A CCHC metal-binding domain in Nanos is essential for translational regulation

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The *Drosophila* Nanos protein is a localized repressor of *hunchback* mRNA translation in the early embryo, and is required for the establishment of the anterior–posterior body axis. Analysis of *nanos* mutants reveals that a small, evolutionarily conserved, C-terminal region is essential for Nanos function *in vivo*, while no other single portion of the Nanos protein is absolutely required. Within the C-terminal region are two unusual Cys-Cys-His-Cys (CCHC) motifs that are potential zinc-binding sites. Using absorption spectroscopy and NMR we demonstrate that the CCHC motifs each bind one equivalent of zinc with high affinity. *nanos* mutations disrupting metal binding at either of these two sites *in vitro* abolish Nanos translational repression activity *in vivo*. We show that full-length and C-terminal Nanos proteins bind to RNA *in vitro* with high affinity, but with little sequence specificity. Mutations affecting the *hunchback* mRNA target sites for Nanos-dependent translational repression were found to disrupt translational repression *in vivo*, but had little effect on Nanos RNA binding *in vitro*. Thus, the Nanos zinc domain does not specifically recognize target *hunchback* RNA sequences, but might interact with RNA in the context of a larger ribonucleoprotein complex.

Keywords: CCHC/hunchback/nanos/translation/zinc

Introduction

In the *Drosophila* embryo, spatially localized gene expression is critical for the generation of the anterior–posterior body axis. One mechanism essential for achieving localized protein expression is regulated translation of maternal RNA (reviewed in Curtis *et al.*, 1995b). *nanos* RNA is localized to the posterior pole of the egg during oogenesis, and Nanos protein, translated from this discrete source, diffuses to form a posterior to anterior gradient. Nanos protein then functions to limit the translation of uniformly distributed maternal *hunchback* RNA in the posterior of

the embryo (Tautz and Pfeifle, 1989). *hunchback* encodes a transcription factor whose graded distribution produces differential expression of target genes along the anterior–posterior axis (Hulskamp *et al.*, 1990). *Cis*-acting sequences required for translational regulation of *hunchback* RNA have been mapped to the 3'UTR and are called the Nanos Response Elements (NREs) (Wharton and Struhl, 1991). Mutations in the Nanos protein and mutations in the *hunchback* NRE both cause ectopic posterior translation of Hunchback protein, producing larvae that completely lack abdominal segments (Tautz and Pfeifle, 1989; Lehmann and Nusslein-Volhard, 1991; Wharton and Struhl, 1991).

Nanos-dependent translational repression requires the *pumilio* gene. In contrast to Nanos, Pumilio protein is present in excess and is distributed throughout the embryo (Barker *et al.*, 1992; Macdonald, 1992). Recently it has been shown that Pumilio protein and a second factor of 55 kDa bind specifically to NRE sequences in embryonic extracts, even in the absence of Nanos protein (Murata and Wharton, 1995). However, the molecular mechanisms that lead to *hunchback* translational repression, including the role of Nanos, remain unclear.

nanos RNAs from several Dipteran species are posteriorly localized in embryos and can substitute functionally for *nanos* in *Drosophila melanogaster* (Curtis *et al.*, 1995a). Despite this functional conservation, most of the Nanos protein sequence has diverged. A 72 amino acid C-terminal region, however, is highly conserved among the Nanos proteins and contains an invariant set of eight cysteine and histidine residues. This protein domain is also conserved in a *Xenopus* gene, Xcat-2, which produces an RNA localized to the vegetal pole of the oocyte (Mosquera *et al.*, 1993). The order of cysteine and histidine residues in the Nanos sequence, along with the established role for many zinc-binding proteins in binding to nucleic acids, suggests the hypothesis that this highly conserved protein domain forms two CCHC zinc-binding sites and that its role in translational regulation includes RNA binding.

Since mutants in *nanos* and in the NREs have the same phenotype, the presence of the CCHC elements suggested the simple model that Nanos is an RNA-binding protein that binds directly to the NREs. To test this possibility, we constructed a series of mutants in the NREs and in Nanos to be used in phenotypic assays *in vivo* in parallel with RNA binding assays *in vitro*. Using deletion and point mutants, we show that the C-terminus of Nanos containing the two conserved CCHC motifs is absolutely required for activity, and that both CCHC motifs are essential. The two CCHC motifs are shown to be independent metal-binding elements, each capable of binding Zn(II) or Co(II) ions. Finally, we demonstrate that Nanos binds to RNA with high affinity; however, Nanos alone cannot discriminate between mutant and wild-type NRE RNAs.

Results

Mutations define the C-terminus as a functional domain of the Nanos protein

Previous comparisons between the *nanos* genes from *D.melanogaster* and three other Dipteran species revealed high conservation in a 72 amino acid C-terminal domain, but little conservation elsewhere among these ~400 amino acid proteins (Curtis *et al.*, 1995a). The heterologous Nanos proteins were functional in *D.melanogaster*, suggesting that much of the Nanos protein activity resides in the C-terminal domain. To test this hypothesis we deleted large portions of the Nanos protein sequence and assayed the mutants for function in an RNA injection rescue assay. In this assay, injection of *in vitro*-transcribed wild-type *nanos* RNA into eggs laid by *nanos* mutant mothers leads to complete rescue of abdominal segmentation, and rescued larvae can hatch and develop into fertile adults (Wang and Lehmann, 1991; Curtis *et al.*, 1995a).

Deletion of amino acids 50–218 ($\Delta 50$ –218) had a strong effect on Nanos function, $\Delta 218$ –287 had no detectable effect, and $\Delta 288$ –314 had a moderate effect (Figure 1). Limited amino acid sequence similarity between the Dipteran Nanos proteins is found in the 50–218 and 288–314 regions, while no similarity is found in the 218–287 region (Figure 1). The *D.melanogaster* N-terminal 1–50 amino acid region is not present in other Nanos proteins, which can substitute for *D.melanogaster* Nanos, suggesting that this region is not critical for function. Together, these observations show that no single region outside the C-terminal 87 amino acid region is absolutely required for Nanos protein function. However, when RNAs encoding the C-terminal 87, 99 or 114 amino acids of Nanos were injected ($\Delta 2$ –314, $\Delta 2$ –301, $\Delta 2$ –287), no rescuing activity was detected. Nanos protein was synthesized from these deletion RNAs, as detected by *in vitro* translation of injection RNAs and by α -Nanos antibody staining of injected embryos (Figure 1). Thus, neither the C-terminal domain alone, nor the C-terminal domain together with the adjacent conserved asparagine- and lysine-rich region (amino acids 288–314), are sufficient for Nanos activity *in vivo*. Structures required for Nanos function therefore reside in the 50–218 and 288–314 regions of the Nanos protein, and removal of both regions, as in $\Delta 2$ –314, eliminates detectable Nanos activity.

To further identify regions of the Nanos protein critical for its function, we analyzed four mutant alleles of *nanos* (*nos*). One allele, *nos^{RC}*, disrupts the splice donor site of the first *nanos* intron [nucleotide 734 G to A in the sequence of Wang and Lehmann (1991)]. This allele fails to produce *nanos* RNA or protein, and is therefore a null mutation (Wang *et al.*, 1994). The other three alleles, *nos^{L7}*, *nos^{RW}* and *nosRD* all affect sequences in the C-terminal region of the Nanos protein (Figure 1). *nos^{L7}*, which behaves as a strong allele for abdominal segmentation but retains oogenesis function, is an in-frame deletion of seven amino acids (I376–I382) just outside of the second CCHC domain, while *nos^{RW}*, a weak allele that produces embryos with variable numbers of abdominal segments, is an aspartic acid to asparagine change affecting one of the residues deleted in *nos^{L7}* (D380N). *nosRD*, like *nos^{RC}*, is a strong allele, eliminating both the abdominal segmentation and oogenesis functions of *nanos* (Wang

Fig. 1. (A) Conserved regions of Nanos, based on interspecific comparisons, are indicated by open boxes (Curtis *et al.*, 1995a). The protein sequence is represented to scale. Only the central 11-residue region (open box) and the C-terminal CCHC domain are conserved in primary sequence. S/T, a region rich in serine and threonine residues. N/K, a region rich in asparagine and lysine residues. (B) Deletions drawn to scale, with relative degree of rescue in the RNA injection assay indicated at right. (C) Sequence of the C-terminal 99 amino acids of Nanos, with positions of the *nanos* point mutations and conserved His and Cys residues (black dots) indicated. *nosRD* changes nt 1943 from G to A, changing a TGC cysteine codon to a TAC tyrosine codon. *nos^{L7}* deletes nt 2009–2029, inclusive, eliminating seven codons (ITMEDAI). *nos^{RW}* changes nt 2020 from G to A, altering a GAT aspartic acid codon to an AAT asparagine codon. (D) RNA injection rescue results. ‘Overall rescue’ is the percentage of larvae with any rescue, i.e. 1–8 segments, out of all developing larvae. ‘Strong rescue’ is the percentage of larvae with 5–8 segments. n = number of larvae scored (results from separate trials were consistent and were pooled); # trials = number of independent RNA preparations tested. Nos protein staining: 4(+) indicate staining intensity roughly equivalent to that observed at the posterior pole of wild-type uninjected embryos. Staining of the injected embryos was centered around the site of injection, with diffuse staining throughout the egg. 1(+) indicates that staining was weak and most easily detected in occasional pole cells that stained positively. Such staining was never observed in uninjected embryos.

et al., 1994). The sequence CX₂C is found in many zinc-binding proteins, and is present in both of the conserved Nanos CCHC motifs. *nosRD* changes the first cysteine residue in the second CX₂C motif to tyrosine (C354Y). We changed the first cysteine in motif 1, C319, to tyrosine by *in vitro* mutagenesis, a change analogous to the *nosRD* C354Y mutation in motif 2. When tested by RNA injection

Fig. 2. Absorbance spectra of Co(II)-complexed Nos polypeptides. (A) UV/visible spectrum of Nos99 complexed with Co(II). (B) Visible spectra of Nos54 and Nos99 mutants. The Nos99 wild-type spectrum is included for reference. Protein concentrations were 10–80 μ M and Co(II) was present in saturating concentrations. The free protein spectra have been subtracted, and extinction coefficients were calculated by dividing the corrected absorbance values by the protein concentration.

assay, *in vitro*-transcribed C319Y RNA had no Nanos rescuing activity (Figure 1). Thus, both CX₂C motifs are essential for Nanos protein function.

The Nanos C-terminus contains two consecutive CCHC metal-binding sites

The results described above, together with the resemblance of Nanos to known zinc finger proteins, suggest the presence of two zinc-binding sites in Nanos. To assess the metal-binding properties of Nanos, wild-type and mutant C-terminal 99 amino acid Nanos polypeptides (amino acids 302–401; Nos99) were purified from *Escherichia coli* overexpression strains and assayed for Co(II) and Zn(II) binding. Co(II) can structurally replace Zn(II) in many metalloproteins, but, unlike Zn(II), Co(II)-bound forms of proteins have pronounced absorbance peaks in the UV and visible regions of the spectrum that facilitate quantitative analysis. In addition, the visible absorbance spectrum of Co(II) is sensitive to the ligand environment and thus helps to establish the identity of coordinating side chains (Shi *et al.*, 1993).

Addition of Co(II) to the Nanos apoprotein (Nos99, Figure 1) caused dramatic UV and visible absorbance changes with peaks at 313, 640 and 713 nm (Figure 2A), establishing that Nos99 contains a metal-binding domain. Furthermore, these absorbance peaks are characteristic of CCHC coordination (Green and Berg, 1989; Krizek *et al.*, 1991; Shi *et al.*, 1993). The simplest interpretation of this result is that Nos99 contains two consecutive CCHC metal-binding units. More complicated model structures however, including the interleaved ligand arrangements identified in the RING finger (Barlow *et al.*, 1994) and

protein kinase C (Hommel *et al.*, 1994), remained possible. To distinguish between the models, a smaller polypeptide containing only the C-terminal 54 amino acids of Nanos (amino acids 347–401, Nos54) was prepared. Nos54, which contains only the second CCHC motif, also binds Co(II), and the visible spectrum qualitatively resembles that of the Nos99 complex (Figure 2). Nos54 thus forms an independent, stable CCHC metal-binding domain, strongly supporting the consecutive CCHC model.

The consecutive CCHC model was tested further by examining the Co(II) spectra of mutant Nos99 polypeptides. The model predicts that Nos99C319Y and Nos99C354Y should lack only the first or second metal coordination site, respectively. Metal binding to the double mutant (Nos99C319,354Y) should be abolished. As shown in Figure 2, both single mutants retain Co(II) binding activity, whereas the double mutant does not. The Co(II) complex spectra of the single Cys mutants are consistent with CCHC coordination. Like Nos54, the mutants exhibit reduced extinction coefficients relative to Nos99, suggesting reduced Co(II):protein ratios relative to wild-type. These results strongly support a consecutive arrangement of independent CCHC metal-binding sites. Since the double mutant is completely inactive in Co(II) binding, non-native metal coordination is not observed.

To determine the stoichiometry of metal binding, apo-Nos polypeptides were titrated quantitatively with Co(II). The endpoints of stoichiometric titrations establish that Nos99 binds two molar equivalents of Co(II), while Nos54, Nos99C319Y and Nos99C354Y each bind one equivalent (Figure 3). We conclude that the C-terminal domain of Nanos contains two independent metal-binding sites, both of which are required for Nanos activity *in vivo*.

Although we have not determined the metal coordinated by Nanos protein *in vivo*, Zn(II) is a likely candidate. Zn(II) binding by Nanos protein *in vitro* was measured indirectly by competition analysis (Figure 4). Nos polypeptides were reconstituted with excess Co(II), and the decrease in absorbance at 313 nm or 640 nm was recorded as a function of Zn(II) concentration. In all cases, competition with Zn(II) was stoichiometric, and the metal:protein ratios determined by Co(II) titration (Figure 3) were recapitulated. Since Zn(II) binding was stoichiometric even in the presence of a 10-fold excess of Co(II), we estimate that Zn(II) binding is preferred over Co(II) by at least three orders of magnitude. Co(II) binding is stoichiometric at ~10 μ M protein, the lowest concentration tested, indicating that the upper limit for the K_d of Co(II) is 1 μ M and that Zn(II) binds with sub-nanomolar affinity. Specificity for Zn(II) over Co(II) is common and may result from losses in ligand field stabilization energy that occur when Co(II) changes from an octahedral aqueous species to a tetrahedral species in proteins (Lippard and Berg, 1994).

Characterization of Zn(II) binding to the Nanos CCHC domain by NMR spectroscopy

NMR studies have shown that zinc promotes folding of classical and CCHC-type zinc fingers (Parraga *et al.*, 1988; Lee *et al.*, 1989; South *et al.*, 1989), and one-dimensional ¹H-NMR spectroscopy of the C-terminal Nos99 polypeptide provides further evidence for Zn(II) coordination. Addition of Zn(II) to the Nos99 apoprotein

Fig. 3. Co(II) binding stoichiometry of the Nanos polypeptides: (A) Nos99; (B) Nos99C319Y; (C) Nos99C354Y and (D) Nos54. Co(II) was titrated into Nos polypeptide samples and the absorbance increase at 313 nm (A and D) or 640 nm (B and C) was recorded. The fraction of sites bound was calculated by normalizing the absorbance changes to the maximum value. The x axes represent the ratio of Co(II) concentration to protein concentration.

Fig. 4. Zn(II) binding stoichiometry of Nos polypeptides. (A) Nos99; (B) Nos99C319Y; (C) Nos99C354Y; (D) Nos54. Zn(II) was titrated into Nos polypeptide samples that were equilibrated with 11 molar equivalents of Co(II). Zn(II) binding was detected by a decrease in Co(II)-dependent absorption at 313 nm (A and D), or 640 nm (B and C). Data calculations were performed as for Figure 3.

results in increased resonance dispersions indicative of structure formation. The chemical shift changes are most distinctive for some aliphatic protons (data not shown) and for His-H2 and -H4 protons (Figure 5A). The His-H2 and -H4 protons are useful indicators of Zn(II) coordination. In the apoprotein, the three His-H2 proton

resonances are present as two overlapped peaks at 8.3 p.p.m. and a single peak at 8.2 p.p.m. Two of these peaks shift to 7.7 and 7.8 p.p.m. upon addition of Zn(II) (Figure 5A, arrows) and attain maximum intensity at a Zn(II):protein ratio of 2:1, as expected for the binding of two molar equivalents of zinc. The appearance of these

Fig. 5. Aromatic proton region from ^1H -NMR spectra of Nos polypeptides. **(A)** Zn(II) titration of Nos99 at pH 6.4. Molar ratios of Zn(II) to protein are shown at left. Arrows indicate the two H2 protons of the His residues coordinated to Zn(II); these increase in intensity as Zn(II) is titrated. Filled squares and asterisks indicate the H2 and H4 protons from a third His, most likely His318 (see text). This His only binds Zn(II) after binding to the other two His residues is saturated at Zn(II):polypeptide ratios of >2 . **(B)** Spectra of the apo-Nos polypeptides compared with spectra after addition of 2 (Nos99) or 1 (Nos54 and Nos99C354Y) molar equivalents of Zn(II). Arrows indicate positions of protons from Zn(II)-liganded His residues. **(C)** pH titration of apo-Nos99. The chemical shifts of His-H2 and -H4 protons are pH sensitive in the absence of Zn(II). **(D)** pH titration of Nos99 reconstituted with 2.4 molar equivalents of Zn(II). The chemical shifts of H2 and H4 protons from two His residues are pH insensitive (arrows), while those from the third His are highly sensitive (filled squares). Asterisks indicate H2 and H4 protons from the third His that are pH-insensitive due to non-specific Zn(II) coordination.

two peaks is concurrent, suggesting that the CCHC sites have comparable Zn(II) affinities or, alternatively, that binding is highly cooperative. The third His-H2 proton only forms detectable metal complexes when excess Zn(II) is added (Figure 5A, asterisk), suggestive of non-specific binding. Zn(II) titrations were also performed on the Nos54 and Nos99C354Y apoproteins. The spectra of the apoproteins and the titration end-points are shown in Figure 5B. For Nos54, the His365 proton peak at 7.85 p.p.m. reaches maximum intensity at a Zn(II):protein ratio of 1:1. Similarly, for Nos99C354Y a His proton peak at 7.8 p.p.m. reached a maximum at a Zn(II):protein ratio of 1:1 (Figure 5B, arrow). The two additional His peaks, most likely representing His318 and His365, do not shift to the 7.8 p.p.m. region. The His protons shifted to ~ 7.8 p.p.m. in Nos54 and Nos99C354Y likely correspond to the 7.7 and 7.8 p.p.m. His protons in the Nos99 spectrum.

To confirm the assignment of the 7.7 and 7.8 p.p.m. proton shifts to His–Zn(II) complexes, pH titration experi-

ments were performed (Figure 5C and D). When His is complexed to metal, the chemical shifts of its H2 and H4 protons are insensitive to moderate changes in pH, while an uncomplexed His will demonstrate pH-dependent chemical shifts with the transition midpoint near the pK_a of free His (~ 6.0) (Parraga *et al.*, 1990). A pH titration of apoNos99 shows that the His-H2 proton (peaks near 8.5 p.p.m.) move upfield with increasing pH (Figure 5C). A similar pH titration for Zn(II)-reconstituted Nos99 is shown in Figure 5D. In this sample the His peaks at 7.7 and 7.8 p.p.m. are pH-insensitive, indicating that a bound metal is blocking protonation. In contrast, the third His proton chemical shift (Figure 5D, filled squares) shows a strong pH-dependence similar to that observed for the His protons in the apo-Nos99 sample (Figure 5C). In addition, the peak resulting from the third His–Zn(II) complex (asterisks in Figure 5D) is relatively pH sensitive, providing further evidence against a role for the third His (likely His318) in zinc coordination. We conclude from these

Fig. 6. Mutations in the *hunchback* NRE sequences and their effects. A wild-type *hunchback* reporter transgene is fully regulated, resulting in larvae with the wild-type cuticle pattern drawn at right (anterior to left) (Wharton and Struhl, 1991). Mutation of all six conserved NRE guanosine residues to uracil (G1–6U) results in no translational repression, and a mutant cuticle pattern identical to that seen in a complete deletion of the NREs (Wharton and Struhl, 1991). The G1–3U mutation targeting the first NRE results in a much stronger mutant phenotype than G4–6U, which affects the second NRE. ‘Phenotype’ illustrates average number of abdominal segments formed by embryos from several independent transgenic lines.

studies that Nos99 contains two histidine residues complexed to Zn(II), and that these contribute to two consecutive, independent CCHC zinc-binding domains.

NRE mutations disrupt translational repression

Since many zinc-binding proteins function by binding to nucleic acids, and because of the known role of the NRE sequence in Nanos-dependent regulation of *hunchback* translation, we tested for the ability of Nanos proteins to bind to NRE-containing RNAs. In preliminary experiments we found that C-terminal Nanos polypeptides bind to RNA *in vitro* in UV crosslinking assays (data not shown). The NRE target sequence for Nanos-dependent translational repression was previously defined as a 32 nucleotide repeat present in the *hunchback* and *bicoid* RNAs, in which only 11 out of the 32 nucleotide positions are conserved (Wharton and Struhl, 1991). In order to determine the importance of the conserved nucleotide sequences, and to create sequences suitable for testing the specificity of Nanos RNA binding *in vitro*, we mutated the NREs and tested their ability to repress translation of *hunchback* mRNA in transgenic animals. When all six conserved guanosines in the NRE core sequences are changed to uracil (G1–6U, Figure 6), the transgene produces a dominant female sterile phenotype in which all abdominal segmentation is repressed, a phenotype indistinguishable from the *nanos* mutant phenotype. To test for residual NRE function we raised the effective Nanos protein concentration in G1–6U transgenic embryos in two ways, by injection of *in vitro*-transcribed *nanos* RNA, and by the introduction of the *torso* or *torsolike* mutations (see Materials and methods). In neither experiment did we observe any rescue of the G1–6U dominant phenotype. G1–6U thus behaves as a complete loss of function NRE mutation.

Mutations in the first (G1–3U) and second (G4–6U) NRE sequences were also tested independently in the same transgenic assay (Figure 6). G1–3U mutant transgenes produced a dominant female sterile phenotype, with all embryos developing into larvae with two to five segments. G4–6U transgenes did not result in female sterility, although examination of the larvae from transgenic females showed that the majority had mild defects in one or two abdominal segments (Figure 6). Thus, both NREs contribute to translational repression *in vivo*, but the first NRE contributes much more activity than the second.

Table I. Competition RNA binding assays

Protein	RNAs	Ratio	<i>n</i>
MBPNos	DX/TUB	9.1 ± 4.4	8
MBPNos	DX/XA	4.5 ± 1.2	7
MBPNos	DX/DX[G1–6U]	1.2 ± 0.3	3
Nos99	DX/TUB	9.1 ± 2.5	3
Nos99	DX/XA	2.5 ± 0	2
Nos99	DX/DX[G1–6U]	0.7 ± 0.1	3
apo-Nos99	DX/TUB	1.6 ± 0.1	2
apo-Nos99	DX/XA	0.8 ± 0.1	2
apo-Nos99	DX/DX[G1–6U]	0.7 ± 0	2

While no sequence similarity between NREs is found outside the conserved 11 nucleotide motifs, these results, in agreement with those of Murata and Wharton (1995) suggest that other non-conserved sequences within the 32 nucleotide NRE influence Nanos-dependent regulation.

Nanos binds RNA non-specifically

To test for RNA binding specificity *in vitro*, Nanos proteins were prepared and assayed in filter binding assays. Full-length Nanos was expressed as a maltose-binding protein fusion (MBPNos) and purified under native conditions by amylose and heparin affinity chromatography. Nos99 was prepared under denaturing conditions as described above, and reconstituted with Zn(II). RNA substrates tested include the wild-type NRE fragment (DX), and three RNAs with no NRE activity *in vivo*: DX[G1–6U], an adjacent fragment of the *hunchback* 3’UTR (XA), and a fragment of the α -tubulin 3’UTR (TUB) (this work, and Wharton and Struhl, 1991).

In filter binding assays we observed that MBPNos and Nos99 bind tightly to the DX RNA (K_d ~50 nM, data not shown). The Nanos RNA binding activity therefore resides in the C-terminal Zn binding domain. To address binding specificity, a filter retention assay was used in which the binding reaction contains two RNAs, both in excess concentration over Nanos protein (Bartel *et al.*, 1991). The ratio of the RNAs retained on the filter reflects the relative affinity (i.e. ratio of the K_d values) of the protein for the two RNAs. The data presented in Table I illustrate that MBPNos and Nos99 behave indistinguishably in the binding assay. MBPNos and Nos99 proteins show modest

Fig. 7. Proposed configuration of the Nanos CCHC zinc-binding motifs.

binding preferences for DX over TUB or XA RNAs (9- and ~3-fold, respectively). However, we find that neither protein discriminates between the wild-type DX and DX[G1–6U] RNAs. Since the DX[G1–6U] mutant is completely inactive *in vivo*, it should affect the binding of proteins that interact specifically with the core NRE sequences. These results suggest that Nanos RNA binding is only modestly sequence specific and does not involve direct recognition of the conserved G residues in the NRE sequences.

To determine whether Nanos RNA binding requires zinc, we tested apo-Nos99 protein in the filter binding assay. Apo-Nos99 protein binds RNA but is unable to discriminate between different substrates (Table I). Filter binding and affinity co-electrophoresis (by the method of Lim *et al.*, 1991) studies show that the affinity of apo-Nos99 for DX and DX[G1–6U] RNAs is moderately reduced (~3- to 5-fold) relative to Zn(II)–Nos99 (data not shown). In addition, the *Nos*RD C354Y mutation, which eliminates one Zn(II) coordination site, significantly reduced binding of the C-terminal polypeptide to RNA substrates in UV crosslinking assays (data not shown). Thus, the zinc-dependent structure promotes the Nanos–RNA interaction to a modest extent. In summary, we observe that full-length MBPNos and Nos99 proteins bind RNA *in vitro* with high affinity but with little specificity, and an intact zinc-binding domain is required for maximal binding affinity and specificity.

Discussion

Our results establish that the C-terminal domain of Nanos is essential for its function, and that this domain binds two molar equivalents of Zn(II). Several observations support a Zn(II)-coordination model in which Zn(II) is bound by consecutive CCHC motifs, C-X₂-C-X₁₂-H-X₁₀-C (motif 1) and C-X₂-C-X₇-H-X₄-C (motif 2), that are separated by a seven amino acid linker (Figure 7). Nos99, which encompasses both motifs, binds two equivalents of Co(II) or Zn(II), and the Co(II) complex has an absorption spectrum characteristic of CCHC coordination. Mutation of the first Cys residue in either motif 1 or 2 reduces metal binding stoichiometry to 1:1, and the combined double mutant does not bind metal. Nos54 encodes only motif 2 and binds one equivalent of Co(II) or Zn(II). Thus, the two Nanos motifs form independently stable Zn(II)-binding sites.

NMR data provide further support for the consecutive CCHC model. Proton resonances from two of the three His residues in Nos99 shift upfield and become pH

insensitive in the presence of Zn(II). In the mutants that bind one metal equivalent, the proton resonance of one out of three His residues is Zn(II) dependent, an observation inconsistent with CCHH or CCCC coordination. Our data do not rule out that His318, and not His335, is coordinated in motif 1, although we consider this configuration (H-C-X₂-C-X₂₂-C) unlikely for two reasons. First, the lack of spacer residues between the His and Cys ligands would be expected to yield a highly strained structure that has not been observed in other proteins. Second, His318 is not conserved in the similar *Xenopus* Xcat-2 protein, whereas all other candidate metal-binding ligand residues are invariant among the Dipteran and *Xenopus* proteins (Curtis *et al.*, 1995a).

The two Zn(II) motifs differ in ligand spacing both from each other and from known CCHC motifs, including the retroviral type (RT) zinc finger (C-X₂-C-X₄-H-X₄-C) (Green and Berg, 1989), and the first finger of the LIM domain (C-X₂-C-X_{16–23}-H-X₂-C) (Freyd *et al.*, 1990; Karlsson *et al.*, 1990; Michelsen *et al.*, 1993; Perez-Alvarado *et al.*, 1994). The ligand spacing in motif 2 differs from the spacing in RT only by the insertion of three residues into the second RT loop, while Nanos motif 1 has insertions in both the second and third loops relative to the invariant RT spacing. The novel spacing between the Zn(II) ligands, as well as the spacing between the two CCHC modules in the Nanos C-terminus is precisely conserved in evolution among the Dipteran Nanos and *Xenopus* Xcat-2 proteins. Hence, the Nanos motifs define a distinct variant of the retroviral-type CCHC Zn(II)-binding domain.

At present, little is known about the structure of the Nanos motifs. Some aspects of the structure are zinc-dependent, as demonstrated by chemical shift changes in the aliphatic and His-H2 and -H4 regions of NMR spectra upon addition of Zn(II) to apo-nos99. By contrast, circular dichroism studies suggest that Zn(II) binding does not change the secondary structure content of the apoprotein (data not shown). One Zn(II)-dependent structure likely to be found in both Nanos motifs is the C-X₂-C-X₂ rubredoxin knuckle, a turn motif characteristic of nearly all zinc-binding domains (Berg, 1990; Schwabe and Klug, 1994). In motif 2, Gly follows the second Cys residue, a sequence especially consistent with the knuckle structure since Gly at this position stabilizes the motif (Green and Berg, 1989).

It is unclear if the two Zn(II) motifs fold to form a single domain or if each is a separate structural unit. The classical TFIIIA-type CCHH nucleic acid-binding fingers, although tandemly arrayed, form separate structural units. There are also examples of domains comprised of two consecutive Zn(II) motifs that interact to form a global structure, including the steroid–thyroid hormone receptor family C8 motif (Schwabe *et al.*, 1993) and the LIM domain C₂HC₅ motif (Perez-Alvarado *et al.*, 1994) which, like Nanos, have consecutive arrangements of metal-binding sites. In the LIM domain, Zn(II) coordination appears to be hierarchical, as binding to the CCHC site requires that the CCCC site first be occupied (Michelsen *et al.*, 1994). In the hormone receptor, tertiary interactions between the two modules lead to co-stabilization (Klug and Schwabe, 1995). It is clear from our mutational studies that each of the Nanos motifs can bind Zn(II) or Co(II)

with high affinity in the absence of binding to the other site. Hence, at the protein concentrations used in our studies, there is no evidence to support either cooperative or hierarchical models for Zn(II) coordination. Binding studies performed at protein concentrations below the K_d value for metal coordination are required to address this issue further.

Structural zinc is important for organizing small protein domains that play diverse functional roles (Schwabe and Klug, 1994; Berg and Shi, 1996). Examples include the DNA- and RNA-binding TFIIIA-type zinc finger (Clemens *et al.*, 1993), the diacylglycerol-binding protein kinase C cysteine-rich domain (Ono *et al.*, 1989; Quest *et al.*, 1994) and the LIM domain, which mediates protein–protein interactions (Feuerstein *et al.*, 1994; Schmeichel and Beckerle, 1994). Proteins containing RT-type CCHC zinc domains commonly interact with single-stranded nucleic acids. The retroviral nucleocapsid proteins have been implicated in both sequence-specific and non-specific RNA binding, primer tRNA annealing, and packaging of genomic RNA into the nucleocapsid core (Darlix *et al.*, 1995). The mammalian CNBP family proteins have tandem CCHC domains and function as sequence-specific ssDNA-binding proteins (Rajavashisth *et al.*, 1989; Tzfati *et al.*, 1995), and the bacteriophage T4 gene 32 protein has a CHCC domain that acts both to bind ssDNA and to bind its own RNA and autoregulate translation (Shamoo *et al.*, 1991). Intact zinc domains are critical to the functions of these protein classes. The loss-of-function phenotypes of the *nosRD* (C354Y) and *nos* (C319Y) mutations demonstrate an *in vivo* functional requirement for both of the Nanos CCHC zinc-binding motifs.

To address the question of a biochemical function for Nanos, we asked whether Nanos is an RNA-binding protein. Indeed, our results demonstrate that Nanos binds RNA with high affinity *in vitro*, and the RNA-binding activity is associated with the C-terminal zinc domain. However, Nanos shows little RNA-binding specificity, and binding is reduced only moderately when zinc is removed from the protein preparation. These *in vitro* effects contrast strongly with the *in vivo* loss-of-function phenotypes of Nanos zinc ligand point mutants. RNA binding is thus likely to be just one component of the function of the Nanos C-terminus. In addition, the C-terminus is not sufficient for activity *in vivo*, and two other regions of the protein, defined by the $\Delta 50$ –218 and $\Delta 288$ –314 deletions, contribute to Nanos translational repression activity.

Murata and Wharton (1995) have shown that Pumilio protein binds specifically to the NRE in embryonic extracts, independent of Nanos protein. Our data, and those of Murata and Wharton (*ibid*), suggest that Nanos is unlikely to be the primary determinant of NRE sequence recognition. These data suggest that Pumilio and/or other RNA-binding factors, and not Nanos, initially recognize the NRE. Nanos might then be recruited, through a combination of protein–protein and protein–RNA interactions, to assemble a highly specific complex. How this putative ribonucleoprotein complex would result in translational repression of *hunchback* RNA remains to be determined.

Materials and methods

Nanos mutant allele sequences

DNA was prepared from adults carrying *nanos* mutant alleles over *Df(3R)DI^{K43}* which removes the *nanos* locus. 3.4 kb of DNA from

each *nanos* mutant was amplified in three overlapping fragments by polymerase chain reaction (PCR). The entire coding regions and intervening sequences of two independent isolates from each allele were sequenced.

RNA injection rescue

Deletion and mutant versions of Nanos were created by manipulating the full-length pN5 *nanos* cDNA (Wang and Lehmann, 1991). $\Delta 50$ –218 was generated by deleting sequences between two *Pst*I sites, and $\Delta 218$ –287 by removing sequences between two *Pvu*II sites. Other deletion and point mutant constructs were generated by site-directed PCR mutagenesis. All constructs were confirmed by dideoxy sequence analysis.

pN5 derivative RNA templates were linearized with *Not*I and transcribed *in vitro* with SP6 polymerase. RNAs were precipitated with ethanol and resuspended in water at a concentration of 2 mg/ml. The integrity of *nanos* templates was tested by *in vitro* translation; all RNAs produced the expected size protein products with similar efficiency. 0- to 1-h-old embryos laid by mothers of the genotype *nos^{RC/nos^{BN}}* were injected at 40% egg length and allowed to develop for 48 h at 18°C. Hatched larvae were collected, and unhatched embryos were hand de-vitelinated for cuticle preparations. For antibody detection of injected Nanos derivatives, a subset of injected embryos were aged for 60–70 min at 18°C, removed from oil by immersion in heptane, fixed in 4% formaldehyde in phosphate-buffered saline (PBS), hand de-vitelinated, dehydrated in methanol and stored at –20°C. After the injection series was completed, all injected embryos were stained in parallel with an antibody directed against the Nanos C-terminal peptide as described (Gavis and Lehmann, 1992).

Construction of bacterial expression plasmids

DNA cassettes encoding wild-type and mutant versions of the C-terminal 99 or 54 amino acid domain of Nanos (Figure 1) were prepared by standard and recombinant (Higuchi, 1990) PCR protocols, respectively, using full-length pN5 *Nanos* cDNA as a template. PCR products were cloned into either pKK223-3 (Pharmacia) or pTACTAC (generously provided by C.S.Craik, U.C.San Francisco) for IPTG-inducible expression in *E.coli* strain KS1000 (New England Biolabs). MBPNos was prepared by inserting the full *nanos* coding sequence into the *Eco*RI site of pMAL-c2 (New England Biolabs).

Overexpression and purification of Nanos polypeptides

MBPNos was produced in Top 10 F' cells (New England Biolabs) and affinity-purified by amylose chromatography according to the manufacturer's recommendations. MBPNos eluted from an amylose column by maltose was loaded on a 1 ml Hitrap Heparin column (Pharmacia), washed with 50 mM HEPES pH 7.5, 0.1 mM EDTA, 10% glycerol, 0.5 mM DTT (buffer A) and the column developed with a 0–1 M NaCl gradient in buffer A. Peak fractions were pooled and dialyzed against a 1000-fold excess volume of 2× RNA-binding buffer (below) without MgCl₂.

Nos99 and Nos54 expression strains were grown at 37°C in 1 l batches of Terrific Broth supplemented with 50 mg/ml ampicillin to an OD₆₆₀ of 0.8, induced for protein expression by the addition of 1 mM IPTG, and grown for an additional 6 h. Cells were harvested by centrifugation and resuspended in denaturing buffer (DB; 10 mM potassium phosphate pH 5.8, 10 mM KCl, 5 mM EDTA, 5 mM DTT, 6 M urea) supplemented with 1 mM PMSF. Cells were disrupted by sonication and lysates were cleared of particulate material by centrifugation and applied to an SP 650M (Toyopearl) cation exchange column that was equilibrated with DB. The column was washed with DB and eluted with a KCl gradient (in DB). Pooled fractions were dialyzed against 2% acetic acid overnight and lyophilized. Dry samples were dissolved in reducing buffer (100 mM DTT, 25 mM EDTA, 6 M urea pH 8.0) and incubated at 65°C for 30 min to ensure full reduction of cysteine residues before preparative reverse-phase HPLC. Reduced Nanos samples were injected onto a Vydac protein and peptide preparative C18 column equilibrated with 0.1% trifluoroacetic acid in water. Protein was eluted with a gradient of acetonitrile/0.1% trifluoroacetic acid, and the major peak absorbing at 230 nm, corresponding to the Nanos polypeptide, was collected, lyophilized and stored at –70°C. Purified Nanos polypeptides were judged to be >98% pure by SDS–PAGE. The protein preparation was found to be essentially devoid of metals by inductively coupled plasma emission spectroscopy (performed by the University of Georgia chemical analysis laboratory). In subsequent manipulations, protein concentrations were determined spectrophotometrically using molar extinction coefficients calculated by the method described by Gill and von Hippel (1989). Amino acid analysis (performed

by the MIT biopolymers laboratory) confirmed that the extinction coefficients were accurate. Overall yields of pure protein were generally 10–20 mg/l of bacterial culture.

Metal ion binding studies

Lyophilized polypeptides were dissolved in refolding buffer (RB; 50 mM Tris–HCl pH 7.2, 50 mM NaCl) immediately before use. Final protein concentrations were generally 20–80 mM. UV and visible absorbance changes resulting from Co(II) or Zn(II) coordination were measured at 25°C in a 1 cm, 1 ml cuvette using an AVIV model 14DS UV/VIS spectrophotometer. In metal binding stoichiometry experiments, absorbance changes were measured at either 313 nm or 640 nm, and metal binding was allowed to reach completion by monitoring the absorbance as a function of time. The cuvette chamber was purged with nitrogen throughout all experiments. For all titrations, dilutions of 1 M CoCl₂ and ZnSO₄ stocks (in RB) were added directly to the cuvette followed by gentle mixing. Changes in the sample volume were accounted for in all calculations. Density measurements confirmed that the metal stock solutions were 1 M.

NMR spectroscopy

NMR samples were prepared by dissolving buffer-free lyophilized Nanos polypeptides in 99.9% D₂O (Cambridge Isotope Laboratories) under an argon atmosphere. Final concentrations for Nos99, Nos54 and Nos99C354Y were ~1 mM. The pH of the samples was immediately adjusted with dilute NaOD or DCl. One-dimensional spectra were acquired with 16K data points on a Varian 500 MHz spectrometer at 25°C. Data were processed using FELIX version 2.30 (Biosym Inc.). The chemical shifts were referenced to the HOD peak, 4.75 p.p.m. To prepare Zn(II)–peptide complexes, aliquots of a 60 mM ZnCl₂ stock solution were titrated into the peptide sample solutions on ice, and the pH was adjusted under normal atmosphere. pH values were not adjusted for the isotope effects.

RNA binding assays

RNA templates were generated by cloning into the *EcoRV* site of pSP72 (Promega) the following 3'UTR fragments: *hunchback DdeI* to *XbaI* filled-in 152 nucleotide fragment from the 3'UTR containing the NREs (DX), *hunchback* 3'UTR *XbaI* filled-in to *AseI* filled-in 221 nt fragment (XA), and α -tubulin *ScaI*–*HpaI* 197 nt fragment containing almost the complete 3'UTR (TUB) (Theurkauf *et al.*, 1986). Templates were linearized with *EcoRI* and transcribed with T7 polymerase in the presence of [α -³²P]UTP.

Filter binding competition assays were performed essentially as described (Bartel *et al.*, 1991), except that 0.5 μ M of each RNA and ~0.1 μ M protein were used. Background RNA retention on the filter in the absence of protein was independent of sequence. RNAs were heated for 3 min at 85°C and cooled on ice before addition to binding reactions (100 μ l). Binding buffer was 10 mM HEPES–KOH pH 7.5, 50 mM KCl, 5% (v/v) glycerol, 1.0 mM MgCl₂, 0.5 mM DTT. Binding was for 30 min at room temperature before filtering. Filters were pre-rinsed with binding buffer plus 1 mM MgCl₂ and rinsed after filtering with 3 ml of the same buffer. RNA retained with protein on the filter was recovered by digestion with 10 μ g/ml proteinase K in 200 mM Tris–HCl pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% (w/v) SDS at 65°C for 2 h, precipitated with isopropanol, and electrophoresed on a denaturing 5% polyacrylamide gel. Dried gels were analyzed using a BAS2000 Bio-analyzer (Fujix) to determine the ratio of RNAs retained, corrected for the ratio of input RNAs.

Analysis of mutant NREs in vivo

PCR was used to mutagenize and amplify the *DdeI*–*XbaI* 152 nt fragment of the *hunchback* 3'UTR and to create flanking *SpeI* sites. Fragments were cloned into the unique *SpeI* site in p2519 (Wharton and Struhl, 1991). p2519 is a modified Carnegie 20 transformation vector carrying a 10 kb genomic *BamHI* fragment of the *hunchback* locus from which the *DdeI* to *XbaI* NRE-containing portion of the 3'UTR is deleted, and which has a unique *SpeI* site engineered in the remaining 3'UTR sequences. P element constructs were co-injected with Pr25.7wc helper plasmid into *ry*⁵⁰⁶ embryos. Several independent transformant lines were obtained for each construct.

To test if the G1–6U mRNA has any residual activity, additional Nanos was provided by injection of *in vitro*-translated *nanos* mRNA at high concentration (2 mg/ml) into embryos carrying the G1–6U *hunchback* transgenic RNA. In addition, G1–6U transgenes were crossed into a *torso*^{WK} or *torsolike*⁶⁹¹ background as described (Wharton and Struhl,

1991), which produces a fate map shift of prospective abdomen towards the posterior of the embryo where Nanos concentration is highest.

Acknowledgements

We thank Robin Wharton for providing *hunchback* reporter constructs and transformed fly strains, and Charlotte Wang for building the pN5 Δ 50–218 and Δ 218–287 cDNA constructs. Anne Williamson provided helpful comments on the manuscript. D.C. was supported by postdoctoral fellowships from the American Cancer Society and the Howard Hughes Medical Institute, D.K.T. by an American Cancer Society postdoctoral fellowship, and F.T. by an NIH postdoctoral fellowship. P.D.Z. is a Howard Hughes Medical Institute postdoctoral fellow of the Life Sciences Research Foundation. This work was initiated with the support from a Science Partnership Fund in the School of Science at MIT. J.R.W. was supported by grants from the Rita Allen Foundation and the NIH (GM-53320) and R.L. was an Associate Investigator of the Howard Hughes Medical Institute.

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Received on August 6, 1996; revised on October 18, 1996