Use of Fluorescence Resonance Energy Transfer To Estimate Intramolecular Distances in the Msx-1 Homeodomain†

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ABSTRACT: We have utilized fluorescence resonance energy transfer (FRET) to investigate the spatial proximities of segments in the Msx-1 homeodomain (Msx). This strategy makes use of a single, invariant tryptophan (Trp-48) in helix III as the donor for FRET. The acceptor molecule, 5-[[[(iodoacetyl)amino]-ethyl]amino]naphthalene-1-sulfonic acid (AEDANS), was incorporated into Msx at positions 6, 10, or 27 which are within the N-terminal arm, and helices I and II since these segments have been implicated in interactions with helix III. Specific incorporation of AEDANS was achieved by using a two-step strategy consisting of site-directed mutagenesis for introducing unique cysteine residues at the selected positions followed by covalent modification of these cysteine residues with AEDANS. Using this approach, we demonstrated energy transfer between Trp-48 and the AEDANS-labeled cysteines at positions 6, 10, and 27 and estimated the distances between the Trp-48 and AEDANS pairs to be 19, 23, and 16 Å, respectively. We further demonstrated that FRET provides a strategy for detecting subtle alterations in protein conformation that result from replacement of specific residues in helix III and the N-terminal arm. Together, these findings show that FRET provides a useful approach for estimating intramolecular distances and for examining the conformation of Msx. Moreover, given the fact that Trp-48 is invariant among all homeodomain sequences, we propose that FRET will provide a general approach for facilitating comparative analyses of homeodomain conformations.

The homeodomain is a DNA binding motif commonly found among proteins which regulate gene transcription during development (Gehring, 1987; Scott *et al.*, 1989; Kessel & Gruss, 1990; Dubole, 1994). It has a highly conserved structure that is comprised of three α-helices and a flexible N-terminal arm (Figure 1). The homeodomain is composed of sixty amino acids, several of which are invariant and comprise a consensus sequence (Figure 2A). Despite its prevalence and extensive conservation, the homeodomain serves as a primary determinant of functional specificity among homeodomain-containing proteins *in vivo* (Kuziora & McGinnis, 1989; Gibson *et al.*, 1990: Mann & Hogness, 1990; Lin & McGinnis, 1992; Zeng *et al.*, 1993). This paradox suggests that certain features of the homeodomain contribute to specificity, and these are likely to include residues other than those which contribute to the consensus sequence (e.g., Figure 1). We have been studying the contribution of nonconsensus residues toward functional specificity by using the Msx-1 homeodomain (Msx) (Catron *et al.*, 1993; Shang *et al.*, 1994a,b; Ebu Isaac *et al.*, 1995). We have shown that the DNA binding properties of Msx and the Hox family, another class of homeodomains, can be distinguished by nonconsensus residues in the N-terminal arm and helix III (Ebu Isaac *et al.*, 1995). These residues influence both DNA binding specificity and affinity (Ebu Isaac *et al., 1995*).

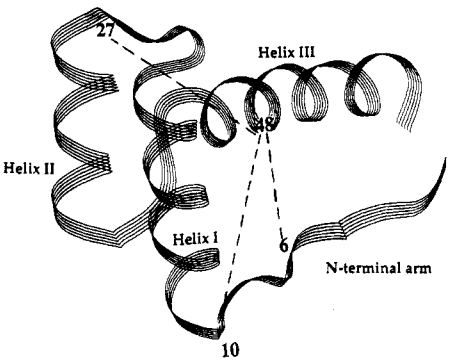

FIGURE 1: Structure of Msx. The computer homology model of Msx has been previously described (Ebu Isaac *et al.*, 1995). Shown here is a ribbon representation illustrating the positions of helices I, II, and III and the N-terminal arm. Also shown are Trp-48 and positions 6, 10, and 27 which were mutated individually to cysteine residues and subsequently modified with AEDANS.

Fluorescence resonance energy transfer (FRET) provides a useful tool for detecting structural changes and for determining the relative distances of chromophores within proteins (Forster, 1948; Stryer & Haugland, 1967). This strategy requires two chromophore moieties, one that serves as the donor and another that serves as the acceptor, and also requires that their emission and absorption spectra overlap. When these are located in close proximity, excitation of the donor results in nonradiative transfer of the quanta to the acceptor, and this is apparent as a reduction in the

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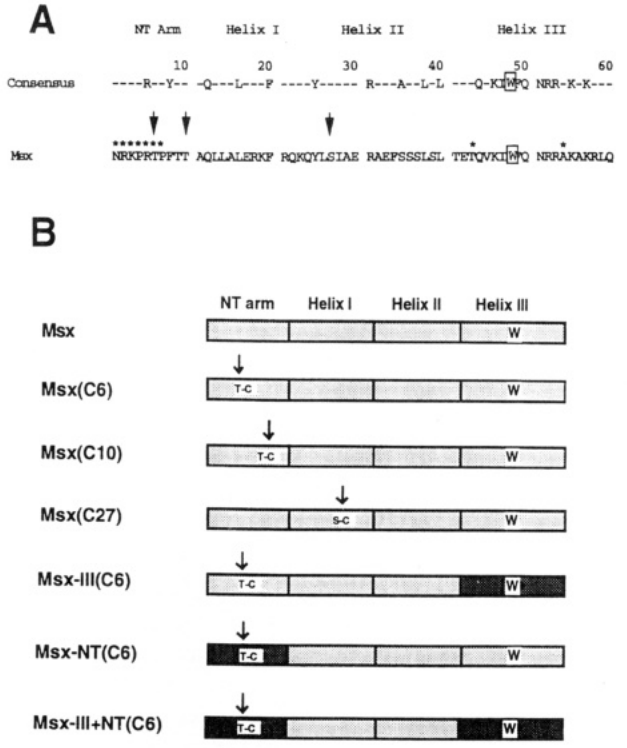

FIGURE 2: Sequence comparison and design of cysteine-substituted Msx polypeptides. (A) The sequence of Msx is shown in comparison with the consensus sequence described by Scott *et al.* (1989), using the single letter amino acid code. Trp-48 (W) is boxed; the arrows show positions 6, 10, and 27 which were substituted with cysteine; the *'s show the residues substituted in Msx(NT) and Msx(III) as in Ebu Isaac *et al.* (1995). The homeodomain residues are designated numerically (residues 10−60), and the locations of the N-terminal arm (NT Arm) and helices I, II, and III are shown. (B) Cysteine substitutions at positions 6, 10, and 27 are shown by the arrows, and Trp-48 is shown by W. The dark hatched boxes indicate amino acid replacements in the NT arm and in helix III [as in Ebu Isaac *et al.* (1995)].

donor emission spectra (i.e., donor quenching). FRET has been used extensively to investigate various intra- and intermolecular interactions in proteins, such as the compactness of a constant fragment in the type-K immunoglobulin light chain (Y. Kawata and K. Hamaguchi, 1991), the conformational changes in actin (Miki, 1991), the dimerization of two DNA binding proteins, Fos and Jun (Patel *et. al.*, 1994), and the *in vivo* interaction of two cAMP subunits (Adams *et. al.*, 1991).

In this study, we used FRET to estimate intramolecular distances within Msx and to detect subtle differences in its conformation that result from specific amino acid substitutions in the N-terminal arm and helix III. Since our strategy utilizes a unique and invariant tryptophan residue (Trp-48) as the donor for FRET, we propose that this approach may be of general utility for investigating intramolecular distances in other homeodomains and for making comparisons among these proteins that may not be readily apparent by using other approaches.

EXPERIMENTAL PROCEDURES

Purification of Polypeptides. The bacterial expression plasmid encoding Msx [*pmsx-1* (*157*−*233*)] was described in Catron *et al.* (1993). The plasmid encoding Msx(C10) [*pCys*^{175}*msx-1*(*164*−*233*)] was described in Shang *et al.* (1994a), and those encoding Msx(III) [*pmsx-1* (*157*−*233*) (T209R; A220M)], Msx(NT) [*pmsx-1* (*157*−*233*) (N166S; R167K; K168R; P169G; P172A; F173Y)], and Msx(III+NT) [*pmsx-1* (*157*−*233*) (N166S; R167K; K168R; P169G; P172A; F173Y; T209R; A220M)] were described in Ebu Isaac *et al.* (1995). Unique cysteine residues were introduced into these *msx* plasmids by PCR mutagenesis as described in Ebu Isaac *et al.* (1995) using overlapping oligonucleotides that contained the appropriate nucleotide substitutions. As in Shang *et al.* (1994a), each of the plasmids encoding Msx polypeptides corresponded to residues 164−233 (to exclude a cysteine residue at residue 163). The sequences of the *msx* plasmids were verified by DNA sequence analysis. The mutated Msx polypeptides were produced in *Escherichia coli* as hexahistidine fusion proteins and purified by nickel affinity chromatography as described in Ebu Isaac *et al.* (1995). Protein purity was >90% for each Msx polypeptide as determined by Coomasie blue staining of an SDS (sodium dodecyl sulfate)−polyacrylamide gel [as in Ebu Isaac *et al.* (1995)].

Fluorescence Labeling of Polypeptides. Labeling of Msx proteins that contained unique cysteine residues was performed in the presence of 6 M guanidine hydrochloride adjusted to pH 7.5−8.0. Prior to labeling, the cysteine residues were reduced by incubation with 2 mM dithiothreitol (DTT) for 30 min at 37 °C. AEDANS (5-[[[(iodoacetyl)-amino]ethyl]amino]naphthalene-1-sulfonic acid) (Molecular Probes) was added to a final concentration of 4 mM, and labeling proceeded at room temperature for 2 h, followed by incubation overnight at 4 °C. The excess label was removed by gel filtration on a NAP-10 column (Pharmacia) equilibrated with 6 M guanidine hydrochloride (pH 7.5−8.0). The AEDANS-labeled Msx proteins were renatured by extensive dialysis against phosphate buffer saline (pH 7.0) containing 10% glycerol (v/v) and 1 mM DTT (buffer A). The proteins were chromatographed in buffer A on a NAP-10 gel filtration column to ensure complete removal of excess fluorophor. The stoichiometry of labeling was determined as the concentration of AEDANS (measured by absorbance at 340 nm with an extinction coefficient of 5700 cm^{-1} M^{-1}) relative to the concentration of Msx. Protein concentration was determined by absorption at 280 nm using an extinction coefficient of 7000 cm^{-1} M^{-1}, Biorad protein assay, and relative fluorescence intensity at 340 nm in 6 M guanidine hydrochloride and in buffer A. The stoichiometries of labeling were routinely at (or very close to) 1.0 mol of AEDANS/mole of Msx protein. Only proteins that were labeled stoichiometrically (i.e., 1:1) were used in these analyses.

Potassium Iodide Quenching. Assays were performed essentially as described in Chapman *et al.* (1992). Briefly, tryptophan emission spectra were recorded for Msx proteins (2 μM) in buffer A as a function of increasing potassium iodide (KI) concentration. Samples were excited at 295 nm in the absence of KI or in the presence of KI which was added in 10 mM increments. The emission spectra were corrected for dilution effects, and the area under the peak was integrated from 310 to 410 nm. The values obtained in the absence of KI (F_0) were divided by the values obtained when KI was added (F) (F_0/F) as shown in the Stern−Volmer plot (Figure 3).

Fluorescence Measurement and Spectroscopy Instrumentation. Fluorescence measurements were made using an Aminco SLM 8100 interfaced with an IBM computer.

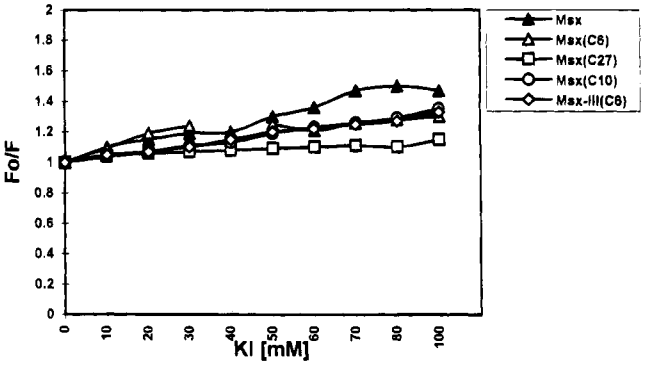

FIGURE 3: Stern–Volmer plot. Shown is the potassium iodide (KI) quenching of Trp-48 in Msx (▲), Msx(C6) (△), Msx(C10) (○), Msx(C27) (□), and Msx-III(C6) (◇). Protein concentrations were 2 μM in buffer A. Samples were excited at 295 nm, and emission spectra were recorded following addition of KI in 10 mM increments. The data are represented as values obtained in the absence of KI (F_0) divided by the values obtained when KI was added (F) (F_0/F).

Samples were excited at 295 nm in buffer A or 6 M guanidine hydrochloride (pH 7.0) using a 5 mm path length microcell (total volume = 450 μL). Energy transfer efficiency was calculated by measuring the integrated area of the tryptophan emission peak between 310 and 390 nm (i.e., donor quenching). Absorbance spectra for protein and fluorophor concentrations were made using a Beckman DU-7 spectrophotometer.

Determination of Distance Measurements Using Fluorescence Resonance Energy Transfer. The relationship between the distance between donor and acceptor, R, and the energy transfer efficiency, E, is given by the following equations:

$$R = R_0(E^{-1} - 1)^{1/6} \quad (1)$$

$$E = 1 - F_{DA}/F_D \quad (2)$$

F_{DA} and F_D are the fluorescence intensities in the presence and absence of an acceptor, respectively.

R_0 is the Forster critical distance at which $E = 0.50$ and is calculated from

$$R_0 = 9786(\kappa^2 n^{-4} Q_D J)^{1/6} \quad (3)$$

where n is the refractive index of the medium between donor and acceptor, and was taken to be 1.4 for proteins in water (Lakowicz, 1983); Q_D (0.11) is the quantum yield of the donor which was calculated for Msx-1 by the method of Chapman *et al.* (1992). κ^2 describes the orientation of the donor and acceptor; the generally accepted value of $^2/_3$ was used on the basis of the assumption of random tumbling of fluorophors (Chapman *et al.*, 1992). The overlap integral J (5.43×10^{-15} cm^3 M^{-1}) was calculated from the degree of overlap between tryptophan emission and AEDANS absorbance spectra using a Microsoft Excel spreadsheet and the equation

$$J = \sum F(\lambda)\epsilon(\lambda)\lambda^4 \Delta\lambda / \sum F(\lambda)\Delta\lambda \quad (4)$$

where $F(\lambda)$ is the donor emission spectra, $\epsilon(\lambda)$ is the extinction coefficient of acceptor, and λ is the wavelength in centimeters.

RESULTS AND DISCUSSION

Strategy for Using Fluorescence Resonance Energy Transfer (FRET) To Study Msx. Trp-48 is invariant in every homeodomain sequence (Figure 2A), and is located in helix III (Figures 1 and 2B). This residue is predicted to play an important role in stabilizing the folded structure of the homeodomain, particularly in controlling the packing of helix I against helix III (Kissinger *et al.*, 1990). We have noted previously that this tryptophan residue is unique in Msx and that its fluorescent properties provide insight into the conformation of Msx and mutated Msx polypeptides (Shang *et al.*, 1994). The goal of the present study was to take advantage of these characteristics by using Trp-48 as an intrinsic donor for FRET analysis.

A suitable acceptor molecule for tryptophan is AEDANS since its absorption spectra overlap directly with the emission spectra of tryptophan. Our strategy was to produce AEDANS-labeled Msx proteins by introducing unique cysteine residues in Msx and covalently modifying them with AEDANS. The positions selected for replacement with cysteine were chosen on the basis of the following considerations: (i) that the residues at these positions were not homeodomain consensus residues (to avoid replacing residues critical for Msx structure or function) and (ii) that cysteine replacements for residues at such positions were relatively conservative substitutions (i.e., by selecting threonine or serine residues). On the basis of these criteria, three positions were selected and cysteine residues were introduced by site-directed mutagenesis. These were position 6 in the N-terminal arm, position 10 in the turn between the N-terminal arm and helix I, and position 27 in the turn between helices I and II (Figures 1 and 2). The design of the Msx polypeptides containing unique cysteine residues at positions 6, 10, or 27 (i.e., Msx(C6), Msx(C10), and Msx(C27), respectively) is shown in Figure 2B.

Cysteine Substitutions Do Not Alter Msx Conformation. Control experiments were performed to determine whether Msx(C6), Msx(C10), and Msx(C27) exhibited grossly altered protein conformation relative to Msx. Circular dichroism analysis demonstrated that the cysteine-substituted Msx polypeptides had similar α-helical content as Msx, and DNA binding analyses showed that their DNA binding affinities and specificities were also comparable (data not shown). Furthermore, since our goal was to utilize donor quenching as a measure of FRET, we confirmed that the quantum yield of Trp-48 was unaltered in the cysteine-substituted Msx polypeptides (data not shown). Finally, we investigated whether the accessibility of Trp-48 in the cysteine-substituted Msx polypeptides was altered relative to that in Msx. As determined by potassium iodide (KI) quenching, the accessibility of Trp-48 was not altered significantly in the cysteine-substituted Msx polypeptides (Figure 3). Therefore, introduction of cysteines at these various positions in Msx did not grossly alter overall conformation or relative exposure of Trp-48.

FRET Provides a Reliable Method for Approximating the Distance between Trp-48 and AEDANS-6. We investigated the feasibility of FRET measuring intramolecular distances by using Msx(C6). Conditions were established to obtain selective and stoichiometric labeling of the unique cysteine residue in Msx(C6). Emission spectra were recorded for unlabeled Msx(C6) and for Msx(C6) labeled with AEDANS (AEDANS-Msx(C6)) between 310 and 500 nm using an excitation wavelength of 295 nm (Figure 4A). These data show that both proteins generated a single peak at the characteristic tryptophan emission maxima of 340 nm;

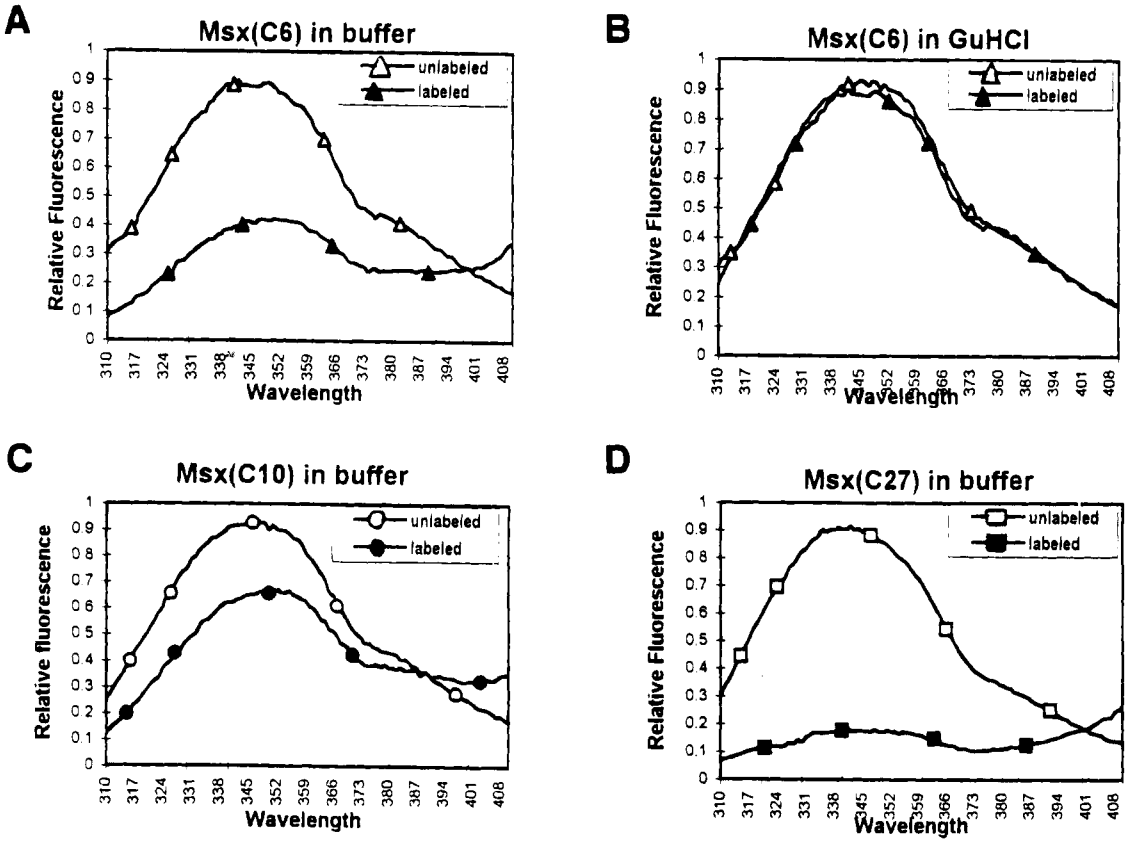

FIGURE 4: Fluorescence energy transfer using Msx(C6), Msx(C10), and Msx(C27). Shown are the tryptophan emission spectra for the indicated proteins in buffer A (A, C, and D) or 6 M guanidine hydrochloride (B). The spectra shown were recorded using 5 μM protein; similar spectra were obtained using 0.5, 1, or 2 μM protein (data now shown). The data are represented as relative fluorescence intensity versus wavelength for the indicated labeled or unlabeled proteins.

however, the peak generated by AEDANS-Msx(C6) was significantly reduced relative to that of the unlabeled protein (Figure 4A). This reduction in tryptophan emission is indicative of energy transfer between Trp-48 and AEDANS at position 6 (AEDANS-6). This conclusion is supported by control experiments which show that only a modest reduction in the Trp-48 emission peak was evident when the spectra were recorded for AEDANS-Msx(C6) under denaturing conditions (i.e., in the presence of 6 M guanidine hydrochloride) (Figure 4B). Furthermore, the observed quenching of tryptophan emission was accompanied by a corresponding increase in AEDANS emission at 490 nm the for labeled protein but not for the unlabeled protein or for the labeled protein in 6 M guanidine hydrochloride (data not shown).

These findings demonstrate intramolecular energy transfer between the donor, Trp-48, and the acceptor, AEDANS-6. The efficiency of energy transfer for this Trp-AEDANS pair was in the range of 57−70% with an average of 62% (Table 1A). The overlap integral between the donor and acceptor, J, was 5.44×10^{-15} cm^3 M^{-1}, and the Forster critical distance, R_0, was determined to be 21.2 Å. This R_0 value is in good agreement with the reported R_0 for a Trp donor/AEDANS acceptor pair which was estimated to be 22 Å (Fairclough & Cantor, 1978; Chapman *et al.*, 1992). We conclude from these observations that energy transfer between Trp-48 and AEDANS-6 provides a reliable measure for estimating the distance between these residues. The average distance was calculated to be 19.6 Å (Table 1A). This value was obtained from three independent labeling experiments performed using varying protein concentrations

Table 1: Energy Transfer Distance Measurements[a]

	transfer efficiency E (%)		estimated distances R (Å)	
	range	average	range	average
A				
Msx(C6)	57−70	62 ± 8.3	18−22	19.6 ± 0.78
Msx(C10)	36−46	40 ± 5.5	21−24	22.7 ± 1.5
Msx(C27)	79−95	90 ± 8.5	15−17	16.5 ± 1.0
B				
Msx-III(C6)	70−87	80 ± 7.4	14−18	16.8 ± 1.3
Msx-NT(C6)	41−45	38 ± 12	21−23	21.5 ± 0.5
Msx-III+NT(C6)	60−70	64 ± 5.3	18−20	19.3 ± 0.75

[a] Energy transfer efficiency (E) and estimated distances (R) were calculated for the indicated proteins as described in the text. Averages were derived from three to five independent experiments; the standard deviation is shown.

(from 0.5 to 5.0 μM). We used the program O version 5.9 (Jones *et. al.*, 1991) to compare the estimated distance obtained by FRET with the predicted distance of two known homeodomains, engrailed and antennapedia. The distance in these two homeodomains was measured from the indole ring of Trp-48 (ϵ-carbon) to the α-carbon of position 6 and were found to be 11.5 and 9.6 Å, respectively. The difference in the estimated distance measured for Msx by using FRET is likely due to the iodoacetamide side chain of AEDANS which is approximately 8.5 Å.

Use of FRET To Compare Intramolecular Distances in Msx. We investigated whether FRET was suitable for surveying the distances between Trp-48 and other positions in Msx. As detailed above, Msx(C10) and Msx(C27) were produced and subjected to cysteine-specific labeling to

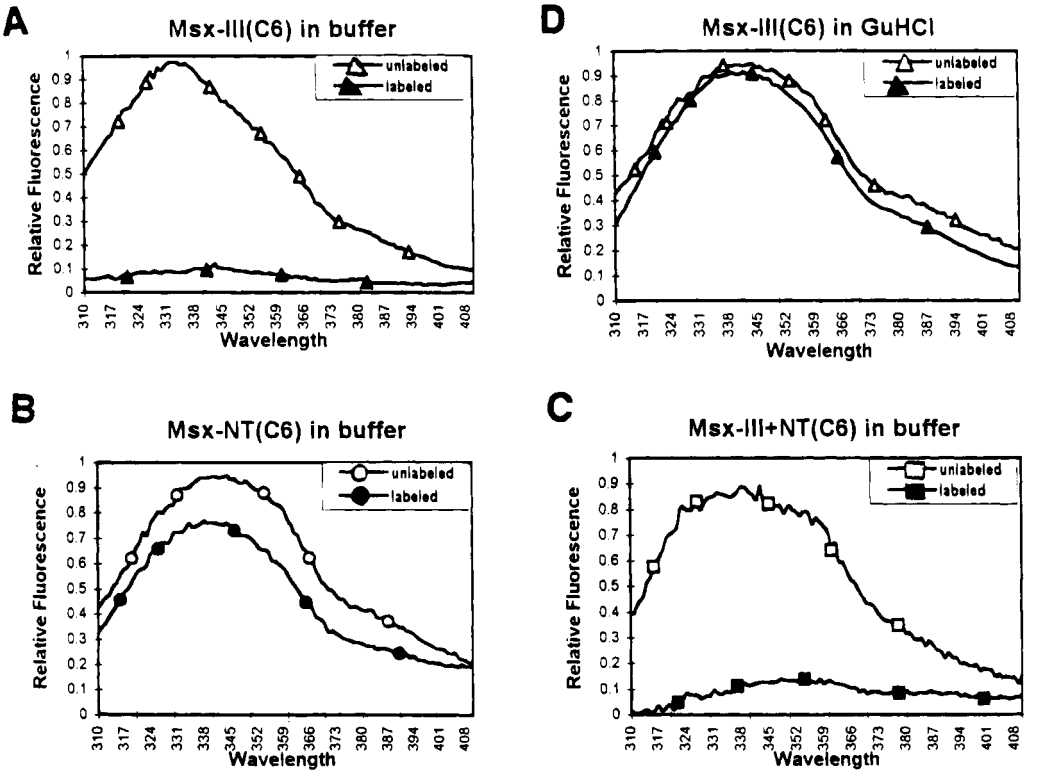

FIGURE 5: Fluorescence energy transfer using Msx-III(C6), Msx-NT(C6), and Msx-III+NT(C6). Shown are the tryptophan emission spectra for the indicated proteins in buffer A (A–C) or 6 M guanidine HCl (D). The spectra shown were recorded using 5 μM protein; similar spectra were obtained using 0.5, 1, or 2 μM protein (data now shown). The data are represented as relative fluorescence intensity versus wavelength for the indicated labeled or unlabeled proteins.

generate AEDANS-Msx(C10) and AEDANS-Msx(C27), respectively. The emission spectra were recorded for unlabeled Msx(C10) or Msx(C27) and for AEDANS-Msx-(C10) or AEDANS-Msx(C27) between 310 and 500 nm using an excitation wavelength of 295 nm (Figure 4C,D). Similar to that observed for AEDANS-Msx(C6), the characteristic tryptophan emission peak at 340 nm was reduced for AEDANS-Msx(C10) and AEDANS-Msx(C27) as compared with the corresponding unlabeled polypeptides (Figure 4C,D). This reduction in tryptophan emission was indicative of energy transfer between Trp-48 and AEDANS at position 10 (AEDANS-10) and AEDANS at position 27 (AEDANS-27) since no such reduction peak was evident when the spectra were recorded in the presence of 6 M guanidine hydrochloride (data not shown). The energy transfer efficiency averaged 40% for AEDANS-Msx(C10) and 90% for AEDANS-Msx(C27) (Table 1A) (however, it should be noted that, at extremes of E, the accuracy of R is not as reliable). The corresponding average distances between these Trp-AEDANS pairs were estimated to be 22.7 Å for AEDANS-Msx(C10) and 16.5 Å for AEDANS-Msx(C27) (Table 1A). These findings suggest that FRET can be used to estimate the intramolecular distances of residues at various positions in Msx relative to Trp-48. This strategy for investigating the spatial proximity of residues within Msx is complementary to other biochemical and biophysical approaches that have been utilized to compare the conformation of Msx with the known structures of other homeodomains (Shang *et al.*, 1994a,b; Kissinger *et al.*, 1990; Billeter *et al.*, 1993).

Use of FRET To Detect Subtle Alterations in Msx Conformation. In a previous study, we identified specific residues in the N-terminal arm and helix III that were responsible for distinguishing the DNA binding properties of Msx from those of Hox homeodomains (Ebu Isaac *et al.*, 1995). We further showed that replacement of these residues (see Figure 2A) in either the N-terminal arm or helix III alone interfered with DNA binding activity, whereas replacement of residues in both homeodomain segments restored native binding activity (Ebu Isaac *et al.*, 1995). These observations suggested a critical interplay between specific residues in the N-terminal arm and helix III. Since no gross alterations in the secondary structure were detected by circular dichroism (Ebu Isaac *et al.*, 1995), we hypothesized that these substitutions produced modest alterations in Msx conformation, which required a more sensitive assay for detection.

We tested the feasibility of using FRET for detecting such subtle differences in Msx conformation. The first step was to introduce unique cysteine residues in the Msx polypeptides which contained the relevant amino acid substitutions in the N-terminal arm (Msx-NT(C6)), helix III (Msx-III(C6)), or both (Msx-III+NT(C6)) (Figure 2B). As described for the other cysteine-substituted Msx proteins, control experiments were performed to ensure that introduction of cysteine at position 6 did not grossly alter protein conformation or the accessibility of Trp-48 (e.g., Figure 3). The cysteine-substituted polypeptides were modified to produce the corresponding AEDANS-labeled derivatives (i.e., AEDANS-Msx-III(C6), AEDANS-Msx-NT(C6), and AEDANS-Msx-III+NT(C6)).

Emission spectra were recorded for unlabeled Msx-III-(C6), Msx-NT(C6), and Msx-III+NT(C6) and for the corresponding labeled proteins AEDANS-Msx-III(C6), AEDANS-Msx-NT(C6), and AEDANS-Msx-III+NT(C6) (Figure 5A–C). Both labeled and unlabeled proteins generated a single peak at the characteristic tryptophan emission maxima of

340 nm; however, the peak generated by each of the AEDANS-labeled proteins was reduced relative to that of the unlabeled proteins (Figure 5A−C). These data are indicative of FRET since transfer is observed when the proteins were assayed in buffer, but not when denatured in 6 M guanidine hydrochloride (e.g., Figure 5D). Although each Trp-AEDANS pair demonstrated energy transfer, the efficiency varied significantly as did their corresponding estimated distances (Table 1B). Specifically, the estimated distance between Trp-48 and AEDANS-6 in Msx-III(C6) (16.8 Å) and Msx-NT(C6) (21.5 Å) differed from that in Msx(C6) (i.e., 19.6 Å), whereas the distance between Trp-48 and AEDANS-6 of Msx-III+NT(C6) (i.e., 19.3 Å) was virtually identical to that of Msx(C6) (Table 1A, B). This finding supports the hypothesis that substitutions of residues in the N-terminal arm and helix III that altered DNA binding activity also produced alterations in the intramolecular distance between residues in these homeodomain segments. Moreover, complementary substitutions which restored appropriate DNA binding activity also restored appropriate spatial proximity. The implication of these observations is that nonconsensus residues in the homeodomain may mediate specific intramolecular interactions which can be detected by FRET.

CONCLUSIONS

In this report, we demonstrate the utility of using FRET to examine the spatial proximity of residues within Msx and to investigate its conformation. These observations are of particular relevance for Msx, for which there is no structure presently available. However, FRET may also be useful for examining homeodomains whose structures are known since this approach is complementary to X-ray crystallography and NMR analyses in that it provides information regarding the dynamic interactions of proteins in solution. Moreover, since an invariant tryptophan (Trp-48) serves as the intrinsic donor, we anticipate that FRET may be of general utility for investigating intramolecular distances in other homeodomains and for making comparisons among these various proteins. It is likely that the selective actions of particular homeodomains are due to subtle differences in their intramolecular interactions rather than gross structural changes. Therefore, we propose that FRET may provide pertinent information regarding the molecular basis of functional specificity.

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