DNA under our experimental conditions.

Surprisingly, in very similar experiments, a 10-fold excess of unlabeled nonspecific DR5 duplex DNA (Fig. 2c) resulted in a large shift to a higher concentration of anisotropy increase (C<sub>iso</sub> = 200 nM), revealing a relatively low specificity of the interaction between SRY<sub>WT</sub> and its target DNA under these low salt conditions. The specificity of the SRY<sub>WT</sub> binding to F-SRBE was next examined in the fluorescence anisotropy assays by using incremental increases in the buffer salt concentration. Increasing salt concentration usually reduces nonspecific protein-DNA affinity more strongly than specific affinity by competing for interaction with the negatively charged phosphate backbone (18, 23, 24). Increasing salt concentration in the absence of competitor DNA (Fig. 2b) yielded profiles exhibiting the same saturating plateau, consistent with a complex of identical stoichiometry, but the apparent affinities decreased. At 50 mM KCl, for example, the C<sub>iso</sub> is 12 nM, whereas at 150 mM KCl, it is ~40 and ~400 nM at 250 mM KCl. As can be seen in Fig. 2b, we note a loss of cooperativity at higher salt concentrations that may arise from a salt effect on protein-protein affinity. A striking effect of increasing the salt concentration on the complex specificity can be seen in Fig. 2c. Closed triangles correspond to the binding of SRY<sub>WT</sub> to 5 nM F-SRBE in the presence of 250 mM KCl, and the open triangles correspond to the same profile in the presence of 50 nM (a 10-fold molar excess) unlabeled nonspecific target DR5. At this salt concentration, we did not observe the large shift of the binding profile to higher concentration observed with DR5 at 50 mM KCl (closed and open squares). Thus, although SRY<sub>WT</sub> binds to its target DNA with low specificity at low salt concentrations, presumably due to substantial electrostatic contacts between the positively charged SRY<sub>WT</sub> and negatively charged DNA, the interaction is of lower overall affinity but becomes much more specific at higher salt concentration.

**DNA Bending by FRET**—Protein-directed DNA bending is proposed to facilitate the assembly of DNA-multiprotein preinitiation complexes giving rise to architectural gene regulation. Such behavior is believed to be a crucial property of SRY (9). Therefore, the complex we observed should bend the target DNA. We have evaluated full-length SRY<sub>WT</sub>-induced DNA bending by FRET. This technique employs the SRBE probe containing a fluorescent donor (fluorescein) at the 5' end of the sense strand and an acceptor (rhodamine X) at the 5' end of the antisense strand. In the absence of protein, for this oligonucleotide, the distance separating the donor-acceptor pair is ~88 Å. Bending is detected as enhanced FRET efficiency due to decreased end-to-end distance. We have analyzed DNA bending by FRET using increasing amounts of full-length SRY<sub>WT</sub>, and thus we have established a titration profile of the DNA bending property. Because the saturating anisotropy plateau was identical at all salt concentrations tested, the stoichiometry and nature of the complex are assumed to be similar. We thus evaluated the DNA bending properties of SRY<sub>WT</sub> at the salt concentration (50 mM KCl) that allowed complete saturation of 50 nM SRBE by SRY<sub>WT</sub> compatible with our available concentrations of protein. Increasing concentrations of SRY<sub>WT</sub> ranging from 0 to 800 nM (Fig. 3a) resulted in a decreased intensity of the donor emission (excited at 450 nm), indicating that FRET occurred upon formation of the SRY<sub>WT</sub>-SRBE complex. We ascribe this result to protein-induced bending of target DNA.

To verify the energy transfer, lifetime measurements on the donor fluorescence were carried out at several concentrations of SRY<sub>WT</sub>. Binding of SRY<sub>WT</sub> to the double-labeled F-SRBE-R caused marked changes to the donor fluorescence lifetime (Fig. 3a). In the free DNA, the mean lifetime of the donor in absence of acceptor (F-SRBE) was 4.2 ns and was unchanged upon protein binding. The presence of the acceptor (F-SRBE-R) did not lead to a reduction in the donor’s mean lifetime for the free DNA, consistent with the distance of separation in the linear oligonucleotide. The addition of increasing amounts of SRY<sub>WT</sub>
to the double-labeled target led to complex decay and a progressive shortening of the amplitude-weighted average lifetime (3.25 and 2.44 ns, respectively, at 50 and 100 nM SRYWT) before reaching a plateau (0.90 ns for 200 nM SRYWT; Fig. 4b). The fluorescence mean lifetime values were used to calculate FRET efficiency (E) and the distance R between the dyes (Table 1). It can be seen from Figs. 3 and 4 that for a 1:1 stoichiometry (50 nM both F-SRBE-R and SRYWT), very little FRET occurs, and thus maximal DNA bending was not achieved. Saturation of the FRET signal and thus the protein-induced DNA bending occur near a ratio of 4 SRY/DNA, indicative of the existence of a multiprotein complex on DNA. It is noteworthy that the FRET experiment also revealed DNA bending at elevated salt concentration, as seen in Fig. 3b, but according to the loss of affinity observed in the presence of 250 mM KCl, the titration was not complete, and the maximal DNA bending could not be achieved at high salt concentration.

We also analyzed the lifetime data at saturating protein concentrations in terms of decay parameters that included FRET. Given the nonhomogeneous character of the decay in the presence of protein evident in the raw data (Fig. 4a), analysis in terms of a unique distance, R, between the probes was not possible. In contrast, analysis in terms of a distance distribution yielded a good fit to the data and a broad distribution centered at 40 ± 25 Å. This is indicative of structural and/or dynamic heterogeneity in the SRYWT-DNA complex.

**DNA Binding and Bending Characteristics of Mutated SRYY129N—**To investigate structural and energetic features important in the function of SRY, we performed the same experiments with an SRY mutant (Y129N) identified in a rare case of partial gonadal dysgenesis. The anisotropy profile for SRYY129N binding to F-SRBE at 50 mM KCl is shown in Fig. 5a (Δ). Notably, the same large increase in the anisotropy values from the initial to the saturating plateau as observed with the SRYWT is consistent with an identical elevated stoichiometry of the SRYY129N/F-SRBE complex. We also noticed that increasing salt concentration had a stronger effect on the binding of

---

**Table I**

| Protein | Concentration (nM) | R (Å) | Width (Å) | R (Å) |
|---------|--------------------|-------|-----------|-------|
| SRYWT   | 800                | 40    | 51        | 43    |
| SRYY129N| 3000               | 52    | 49        | 43    |

*Parameters recovered from the analysis of the lifetime data using a model that included the average distance between donor and acceptor and the width of a Gaussian distribution. Calculated from the energy transfer efficiency, E, using the values of the amplitude-weighted average lifetimes obtained from fits of the data to a two-component discrete model, where E = 1 - (r_D/R_D + R^2).

---

**Fig. 4.** FRET is confirmed by time-resolved fluorescence. a, frequency response profiles of 50 nM F-SRBE-R at 50 mM KCl in the absence of SRY (•, phase; △, modulation), in the presence of 800 nM SRYWT (○, phase; △, modulation), and in the presence of 3000 nM SRYY129N (▲, phase; ○, modulation). b, average (amplitude-weighted) fluorescence lifetime obtained from triple exponential fit of the frequency response profiles for F-SRBE-R in the presence of increasing concentrations of SRYWT (■) and SRYY129N (▲).

**Fig. 5.** Full-length SRYY129N interacts with F-SRBE-R. a, salt dependence of the SRYY129N-DNA interaction. Profiles for titrations of F-SRBE obtained in the presence of 50 (■), 100 (▲), 150 (○), 200 (●), and 250 mM KCl (■) are shown. The profiles were obtained in TEGD buffer at 4 °C, and the DNA concentration was 5 nM. b, profiles for titrations of 5 nM F-SRBE by SRYWT (○) and SRYY129N (■) in the presence of 50 mM KCl are shown. c, profiles for titrations of 5 nM F-SRBE by SRYWT (■) and SRYY129N (▲) in the presence of 100 mM KCl are shown.
the emission spectra of a representative titration experiment. It is clear from this large decrease in donor fluorescence that FRET occurred. Analysis of the lifetime data (Fig. 4a) in the presence of saturating mutant protein in terms of a distributed D-A distance again yielded a broad distribution with a slightly larger mean value than that seen for SRYWT (Table I). Consistent with a lower overall affinity for the mutant protein, the FRET titration was shifted to a higher protein concentration (Fig. 4b). These results indicate that although the affinity and cooperativity are lower for the mutant protein than for SRYWT, the mutant protein, when bound, induces similar structural changes in DNA.

**DISCUSSION**

We have used fluorescence anisotropy to examine the interaction of full-length human SRY and its target DNA under equilibrium binding conditions. Our results indicate that full-length human SRY forms a complex of high stoichiometry (6 or 7) with its target DNA. Moreover, we have demonstrated a strong salt dependence on both the affinity and specificity of the interaction. At 50 mM KCl, the apparent affinity, as judged by the concentration of half saturation of full-length human SRYWT for its target DNA, is comparable with that determined by electrophoretic mobility shift analysis under 60 mM NaCl by Trimmer et al. (15), namely, in the nanomolar range. Nevertheless, at this salt concentration, the interaction exhibits a low degree of specificity. Increasing the salt concentration improves the specificity of the interaction, and under buffer conditions near physiological salt concentrations, SRYWT exhibited a reasonable discrimination between its potential target DNA and a nonspecific oligonucleotide. We note that the increase in specificity is accompanied by a loss of both cooperativity and affinity. Thus, under near physiological salt concentrations, the affinity of SRYWT for its target DNA seems to be closer to the micromolar range rather than the nanomolar range.

The influence of the salt concentration on the specificity of the SRYWT-DNA interaction is similar to that observed for the interaction between the estrogen receptor and its target DNA (18, 24), but one main difference should be noted. Whereas salt concentration affects the affinity, specificity, and stoichiometry of estrogen receptor-DNA interactions, the stoichiometry of SRYWT-DNA complex appears to remain unchanged over the range of salt concentrations tested because anisotropy plateau values remain constant. We evaluated the stoichiometry of the SRYWT-DNA complex to be far from 1:1, rather 6:1 or 7:1, but the lack of accuracy of the stoichiometric titration does not allow its precise determination. The unknown stoichiometry precludes us from performing a complete thermodynamic analysis. In fact, the characterization of the stoichiometry of biomolecular complexes can be one of the most difficult tasks in advancing our understanding of these interactions. A number of techniques are available, but each presents its own advantages and disadvantages. Analytical ultracentrifugation would require availability and micromolar concentrations of protein, and several hours would be required for equilibrium measurements. The limited stability of the protein precludes such an approach. Moreover, resolving the difference between complexes of 6:1 versus 7:1 stoichiometries with this method would not necessarily prove to be trivial, given the one-third power dependence of the translational diffusion coefficient on molecular mass. Size exclusion chromatography would require a long column and thus unreasonably large amounts of proteins in order to achieve, if possible, sufficient resolution. We have previously used fluorescence correlation spectroscopy with photon counting histogram analysis to perform a direct measure of the stoichiometry of the estrogen receptor-co-activator complex (19). In the case of the interaction of SRYWT with DNA, fluorescence correlation spectroscopy is not appropriate because the relatively low affinity of the complex would require over 200 nM labeled SRYWT for 100% complexation, and this is outside the single molecule limit. Moreover, the difference between 6 and 7 molecules/DNA should only yield a 15% variation in the molecular brightness, which is probably difficult to discriminate with confidence, particularly outside the single molecule limit.

Higher order SRBE-SRYWT complexes have been reported previously using electrophoretic mobility shift analysis by several authors (9, 11, 15, 25). In electrophoretic mobility shift analysis, in addition to the classical 1:1 complex, others complexes of lower mobility or no mobility could be observed. In almost all cases, these complexes were not taken into account for the determination of the affinity by the authors and were assumed to correspond to nonspecific complexes (9, 11, 15). Nevertheless, Teo et al. (25) also demonstrated the tendency of HMG boxes of SRY and other members of the high mobility group protein to oligomerize when bound to DNA. Furthermore, the addition of N- and C-terminal extensions to the high mobility group protein to oligomerize when bound to DNA. Thus, oligomerization appears to be a common feature of HMG box-containing proteins and could be directly related to the function. Indeed, our FRET evaluation of the DNA binding properties of full-length SRYWT reveals a higher FRET efficiency than observed for the HMG box-DNA complex, consistent with an increased bend angle as compared with the literature, including the one determined by a very similar approach (11). Such a discrepancy could be due in part to the structural differences between complexes of truncated and full-length proteins because the addition of a C-terminal extension to the HMG box of LEF-1 increases the bend angle (26). However, it could also be related to the stoichiometry of the complex. Indeed, our titration of DNA bending by FRET revealed that maximal bending was achieved with a protein to DNA ratio significantly higher than 1:1, whereas the previously reported bend angles were determined using the classical 1:1 ratio, despite the existence of a lower mobility complex in the permutation gel experiment (9). Only Lneniek-Allen et al. (26) noticed that the second shifted complex of LEF-1 HMG box exhibited an increased bend, from 57° to 125°.

The physiological relevance of such an oligomerization on DNA remains to be clarified, but it is noteworthy that the differences observed between SRYWT and the Y129N mutant concerned mainly the degree of binding cooperativity and the magnitude of salt-induced shifts. It is therefore likely that the differences in salt effects arise from differences in the salt dependence of protein-protein interactions rather than differences in protein-DNA binding. This and the decreased binding cooperativity of the mutant point to a defect in the protein-protein interactions induced by the mutation.

From a structural viewpoint, the consequences of the substitution are difficult to rationalize because the structure of full-length SRYWT remains to be determined; only the structure of monomeric SRY-HMG in complex with DNA is available. Basing our interpretation on this structure, the point mutation Y129N, due to the C-terminal location of the Y129, would not affect the packing of residues within the protein core that would be expected to destabilize the protein. Indeed, our mutant protein is stable, and its DNA binding and bending properties, at least under low salt concentration conditions, indicate that it is active.
The modified protein-protein interaction induced by the substitution strongly suggests the implication of Y129 in protein association, although the effect may be indirect because Y129, in the NMR structure, appears to be orthogonally oriented and in direct contact with the DNA bases. Its proper location could be of crucial importance for the correct orientation and positioning of the remaining C-terminal region of SRY, which in turn could potentiate protein-protein association. Moreover, Y129 is the last ordered residue observed in the NMR structure, likely reflecting the need for further stabilization of the C-terminal region of SRY.

Although the tendency of full-length human SRY to oligomerize will require in vivo confirmation, one should bear in mind that, in many cases, formation of higher order oligomeric structures contributes crucially to protein functionality and regulation (27). Single mutations in the tetramerization domain of the tumor suppressor p53, for example, can inactivate the protein in a manner similar to that seen with mutations in the DNA-binding domain (for review, see Ref. 28). Oligomerization could also result in transcriptional silencing such as that observed for TEL, a frequent target of chromosomal translocations, which oligomerization mediates the spreading of transcriptional repression complexes along the chromosome (29). Because human SRY appears devoid of any transcriptional activation domain, and no gene positively regulated by SRY has yet been identified, active regulation of transcription by SRY remains a matter of debate. A repressive mechanism, similar to the yeast HMG box-containing hypoxic repressor Rox1 (30), cannot be excluded. This would perfectly match the hypothesis drawn by Fellous and co-workers (31, 32), who proposed that SRY acts as a repressor of the expression or activity of an autosomal recessive or X-linked locus termed Z that acts as a negative regulator of the male determining pathway.

We have demonstrated here that 1) full-length human SRY binds to an oligomeric complex to its target DNA and induces a strong bend, 2) SRY protein-protein interactions are linked energetically to protein-DNA interactions, 3) increasing salt concentration leads to a decrease in DNA binding affinity and cooperativity linked to a loss of protein-protein interaction, and 4) a functional mutant of SRY resulting in a sex reversal phenotype exhibits similar stoichiometry and DNA bending characteristics but impaired protein-protein cooperativity in DNA binding.

Thus, SRY function in vivo may be significantly more complex than a simple interaction of its HMG box with target DNA. It is possible that the overall architecture of these higher order complexes underlies the fundamental mechanism of SRY action in sexual differentiation.

REFERENCES

1. Sinclair, A. H., Berta, P., Palmer, M. S., Hawkins, J. R., Griffiths, B. L., Smith, M. J., Foster, J. W., Frischau, A. M., Lovell-Badge, R., and Goodfellow, P. N. (1990) Nature 346, 240–244
2. Berta, P., Hawkins, J. R., Sinclair, A. H., Taylor, A., Griffiths, B. L., Goodfellow, P. N., and Fellous, M. (1990) Nature 348, 448–450
3. Baxevanis, A. D., and Landsman, D. (1995) Nucleic Acids Res. 23, 1604–1613
4. Harley, V. R., Jackson, D. J., Hextall, P. J., Hawkins, J. R., Berkóvits, G. D., Sockanathan, S., Lovell-Badge, R., and Goodfellow, P. N. (1992) Science 235, 453–456
5. Haqq, C. M., King, C. Y., Donahoe, P. K., and Weiss, M. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1097–1101
6. Giese, K., Pagel, J., and Grosschedl, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3368–3372
7. Harley, V. R., and Goodfellow, P. N. (1994) Mol. Reprod. Dev. 39, 184–193
8. Ferrari, S., Harley, V. R., Punizzia, A., Goodfellow, P. N., Lovell-Badge, R., and Bianchi, M. E. (1992) EMBO J. 11, 4497–4506
9. Pontiggia, A., Rimini, R., Harley, V. R., Goodfellow, P. N., Lovell-Badge, R., and Bianchi, M. E. (1994) EMBO J. 13, 6115–6124
10. Werner, M. H., Huth, J. R., Groenborn, A. M., and Clore, G. M. (1995) Cell 81, 705–714
11. Ukiyama, E., Jancso-Radek, A., Li, B., Milos, L., Zhang, W., Phillips, N. B., Morikawa, N., King, C. Y., Chan, G., Haqq, C. M., Radek, J. T., Poulat, F., Donahoe, P. K., and Weiss, M. A. (2001) Mol. Endocrinol. 15, 363–377
12. Nasrin, N., Buggs, C., Kong, X. F., Carnazza, J., Goebl, M., and Alexander-Bridges, M. (1991) Nature 349, 317–320
13. Schaffler, A., Barth, N., Winkler, K., Zietz, B., Rummel, P., Knuechel, R., Scholmerich, J., and Palitzch, K. D. (2000) J. Clin. Endocrinol. Metab. 85, 2287–2292
14. Peters, R., King, C. Y., Ukiyama, E., Falsafi, S., Donahoe, P. K., and Weiss, M. A. (1995) Biochemistry 34, 4569–4576
15. Trimmer, E. E., Zambole, D. B., Lippard, S. J., and Essigmann, J. M. (1998) Biochemistry 37, 352–362
16. Descolezeaux, M., Poulat, F., de Santa Barbara, P., Soulillier, S., Jay, P., Berta, P., and Boizet-Bonhoure, B. (1998) Biochim. Biophys. Acta 1397, 247–252
17. Grillo, A. O., Brown, M. P., and Royer, C. A. (1999) J. Mol. Biol. 287, 538–554
18. Boyer, M., Poujol, N., Margeat, E., and Royer, C. A. (2000) Nucleic Acids Res. 28, 2494–2502
19. Margeat, E., Poujol, N., Boulahtouf, A., Chen, Y., Muller, J. D., Gratton, E., Cavailles, V., and Royer, C. A. (2001) J. Mol. Biol. 306, 433–442
20. Brown, S., Yu, C., Lanzano, P., Heller, D., Thomas, L., Warburton, D., Kitajewski, J., and Stadtmauer, L. (1998) Am. J. Hum. Genet. 62, 189–192
21. Domenice, S., Yamie Nishi, M., Correa Billerbeck, E. A., Latronico, A. C., Aparecida Medeiros, M., Russell, A. J., Vass, K., Marino Carvalho, F., Costa Frade, E. M., Prado Arnhold, I. J., and Bilharinho Mendonca, B. (1998) Hum. Genet. 102, 213–215
22. McElreavey, K., Vilain, E., Barboux, S., Fuqua, J. S., Fechner, P. Y., Souleyreau, N., Doco-Feny, M., Gabriel, R., Quereux, C., Fellous, M., and Berkovitz, G. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8590–8594
23. Recorid, M. T., Jr., Ha, J. H., and Fisher, M. A. (1991) Methods Enzymol. 208, 291–343
24. Ozeris, M. S., Hill, J. J., Ervin, K., Wood, J. R., Nardulli, A. M., Royer, C. A., and Gorski, J. (1997) J. Biol. Chem. 272, 30405–30411
25. Teo, S. H., Grasser, K. D., and Thomas, J. O. (1995) Eur. J. Biochem. 230, 943–950
26. Lieniek-Allen, M., Read, C. M., and Crane-Robinson, C. (1996) Nucleic Acids Res. 24, 1047–1061
27. Engel, J., and Kammerer, R. A. (2000) Matrix Biol. 19, 283–288
28. Chene, P. (2001) Oncogene 20, 2611–2617
29. Kim, C. A., Phillips, M. L., Kim, W., Gingery, M., Tran, H. H., Robinson, M. A., Fakharn, S., and Bowie, J. C. (2001) EMBO J. 20, 4173–4182
30. Deckert, J., Khalaf, R. A., Huang, S. M., and Zitomer, R., S. (1999) Nucleic Acids Res. 27, 3518–3526
31. McElreavey, K., Vilain, E., Abbas, N., Herskovitz, I., and Fellous, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3368–3372
32. Veitia, R. A., Salas-Cortes, L., Otolenghi, C., Pailhous, E., Cotinot, C., and Fellous, M. (2001) Mol. Cell. Endocrinol. 179, 1–16
Equilibrium Binding Assays Reveal the Elevated Stoichiometry and Salt Dependence of the Interaction between Full-length Human Sex-determining Region on the Y Chromosome (SRY) and DNA
Stephanie Baud, Emmanuel Margeat, Serge Lumbroso, Françoise Paris, Charles Sultan, Catherine Royer and Nicolas Poujol

J. Biol. Chem. 2002, 277:18404-18410.
doi: 10.1074/jbc.M112366200 originally published online March 4, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M112366200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 32 references, 7 of which can be accessed free at http://www.jbc.org/content/277/21/18404.full.html#ref-list-1