Regulation of DNA Binding Activity and Nuclear Transport of B-Myb in Xenopus Oocytes*

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DNA binding activity and nuclear transport of B-Myb in Xenopus oocytes are negatively regulated. Two distinct sequence elements in the C-terminal portion of the protein are responsible for these different inhibitory activities. A C-terminal Xenopus B-Myb protein fragment inhibits the DNA binding activity of the N-terminal repeats in trans, indicating that intramolecular folding may result in masking of the DNA binding function. Xenopus B-Myb contains two separate nuclear localization signals (NLSs), which, in Xenopus oocytes, function only outside the context of the full-length protein. Fusion of an additional NLS to the full-length protein overcomes the inhibition of nuclear import, suggesting that masking of the NLS function rather than cytoplasmic anchoring is responsible for the negative regulation of Xenopus B-Myb nuclear transfer. During Xenopus embryogenesis, when inhibition of nuclear import is relieved, Xenopus B-Myb is preferentially expressed in the developing nervous system and neural crest cells. Within the developing neural tube, Xenopus B-Myb gene transcription occurs preferentially in proliferating, non-differentiated cells.

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involved in masking DNA binding and nuclear transport activities. Analysis of the spatial expression characteristics of B-myb during Xenopus embryogenesis reveals neural specific expression and links B-myb gene transcription to proliferative activity within the developing neural tube.

EXPERIMENTAL PROCEDURES

Plasmids and Cloning Procedures—The plasmid pSP64T XB-Myb (2) was used to generate XB-Myb C-terminal mutants with the EcoHI/mung bean nuclease deletion system (Stratagene, La Jolla, CA). The corresponding mutant proteins were generated by in vitro transcription/translation for use in the electrophoretic mobility shift assays.

A BamHI site upstream of the XB-myb translation start ATG was generated by PCR1 using sense (5′ TCT GTG ATC CGG AGA AGT TCC CCG GGG 3′) and antisense (5′ TAT TTC ACC GCA CCT AGT TCC CAA TAC 3′) primers. The resulting PCR fragment spanning nucleotides 46–540 of XB-myb cDNA was cloned into the pGEM-T™ vector (Promega, Madison, WI) and sequenced. From this plasmid a DNA fragment was generated by cutting with BamHI and ClaI was generated. In parallel a DNA fragment was generated from full-length and truncated pSP64T XB-Myb by restriction digestion using ClaI and SalI. Both fragments (BamHI-ClaI and ClaI-SalI) were used for subsequent ligation into vector, pGEM-T™ (Promega, Madison, WI). Sequencing chimera of GST and full-length or truncated XB-Myb were sequenced to confirm the in-frame fusion. These constructs were expressed in XL-1 blue Escherichia coli, followed by purification of the fusion proteins according to the procedure of Kirov (20).

In order to prepare proteins for oocyte microinjection, the full-length and truncated DNAs were transferred into the Mgo-tag vector pc52MT (21) by cutting the GST clones with BamHI/SalI, performing a fill-in reaction, and cloning into XhoI-filled pc52MT vector. Site-directed mutagenesis of the NLS1 was generated by PCR with the sense primer (5′ TCT GTG ATC CGG AGA AGT TCC CCG GGG 3′) and the antisense primer (5′ TCT TGA CAG TGT ACA CCT GGA GTA GGT 3′) using the Quick Change System (Stratagene, La Jolla, CA). These constructs were manually sequenced using either Sequenase (U. S. Biochemicals) or an Applied Biosystems sequencing system, using T7 dye terminator cycle sequencing.2

Electrophoretic Mobility Shift Assays—A double-stranded DNA fragment containing the Myb-specific DNA-binding motif (2) was labeled by fill-in reaction using the Klenow fragment of DNA polymerase and [α-32P]dCTP (22). 6.25 fmol of labeled DNA was incubated with in vitro translated proteins in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 10 mM dithiothreitol) in a 20-μl final volume for 20 min at 25 °C. Non specific DNA binding was diminished by competition with both 50 ng/ml poly(dIdC) (Boehringer Mannheim, Germany) and 50 ng/ml M13 single-stranded DNA. DNA binding activity was competed specifically with an 800-fold molar excess of non-labeled, double-stranded DNA fragment containing the DNA-binding motif of Myb. The complex was resolved in 8% non-denaturing polyacrylamide:bisacrylamide (29:1) gels, 0.25% (w/v) TBE buffer (44.5 mM Tris borate, pH 8.4, 1 mM EDTA) and analyzed by use of a PhosphorImager (Molecular Dynamics, Krefeld, Germany).

In Vitro Protein Expression—The combined in vitro transcription/translation (TNT) system (Promega) was used to generate the 35S-labeled in vitro translated proteins. Reactions were performed according to the Promega TNT protocol, and [35S]methionine (Amersham, Buckinghamshire, UK) was used for radiolabeling. In vitro translation products were analyzed by SDS-PAGE and phosphorimaging (Molecular Dynamics, Krefeld, Germany).

Microinjection of Xenopus Oocytes and Analysis of Nuclear Translocation—Oocytes were removed from adult Xenopus laevis. Stage VNI oocytes (23) were isolated manually or by collagenase treatment (Worthington, Freeholds, NJ) and kept in normal strength modified Barth saline, 1 × MBSH (24). Approximately 50 nl of each of the different 35S-labeled fusion protein solutions (in the rabbit reticulocyte lysate) were injected into the cytoplasm of oocytes. After injection, the

1 The abbreviations used are: PCR, polymerase chain reaction; GST, glutathione-S-transferase; NLS, nuclear localization signal; PAGE, polyacrylamide gel electrophoresis; TRD, transport regulatory domain; XB-Myb, Xenopus B-Myb.

2 During the cloning procedure, we noted an error in the published sequence. This change implicates 26 amino acid residues at the C terminus. The corrected sequence has been submitted to GenBank (accession number M78570).

The oocytes were incubated for between 2 and 20 h in 1× MBSH at 18 °C. Nuclei and cytoplasmic fractions were manually dissected in ice-cold NET-2 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Nonidet P-40 supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mg/ml pepstatin), collected on ice (20 cytoplasmic and nuclear fractions per sample), and homogenized. The homogenates were centrifuged 3–4 times in a microcentrifuge (14,000 × g, 3 min) and the supernatant used for immunoprecipitation analysis.

Immunoprecipitation—Nuclear and cytoplasmic fractions were subjected to immunoprecipitation using monoclonal antibody against the c-myc epitope (Santa Cruz Biotech, Santa Cruz, CA). The myc antibody was bound to Protein G-GammaBind Plus Sepharose (Pharmacia), for 2 h at room temperature in NET-2 buffer. The PGS antibody pellets were washed three times with NET-2 buffer. Subsequently, supernatants of homogenized cytoplasmic or nuclear fractions of injected oocytes (see above) were added and incubated for 90 min at 4 °C. The immunoprecipitate was washed four times with NET-2 buffer, dried, and dissolved in 40 μl of SDS sample buffer. The samples were heated to 100 °C, loaded on a 10% SDS-polyacrylamide gel, and analyzed using a PhosphorImager.

Whole-mount in Situ Hybridization and Histology—Whole-mount in situ hybridization was carried out according to the procedure of Harland (25), using a digoxigenin-labeled antisense XB-myb RNA probe. RNA was generated using Bsp10I1-linearized p7773 XB-Myb and T7 RNA polymerase.

For sectioning, stained and postfixed embryos were gelatin-embedded and Vibratome-sectioned at 30 μm thickness and photographed under a phase-contrast microscope.

Western Blot Analysis—The myc-tagged in vitro translated proteins were resolved on a 10% SDS-polyacrylamide gel, electroblotted to nitrocellulose membrane, probed with the human c-myc monoclonal antibody (Santa Cruz Biotech) and detected using the ECL chemiluminescent detection system (Amersham). The x-ray films were quantified using a Bio-Rad densitometer (Bio-Rad Laboratories, Munich, Germany).

RESULTS

Spatial Expression Characteristics of B-myb during Xenopus Embryogenesis—B-myb is expressed during Xenopus embryogenesis, as revealed by Northern and Western blot analysis (2, 19). In order to characterize the spatial distribution of B-myb encoding transcripts, staged Xenopus embryos were subjected to whole-mount in situ hybridization with a B-myb-specific antisense RNA probe (Fig. 1). During neurula stages, B-myb is found to be exclusively expressed within the developing central nervous system. At the open neural plate stage, B-myb-specific signals are most prominent in the eye anlagen, as well as in the anterior portion of the neural plate (Fig. 1A). Upon closure of the neural tube, B-myb continues to be expressed in fore-, mid-, and hindbrain, as well as in the entire optic vesicle and in neural crest cells (Fig. 1, C and E). During further development up to the tadpole stage, this pattern is generally maintained (Fig. 1, B and D). In the embryonic eye, B-myb expression becomes restricted to the ciliary margin (Fig. 1F), a group of undifferentiated, proliferative cells that give rise to all major cell types of the retina (Fig. 1F) (26). A transverse section at the level of the hindbrain reveals that, within the neural tube, B-myb is preferentially expressed in the ventricular zone, which also contains proliferating, non-differentiated cells (Fig. 1G).

In summary, B-myb expression in the developing Xenopus embryo is specific to the developing central nervous system and to structures derived therefrom, such as the eye and neural crest/branchial arches. As development proceeds, B-myb in both the tadpole eye and the tadpole hindbrain is found to be preferentially expressed in proliferating, non-differentiated cells.

C-terminal Elements of B-Myb Inhibit DNA Binding in cis and in Trans—We have previously reported that full-length Xenopus B-Myb, either as a recombinant protein isolated from bacteria, or in its native form from Xenopus oocytes and em-
bryos, is inhibited in its DNA binding capacity. Removal of the C-terminal portion of the protein relieves this inhibition (2, 19). Extending these studies, we have now generated a more systematic set of C-terminal deletion mutants of B-Myb and have assayed for their DNA binding activity in order to map the inhibitory domain more precisely. Truncated versions of XB-Myb were produced by in vitro translation and analyzed in electrophoretic mobility shift experiments with a radiolabeled oligonucleotide containing the Myb DNA-binding site (Fig. 2). Assays were performed both in the presence and in the absence of a competitor oligonucleotide encompassing the Myb DNA-binding site (Fig. 2). Assays were performed both in the presence and in the absence of a competitor oligonucleotide encompassing the Myb DNA-binding site (Fig. 2).

Fig. 1. XB-myb is expressed in the developing nervous system during Xenopus embryogenesis. Whole-mount in situ hybridization experiments were carried out with XB-myb antisense RNA as a probe and staged Xenopus embryos. A, neural fold (stage 17) Xenopus embryo; arrowheads indicate eye anlagen. B and C, lateral view (B) and anterior view (C) of a tailbud (stage 24) Xenopus embryo. o.v., optic vesicle; n.c., neural crest; mes., mesencephalon; pro., prosencephalon. D, somite Xenopus embryo (stage 28); e., eye; b.a., branchial arches; ot.v., otic vesicle. E, transverse section of a stage 22 Xenopus embryo; mes., mesencephalon; op. ves., optic vesicle. F and G, transverse sections of a stage 29/30 Xenopus embryo; mes., mesencephalon; c.m., cerebral margin; ot.v., otic vesicle; rom., rhombencephalon. Note preferential staining within the ventricular zone of the rhombencephalon.
import in a manner similar to TFIIIA, which is used as a cytoplasmic retention control (Fig. 6B). This finding demonstrates that the N-terminal half of XB-Myb bearing the DNA-binding domains is not required for the inhibition of nuclear import and is therefore not likely to be involved in masking of the XB-Myb NLS function.

We further investigated the molecular mechanism responsible for the negative regulation of Xenopus B-Myb nuclear transport. Two principal modes of inhibition were considered, one involving interaction of the nuclear transport regulatory domain with a cytoplasmic anchor, and a second one relying on either masking of the NLS function via interaction with a molecule that is physically distinct from B-Myb, or via intramolecular folding.

If an anchoring mechanism is responsible for cytoplasmic retention of B-Myb, transfer of the nuclear transport regulatory domain to a different nuclear protein constitutively imported in Xenopus oocytes should result in its cytoplasmic retention. In order to test this possibility, ribosomal protein L5, which is constitutively transported to the nucleus in Xenopus...
oocytes (27), was fused to either full-length or truncated versions of *Xenopus* B-Myb. All of these constructs are imported into the nucleus after injection into the cytoplasm of *Xenopus* oocytes (Fig. 7). These findings demonstrate the dominance of a functional NLS over the cytoplasmic retention domain. It is therefore unlikely that interaction of the nuclear transport regulatory domain with a cytoplasmic anchor is responsible for the cytoplasmic sequestration of full-length *Xenopus* B-Myb as observed in *Xenopus* oocytes.

**DISCUSSION**

We report herein on the expression of B-myb during *Xenopus* embryogenesis and on the protein domains involved in DNA binding and nuclear transport, two aspects related to the regulation of XB-Myb function in transcription. The C-terminal portion of *Xenopus* B-Myb contains two distinct sequence elements responsible for the negative regulation of DNA binding and nuclear transport activities, respectively. The region between amino acid residues 425 and 458 is involved in the inhibition of DNA binding, presumably through intramolecular interaction between the N and C termini. The last 88 amino acid residues of the C terminus (TRD) negatively regulate nuclear transfer; this inhibition is likely to involve masking of the two separate NLSs, either by intra- or by intermolecular interactions.

Expression of XB-myb is preferentially detected in proliferating, non-differentiated neural cells in the developing *Xenopus* embryo. This correlates with the bona fide function of XB-Myb in cell division, as has been proposed for mammalian B-Myb (28). In mouse, B-myb is also mainly expressed in the developing central nervous system, especially in highly proliferating cells (5). XB-myb is expressed initially as a continuous unit in the anterior neural plate, later becoming more strongly expressed in the lateral regions of the anterior neural plate and fading from the median region. These two domains of expression will give rise to the eye primordia (29). The expression of XB-myb in the developing eye anlage correlates with those of Pax 6 (30) and ET (29). Studies involving these genes have shown the existence of a single retina field, which splits into two distinct primordia (29). Sections of neurula-stage embryos show that XB-myb is initially detected in the whole retinal neuroepithelium. At later stages it becomes restricted to the cells of the retinal ciliary margin, the multipotent retinal progenitor cells (26). It remains to be demonstrated whether XB-Myb also regulates retinal proliferation.

We show that a serine/threonine-rich region (amino acids 369–545) that includes two NLSs is required for efficient nuclear import of XB-Myb and the negative regulation of DNA binding. This region is highly conserved among Myb family members, including human, mouse, *Xenopus*, and chicken B-Myb, implying that it has a conserved function. Such a domain is a potential target of phosphorylation, and it displays two ankyrin-like repeats (31). The ankyrin motif has been related to both cell cycle control and differentiation (31), and these repeats have also been implicated in protein-protein interac-

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**Fig. 3.** The C-terminal XB-Myb fragment inhibits DNA binding activity in *trans*. A, electrophoretic mobility shift assay with full-length (FL) XB-Myb, an N-terminal fragment (N) that contains the DNA-binding repeats (DNA-BD), and an internal fragment (C) that contains the DNA-binding regulatory domain (DBRD). N + C is a mixture of the two latter proteins. Cx denotes the position of the specific complex, F the position of the free probe. B, titration of the in *trans* inhibitory effect of the internal fragment (C) on the DNA binding activity of the N-terminal fragment (N). C, Western blot of the proteins utilized in the experiment shown in panel B.
Such interactions have indeed been shown in mammalian cells in culture for c-Myb (33), and in Xenopus oocytes for B-Myb.3

The region responsible for DNA binding inhibition lies at the C terminus of XB-Myb (amino acids 425–458). Several observations on the activation of B-Myb correlate with results obtained for the regulation of c-Myb activities. Deletion of the C terminus of c-Myb increases its transcriptional activation capacity (34–36). In addition, the suppressor activity of the negative regulatory domain in c-Myb functions in cis and in trans (36). The negative regulatory function of the C terminus in B-Myb has been detected at different levels of B-Myb activity. The transactivation potential of B-Myb in transient transfection assays has been found to be inhibited at several cell lines. The inhibition can be relieved either by introducing C-terminal truncations or by coactivation of the cell cycle-regulated protein kinase cdks2, which appears to use the C terminus of B-Myb as a direct substrate (16–18). We previously reported that maternal Xenopus B-Myb became hyperphosphorylated upon meiotic maturation of oocytes, but that this did not affect binding to DNA (19). Our finding that the C-terminal portion of Xenopus B-Myb inhibits the DNA binding activity of the N-terminal portion in trans supports a model whereby a direct intramolecular interaction is responsible for the negative regulation of DNA-complex formation observed. This mechanism would not require additional factors interacting with the DNA-binding regulatory domain, although some of the interacting factors observed for XB-Myb in oocytes3 might function in the relief of inhibition and promote transcriptional activation later on in embryogenesis.

Intramolecular inhibition of DNA binding has been described for other proteins. An excellent example is the study of the Ets family of transcription factors. DNA binding by Ets-1 is prevented by intramolecular interactions involving the N terminus, the ETS domain, and nearby sequences. DNA-binding inhibition is relieved by conformational changes that occur in the presence of DNA. These changes are thought to promote

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cooperative binding of a stabilizing protein partner (37, 38). Although intramolecular interactions participate in DNA-binding inhibition of XB-Myb, in contrast to Ets-1, the inhibition takes place even in presence of the DNA target. Our results open the way for further investigations about the nature of positive regulation of B-Myb mediated by a protein partner or by posttranslational modifications.

In full-grown oocytes XB-Myb is cytoplasmic, and during embryogenesis XB-Myb localizes in the nucleus. We analyzed the mechanism of negative regulation of XB-Myb in oocytes that would prevent XB-Myb functioning as a transcription factor. We have shown that the nuclear-cytoplasmic distribution of XB-Myb involves at least two different protein elements: the TRD, responsible for the cytoplasmic retention of the protein, and two NLSs, which are necessary for efficient nuclear import of XB-Myb. Our data suggest that both NLSs are functionally cooperative and would be the bona fide domains involved in nuclear import in the embryo. According to our data, the molecular mechanism responsible for the negative regulation of XB-Myb nuclear targeting in oocytes is likely to involve masking of the NLS. We showed the dominance of an additional NLS over the TRD in the full-length protein, indicating that the

FIG. 6. The C terminus but not the DNA-binding domain is involved in the inhibition of XB-Myb nuclear transfer. A, temperature dependence of the nuclear transport of an internal XB-Myb protein fragment that contains NLS1 and NLS2. Microinjected oocytes were incubated for 5 h at either 4 °C or 18 °C prior to separation of nuclear and cytoplasmic fractions. TFIIIA, which is retained in the cytoplasm, was coinjected with the XB-Myb variants as an internal control for cytoplasm contamination in nuclear fractions (A and B). B, the transport regulatory domain inhibits active transport of the internal XB-Myb fragment in Xenopus oocytes.

FIG. 7. Fusion of an additional NLS function relieves cytoplasmic retention of XB-Myb in Xenopus oocytes. Different portions of XB-Myb were fused to ribosomal protein L5, which is constitutively transported to the nucleus of Xenopus oocytes; the structure of the different fusion constructs is indicated. Oocyte microinjections and protein processing were performed as described in Fig. 4. A and B, fusion of a partial or of the entire TRD from XB-Myb does not interfere with nuclear transport of ribosomal protein L5 in Xenopus oocytes. C, fusion of full-length XB-Myb to ribosomal protein L5 does not interfere with nuclear transport in Xenopus oocytes.
extra signal promotes XB-Myb nuclear entry. This finding excludes the possibility of a cytoplasmic anchoring mechanism. Thus, the retention of XB-Myb in the oocyte cytoplasm could be mediated either by intranuclear or intermolecular interactions that mask the NLS.

If the NLS function in Xenopus B-Myb is inhibited by interaction with other proteins that mask the B-Myb NLSs, such interacting proteins should be present in Xenopus oocyte extracts. As an initial step toward the identification of such interacting proteins, radiolabeled oocyte proteins were incubated with full-length and truncated versions of immobilized Xenopus B-Myb. Putative inhibitors should bind to full-length or shortened versions of Xenopus B-Myb that were not able to travel to the nuclear compartment, as analyzed in the oocyte injection assays (see above). Such experiments do indeed detect proteins that interact with a recombinant Xenopus B-Myb/GST fusion, but not with the GST extension alone. However, there was no clear correlation between inhibition of nuclear transfer and binding of any of these proteins (data not shown).

B-Myb phosphorylation could be part of the mechanism that regulates DNA binding and/or nuclear transfer, two activities that define prerequisites for transcription activation by B-Myb. However, phosphorylation of B-Myb during oocyte maturation was not found to be sufficient to activate the DNA binding activity of B-Myb (19). Furthermore, the distribution of B-Myb between nucleus and cytoplasm does not correlate with cell cycle activities (39). Thus, the regulation of B-Myb as a transcriptional activator is not likely to be due to a simple, phosphorylation-mediated switch between binding and not binding to B-Myb-dependent promoters, or between cytoplasmic and nuclear localization, but seems to rely on a more complex mechanism. This mechanism might involve all three processes. Further investigation will elucidate the role of hyperphosphorylation in the regulation of XB-Myb activities.

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