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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.

The Myb-type DNA binding repeat motif defines a growing family of proteins in animals, plants, and lower eukaryotes. Three members of this family, termed c-Myb, A-Myb, and B-Myb, have been described in vertebrates (reviewed in Ref. 1). Though structurally related, these three vertebrate Myb variants appear to fulfill very different biological functions. On the basis of their expression characteristics, it has been proposed that c-Myb and A-Myb exert cell type-specific activities, whereas B-Myb seems to serve a more general function.

B-*myb* expression in *Xenopus* is maternal, and transcripts can be detected throughout embryogenesis as well as in several adult tissues (2). The human and murine B-*myb* encoding genes are also widely expressed in different tissues during embryogenesis, with differential expression during testis development (3–5). Interestingly, B-*myb* expression in mammals has been linked to proliferative activity (5). Earlier B-Myb promoter studies have already identified a negative regulatory element that is responsible for repression of B-*myb* transcription in G_0 via the activity of the E2F DNA-binding protein (6). B-*myb* expression is induced at the G_1/S transition of the cell cycle (7–10). Furthermore, ectopic expression of B-*myb* can induce DNA synthesis in certain cell types (11). Taken together, these observations strongly suggest that B-Myb has a general function in cell division.

Mapping of functional domains in human B-Myb identified a DNA-binding domain located at the N terminus, followed by a transcriptional activation domain; it has been reported that the carboxyl portion of the human protein contains two separate nuclear localization signals (12, 13). B-Myb can activate transcription through two very different mechanisms. The first is independent from its DNA-binding domain and operates, probably indirectly, via the heat shock element (14), whereas the second depends on the B-Myb DNA-binding domain and works on promoters that contain B-Myb DNA-binding sites (15). The latter mechanism has been found to depend on the cell-type utilized in transient transfection studies, and this has been suggested to reflect the requirement for a specific cofactor in transactivation (3). The direct physical interaction of B-Myb with another protein may also negatively regulate its activity; inhibition of B-Myb-dependent transactivation correlates with specific binding of p107, an Rb-related protein (11).

More recently, it has been demonstrated that the transactivation function of B-Myb is subject to post-translational regulation in a cell cycle-dependent manner. Phosphorylation by cdk2 in the C-terminal portion of the protein is required to activate the transactivation function of full-length B-Myb (16–18). A similar effect in transient transfection can be reproduced by C-terminal truncations (17, 18). It therefore appears that the B-Myb C terminus negatively regulates transactivation and that this repression is relieved by cell cycle-dependent phosphorylation. However, the exact molecular events that are initiated by this modification remain to be solved.

We have previously described that both DNA binding activity and nuclear transfer of the full-length B-Myb protein are repressed in stage V/VI *Xenopus* oocytes and that DNA binding activity is relieved upon C-terminal truncation of the protein. Furthermore, we were able to demonstrate that maternal B-Myb becomes phosphorylated upon meiotic maturation, but that these phosphorylation events were not sufficient to activate DNA binding of the full-length *Xenopus* B-Myb protein (2, 19).

This study elucidates the molecular mechanisms that are responsible for the negative regulation of DNA binding activity and nuclear transport of *Xenopus* B-Myb. Deletion mutagenesis identifies two distinct elements within the C terminus of *Xenopus* B-Myb that are responsible for repression of DNA binding and nuclear transport, respectively. Two independent, functional nuclear localization signals were mapped in the C-terminal portion of B-Myb. We also demonstrate that the C terminus of the *Xenopus* B-Myb protein can inhibit the DNA binding activity of the N-terminal portion in *trans*. These and other observations suggest that intramolecular folding may be

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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 *Exo*III/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 *Bam*HI site upstream of the XB-*myb* translation start ATG was generated by PCR[1] using sense (5′ GTC CGG ATC CGC AGA ***ATG*** TCC CGG CGG 3′) and antisense (5′ TAT TTC AGC CCA TCG ATT 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 derived by cutting with *Bam*HI and *Cla*I was generated. In parallel a DNA fragment was generated from full-length and truncated pSP64T XB-Myb by restriction digestion using *Cla*I and *Sal*I. Both fragments (*Bam*HI-*Cla*I and *Cla*I-*Sal*I) were used for simultaneous ligation into pGEX-5X-1 vector (Pharmacia, Uppsala, Sweden). The resulting 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 Myc-tag vector pCS2MT (21) by cutting the GST clones with *Bam*HI/*Sal*I, performing a fill-in reaction, and cloning into *Xho*I/filled-in pCS2MT vector. Site-directed mutation of the NLS1 was generated by PCR with the sense primer (5′ TCT GTG CTG AAA CAA CAC AAC AAC AGA AAC ATT ACC CTG TCA CCT GTT ACA G 3′) and the antisense primer (5′ CTG TAA CAG GTG ACA GGG TAA TGT TTC TGT TGT TGT GTT GTT TCA GCA CAG A 3′) using the Quick Change system (Stratagene, La Jolla, CA).

All the constructs were manually sequenced using either Sequenase (U. S. Biochemicals) or an Applied Biosystems sequencing system, using *Taq* 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-^{32}P]dCTP (22). 6.25 fmol of labeled DNA were incubated with *in vitro* translated proteins in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol) in a 20-μl final volume for 20 min at 25 °C. Nonspecific DNA binding was diminished by competition with both 50 ng/ml poly(dI·dC) (Boehringer Mannheim, Germany) and 50 ng/ml M13 single-stranded DNA. DNA binding activity was competed specifically with an 800-fold molar excess of nonlabeled, 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[time] 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 ^{35}S-labeled *in vitro* translated proteins. Reactions were performed according to the Promega TNT protocol, and [^{35}S]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 Transport—Oocytes were removed from adult *Xenopus laevis*. Stage V/VI 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 ^{35}S-labeled fusion protein solutions (in the rabbit reticulocyte lysate) were injected into the cytoplasm of oocytes. After injection, 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 9E10 epitope of human c-Myc protein (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 *Bsp*106I-linearized pT7T3 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 chemiluminiscent 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. 1*A*). 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. 1*F*), a group of undifferentiated, proliferative cells that give rise to all major cell types of the retina (Fig. 1*F*) (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. 1*G*).

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-

[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 implies an addition of 26 amino acid residues at the C terminus. The corrected sequence has been submitted to GenBank (accession number M75870).

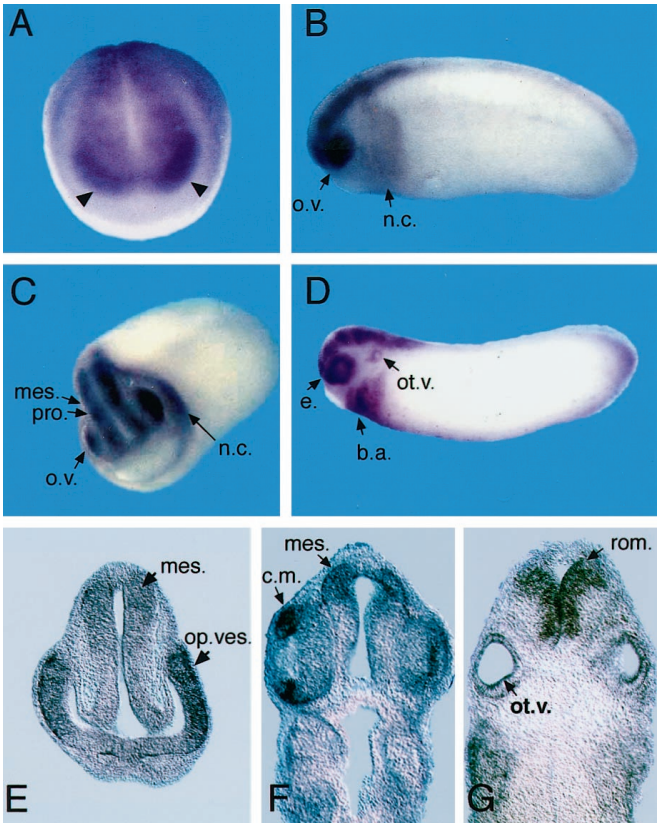


FIG. 1. **XB-*myb* is expressed in the developing nervous system during *Xenopus* embryogenesis.** Whole-mount *in situ* hybridization experiments were carried out with XB-*myb* antisense RNA as a probe and staged *Xenopus* embryos. *A*, neural fold (stage 17) *Xenopus* embryo; *arrowheads* indicate 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.*, ciliary margin; *ot.v.*, otic vesicle; *rom.*, rhombencephalon. Note preferential staining within the ventricular zone of the rhombencephalon.

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 B-Myb recognition site, in order to test for specificity of complex formation. C-terminal truncations of up to 275 amino acids do not relieve inhibition of DNA binding, but deletion of 308 amino acids recovers maximal DNA binding activity. Thus, a protein element located between the amino acids in position 425 and 458 is sufficient to fully inhibit the specific DNA binding capacity of the N-terminal XB-Myb repeats; we refer to this region as the DNA-binding regulatory domain.

The fact that both full-length XB-Myb from *Xenopus* oocytes/embryos and the recombinant, bacterially expressed protein are inactive in DNA binding had already suggested that the inhibitory mechanism does not rely on a specific corepressor protein. Alternatively, inhibition of DNA binding could be a consequence of intramolecular folding and interaction between C- and N-terminal domains of XB-Myb. We therefore tested the possibility that a C-terminally derived fragment containing the entire DNA-binding regulatory domain inhibits the DNA binding activity of the N-terminal fragment in *trans*. Electrophoretic mobility shift assays with a constant amount of the N-terminal DNA-binding domain in the presence of increasing amounts of the C-terminal fragment containing the DNA-binding regulatory domain result in gradually reduction of DNA-complex formation (Fig. 3). Taken together, these results indicate that a sequence element in XB-Myb located between residues 425 and 458 negatively regulates the DNA binding activity of XB-Myb by intramolecular folding and mediates direct interaction between N- and C-terminal domains of XB-Myb.

Positive and Negative Regulatory Elements for the Nuclear Transport of B-Myb in Xenopus Oocytes—We previously reported that neither full-length XB-Myb nor the N-terminal half of the protein are translocated to the nucleus of *Xenopus* oocytes (19). For a more detailed analysis, a systematic series of progressive C-terminal deletion mutants of XB-Myb was produced by *in vitro* translation and injected into the cytoplasm of stage V/VI *Xenopus* oocytes. After 18 h of incubation, nuclear and cytoplasmic fractions were manually separated and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4).

As reported earlier, full-length XB-Myb was not found to be imported into the nucleus, and only background levels of the protein could be recovered from the nuclear fraction (19). Our work shows that a C-terminal deletion, spanning amino acids 655–733, partially activates nuclear transport. A bigger deletion of the C terminus, encompassing amino acids 543–733, enhances nuclear transfer activity. Further removal of sequence elements containing one of two putative basic NLSs strongly reduces nuclear transport, while additional deletion of the second putative NLS fully inhibits the same process. A protein fragment containing both of the putative NLSs but lacking the C-terminal negative regulatory domain, as well as the N-terminal half of XB-Myb, carries full nuclear import activity.

The activity of the two putative NLSs was therefore analyzed in more detail. Kinetic analysis of the nuclear import with XB-Myb lacking the C-terminal inhibitory domain reveals a significant level of nuclear transfer even after 2 h (Fig. 5). Conversely, mutation of NLS1 or deletion of NLS2 results in significantly reduced import kinetics. Site-directed mutagenesis of NLS1 in a deletion mutant that already lacks NLS2 leads to a further reduction of import to background levels. Taken together, these observations define two physically separate, but functionally cooperative, nuclear localization signals in XB-Myb, which are negatively regulated by the C-terminal portion of the protein.

Analysis of the Molecular Mechanism for B-Myb Cytoplasmic Retention in Xenopus Oocytes—The experiments on the regulation of B-Myb DNA binding activity described above argued for an intramolecular interaction of N- and C-terminal portions of the protein that might directly involve the DNA-binding N-terminal repeats. If such an interaction were to occur, it could also be responsible for the inhibition of nuclear transfer, for example by masking the NLS function. To test this possibility, the XB-Myb internal fragment sufficient for efficient nuclear transfer (residues 349–545, Fig. 4) was characterized in further detail. The molecular mass of the corresponding protein (roughly 46 kDa) is within the protein size range that is believed to allow passive nuclear import by diffusion. However, its import is strongly temperature-dependent (Fig. 6*A*), arguing for the existence of an active import mechanism. Fusion of the C-terminal transport regulatory domain completely ablates

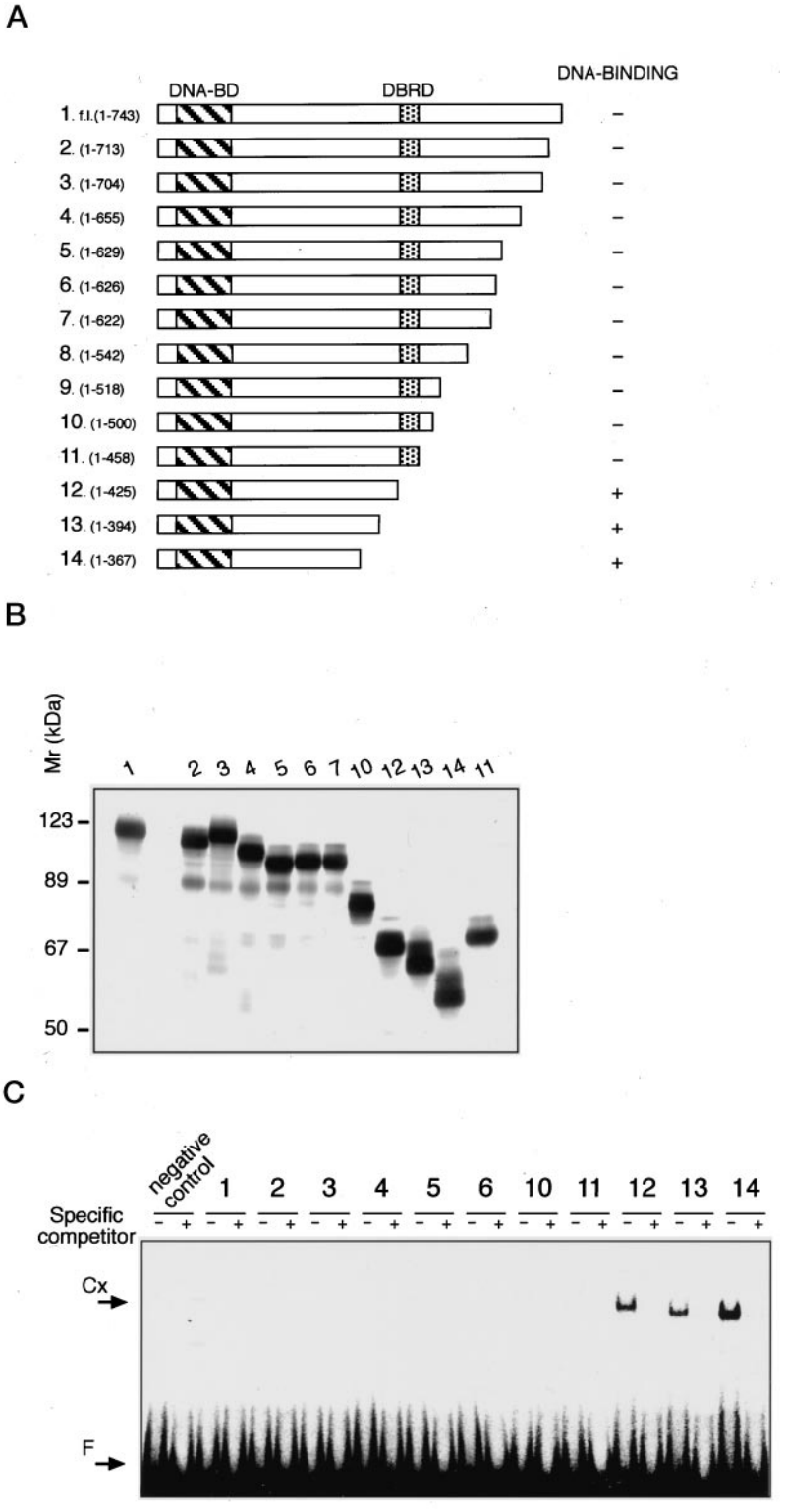


FIG. 2. **Mapping of the DNA-binding regulatory domain in *Xenopus* B-Myb.** The DNA binding activity of a progressive series of C-terminal XB-Myb deletion mutants analyzed by electrophoretic mobility shift assays with the Myb-specific consensus DNA recognition site. *A*, schematic representation of C-terminal deletion mutants utilized; deletion end points as well as DNA binding activities are indicated. The positions of the DNA-binding repeats (*DNA-BD*) (40, 41) and of the DNA-binding regulatory domain (*DBRD*) as determined in this series of experiments are indicated. *B*, SDS-polyacrylamide gel electrophoresis of the *in vitro* transcription/translation products; numbering according to the schematic representation as shown in *panel A*. Proteins were radiolabeled by incorporation [^{35}S]methionine. *C*, electrophoretic mobility shift assay with the ^{32}P-radiolabeled Myb consensus DNA recognition site. Proteins were as shown in *panels A* and *B*. *Cx* denotes the position of the specific complexes, *F* the position of the free probe. Assays were performed either in the presence (+) or absence (−) of specific competitor DNA.

import in a manner similar to TFIIIA, which is used as a cytoplasmic retention control (Fig. 6*B*). 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*

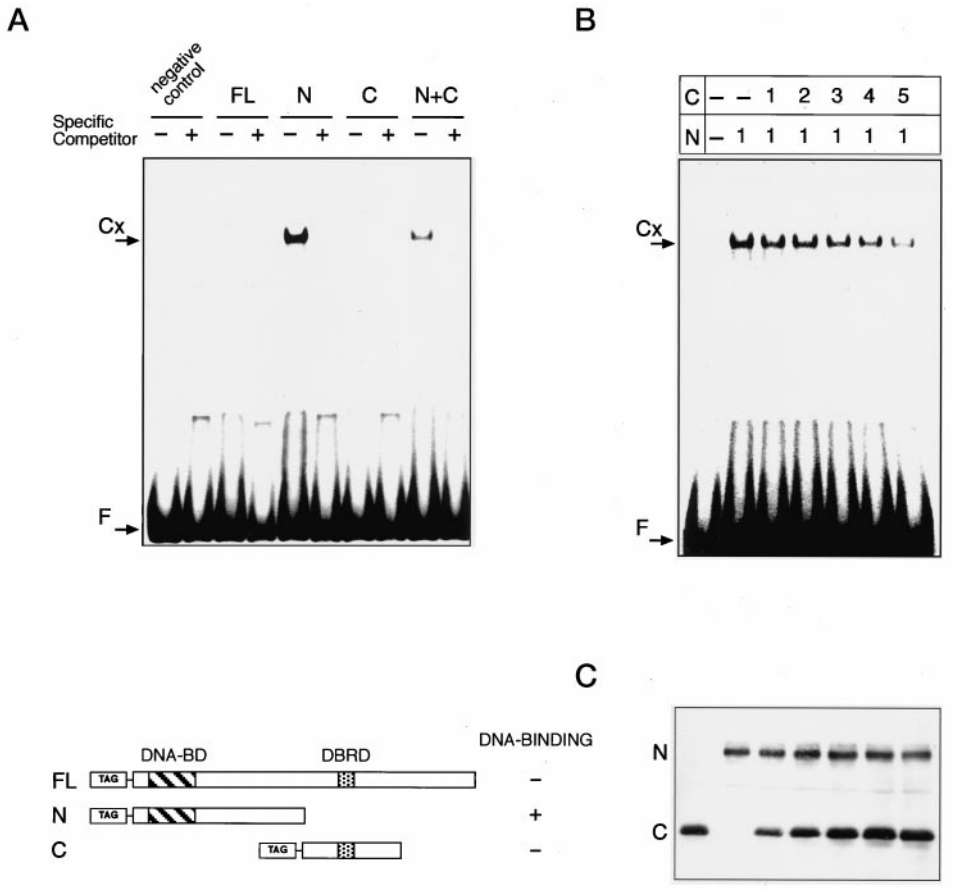


FIG. 3. **The C-terminal XB-Myb fragment inhibits DNA binding activity in *trans*.** *A*, electrophoretic mobility shift assay with full-length (*FL*) XB-Myb, an N-terminal fragment (*N*) that contains the DNA-binding repeats (*DNA-BD*), and an internal fragment (*C*) that contains the DNA-binding regulatory 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*.

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 raise 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-

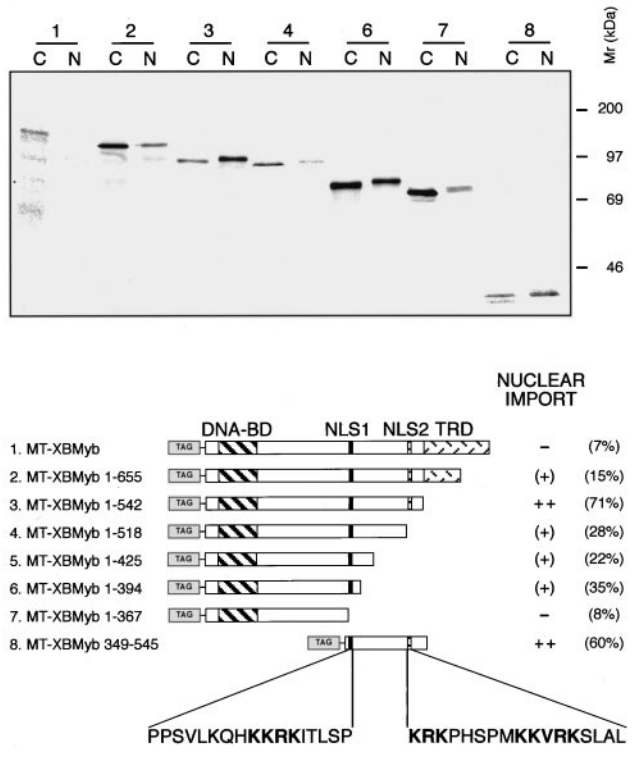


FIG. 4. **Mapping of the nuclear transport regulatory domain in *Xenopus* B-Myb.** ^{35}S-Labeled XB-Myb variants produced *in vitro* were microinjected into the cytoplasm of *Xenopus* oocytes. After 18 h of incubation, nuclear (*N*) and cytoplasmic (*C*) fractions were separated manually and analyzed for XB-Myb protein content by immunoprecipitation and SDS-gel electrophoresis. The nuclear accumulation is indicated as a percentage of total protein recovery in the nuclear fraction. The schematic representation shows the different deletion mutants utilized. *TRD*, transport regulatory domain; *NLS1&2*, nuclear localization signals 1 and 2.

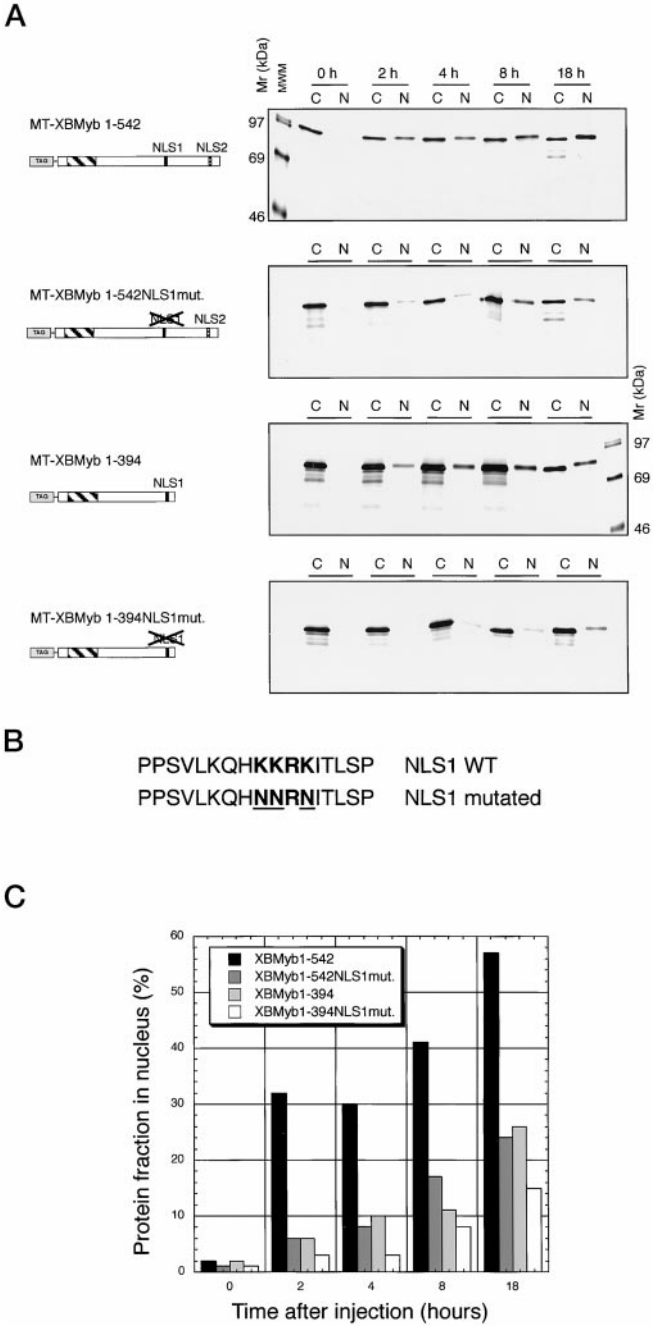


FIG. 5. **XB-Myb carries two physically separate and functionally cooperative nuclear localization signals.** *A*, kinetics of the nuclear transfer of N-terminal XB-Myb fragments containing mutations in either one of the two NLSs, or in both. Oocyte microinjections and protein recovery were performed as described in the legend to Fig. 4. The duration of oocyte incubation after protein microinjection is indicated above the assays. *B*, site-directed mutagenesis was performed in the NLS1. Three lysine residues were replaced by asparagines. *C*, quantification of the nuclear transfer as a percentage of total protein recovery in the nuclear fraction given in the form of a bar diagram for the four different protein variants as indicated.

tions (32). 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 in several cell lines. The inhibition can be relieved either by introducing C-terminal truncations or by coactivation of the cell cycle-regulated protein kinase cdk2, 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 oocytes[3] 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

[3] G. Humbert-Lan and T. Pieler, unpublished results.

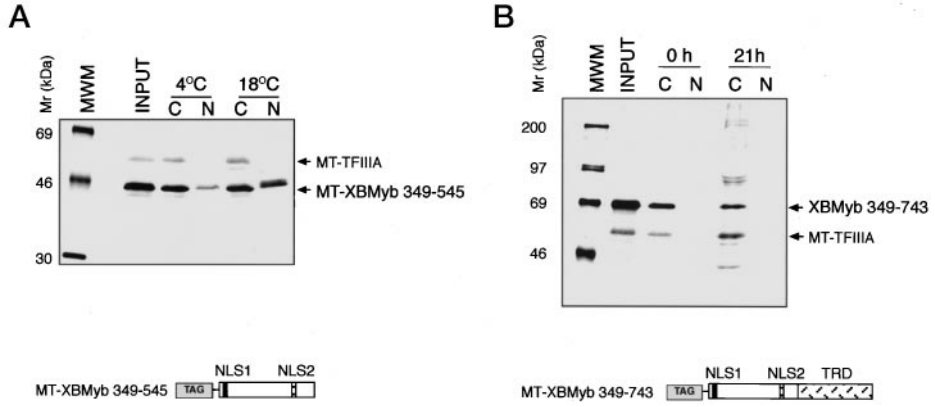


FIG. 6. **The C terminus but not the DNA-binding domain is involved in the inhibition of XB-Myb nuclear transfer.** *A*, temperature dependence of the nuclear transport of an internal XB-Myb protein fragment that contains NLS1 and NLS2. Microinjected oocytes were incubated for 5 h at either 4 °C or 18 °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.

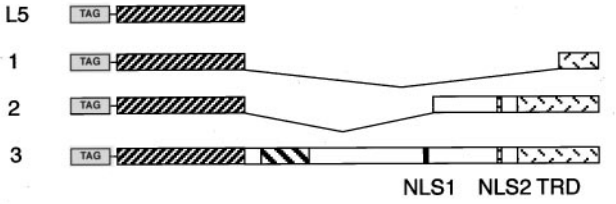
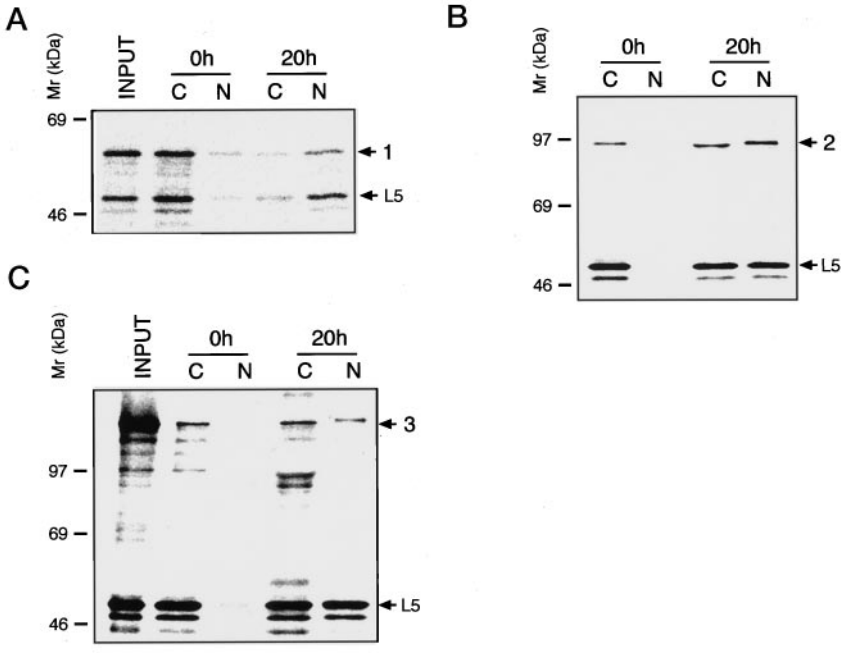


FIG. 7. **Fusion of an additional NLS function relieves cytoplasmic retention of XB-Myb in *Xenopus* oocytes.** Different portions of XB-Myb were fused to ribosomal protein L5, which is constitutively transported to the nucleus of *Xenopus* oocytes; the structure of the different fusion constructs is 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.



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

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 intra- 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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