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Multiple nuclear localization signals of the B-*myb* gene product

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Abstract

Nuclear entry of the B-*myb* gene product (B-Myb) is dependent on multiple nuclear localization signals (NLS's). Mutagenesis of the putative NLS's of B-Myb has identified two separate NLS's, NLS1 and NLS2. Each of the two NLS's is essential for efficient nuclear targeting. NLS2 contains two interdependent basic domains separated by 8 intervening spacer amino acids, and both basic domains are required for nuclear entry. Thus, NLS2 belongs to a class of bipartite NLS's. Like the NLS's in yeast transcription factor SWI5, NLS2 contains a putative *cdc*2 kinase site. However, unlike the case of SWI5, phosphorylation at this site did not affect the nuclear targeting of B-Myb.

Key words: B-*myb* gene; Transcriptional regulator; Nuclear localization signal; Phosphorylation

1. Introduction

The c-*myb* proto-oncogene encodes a nuclear phosphoprotein (c-Myb) that is mainly expressed in immature haematopoietic cell lineages (for review, see [12]). c-Myb is important in the regulation of proliferation and differentiation of haematopoietic progenitor cells [3,7,15,27]. c-Myb binds to specific DNA sequences and functions as a transcriptional activator [1,16,19,20,29]. Three functional domains, which are responsible for DNA binding, transcriptional activation, and negative regulation, respectively, were identified in c-Myb [25].

Two c-*myb*-related genes, A-*myb* and B-*myb*, were isolated [21]. The sequences of A-*myb* and B-*myb* genes show extensive homology to c-*myb*, suggesting that both gene products might be important in cellular proliferation and differentiation like c-Myb. The expression of the B-*myb* gene was observed in not only the hematopoietic cell lineage but also a variety of cells, including fibroblasts [21]. In addition, the B-*myb* mRNA levels increase in the G_1 periods of the cell cycle [8,22], and are down-regulated during macrophage differentiation [22]. These results support the idea that B-Myb is also involved in the regulation of cellular proliferation and differentiation. Previously we showed that B-Myb acts as a *trans*-activating factor [13,17]. In contrast, it was reported that the chicken and mouse B-Myb failed to *trans*-activate several Myb-responsive gene constructs, suggesting that B-Myb functions as a repressor [6,28]. Our recent analyses to resolve this discrepancy indicate that B-Myb has a *trans*-activating capacity in a cell type-specific manner (data not shown). The subcellular distribution of B-Myb is mainly controlled by the central large domain and C-terminal basic region [18], but the essential regions for nuclear localization are still obscure.

In this paper we have identified two nuclear localization signals (NLS's) required for the nuclear entry of B-Myb and examined some characteristics of these two NLS's.

2. Materials and methods

2.1. Preparation of a GST-B-Myb fusion protein and B-Myb-specific polyclonal antibodies

The *Bgl*II–*Bam*HI fragment prepared from pact-B-*myb* [13] was subcloned into the *Bam*HI site of the pGEX-1 vector [26], which allows the expression of a foreign polypeptide as a fusion protein with glutathione *S*-transferase (GST). Since the presence of the C-terminal portion of B-Myb interfered with the solubilization of GST-B-Myb, the C-terminal coding region was deleted as follows. The *Acc*III site (nucleotide 1872; nucleotide numbers are as in [21]) was changed to a blunt end followed by ligation, which caused a frame shift so that a termination codon was introduced 6 amino acids codons past this *Acc*III site. The generated plasmid, pGST-B-MybΔC, encoded a fusion protein containing the 322-amino acids region of B-Myb (amino acids 260–581). Expression and purification of GST-B-Myb were essentially as described by Smith and Johnson [26] except for the induction of fusion proteins at 25°C overnight. An antiserum was raised in a rabbit against the GST-B-Myb fusion protein, and purified using affinity colums in which GST or GST-B-Myb was used as the ligand.

2.2. Plasmid construction

All plasmids designed to express mutant B-Myb proteins in cultured cells were generated from the B-Myb expression vector, pact-B-*myb*, in which the 5′ regulatory region of the chicken cytoplasmic β-actin gene is linked to the human B-*myb* cDNA [13]. Oligonucleotide-directed mutagenesis [11] was used to make the B-Myb mutant expression vectors. ΔNLS1 was generated by an in-frame deletion of nucleotides 1358–1378 using oligonucleotides containing sequences on either side of the region to be deleted. M2, M4, and ΔNLS2 were constructed by the introduction of a stop codon at nucleotides 1847, 1889, and 1817, respectively. To construct M6, nucleotides 1817–1846 were deleted, and then the *Sca*I–*Mro*I fragment (nucleotides 1656–1871) was replaced by the *Sca*I–*Mro*I fragment of M4. ΔNLS1 + M2, ΔNLS1 + M4, ΔNLS1 + M6, and ΔNLS1 + 2 were generated by combination of M2, M4, M6, and ΔNLS2 with the ΔNLS1 mutant, respectively. To make the potential *cdc*2 kinase site mutants (S/A, S/D, and S/E), serine-577

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(nucleotides 1856–1858) was converted into alanine, aspartic acid, and glutamic acid, respectively.

2.3. DNA transfection and CAT assay

CAT co-transfection experiments using African green monkey kidney cells (CV-1), the CAT reporter plasmid pA10CAT6MBS-I or p*myc*CAT, and the B-Myb expression vector pact-B-*myb* were performed as described [18,20].

2.4. Western blotting of B-Myb mutants and immunofluorescence studies

Western blotting to detect B-Myb protein expressed in the transfected cells was performed by using anti-B-Myb rabbit polyclonal antibodies αB-MybAb as described [18]. The intracellular distribution of B-Myb was analyzed by using αB-MybAb as described [18], except that cells were fixed with 3% formaldehyde and permeabilized with 0.5% Triton X-100.

3. Results

3.1. Identification of two NLS's

By using various deletion mutants of B-Myb, we had demonstrated that the C-terminal basic region (amino acids 564–584: termed NLS2) and the central portion (amino acids 373–464) are important for nuclear entry of B-Myb [18]. In fact a deletion of NLS2 considerably, but not completely, impaired nuclear uptake of B-Myb. To abolish the nuclear entry of B-Myb, a deletion of the central portion in addition to NLS2 is required. In this central portion, the basic region (KRQRKRR) (amino acids 411–417: termed NLS1) is located. By the calculations as described by Hoop and Woods [10] and Emini et al. [5], both NLS1 and NLS2 were predicted to be hydrophilic and on the surface of the molecule, suggesting that both regions are good candidates for NLS (Fig. 1A). To examine directly whether NLS1 is critical for nuclear entry of B-Myb, a plasmid expressing a mutant lacking only NLS1 (ΔNLS1) was constructed (Fig. 1A,B). At the same time, this mutation was combined with the deletion mutation of NLS2 (ΔNLS2) to generate the mutant ΔNLS1 + 2, since a deletion of NLS1 alone was expected to only partially affect the nuclear targeting of B-Myb (Fig. 1A,B). The plasmids expressing B-Myb mutants were transfected into CV-1 cells, and the expressed B-Myb proteins were detected by

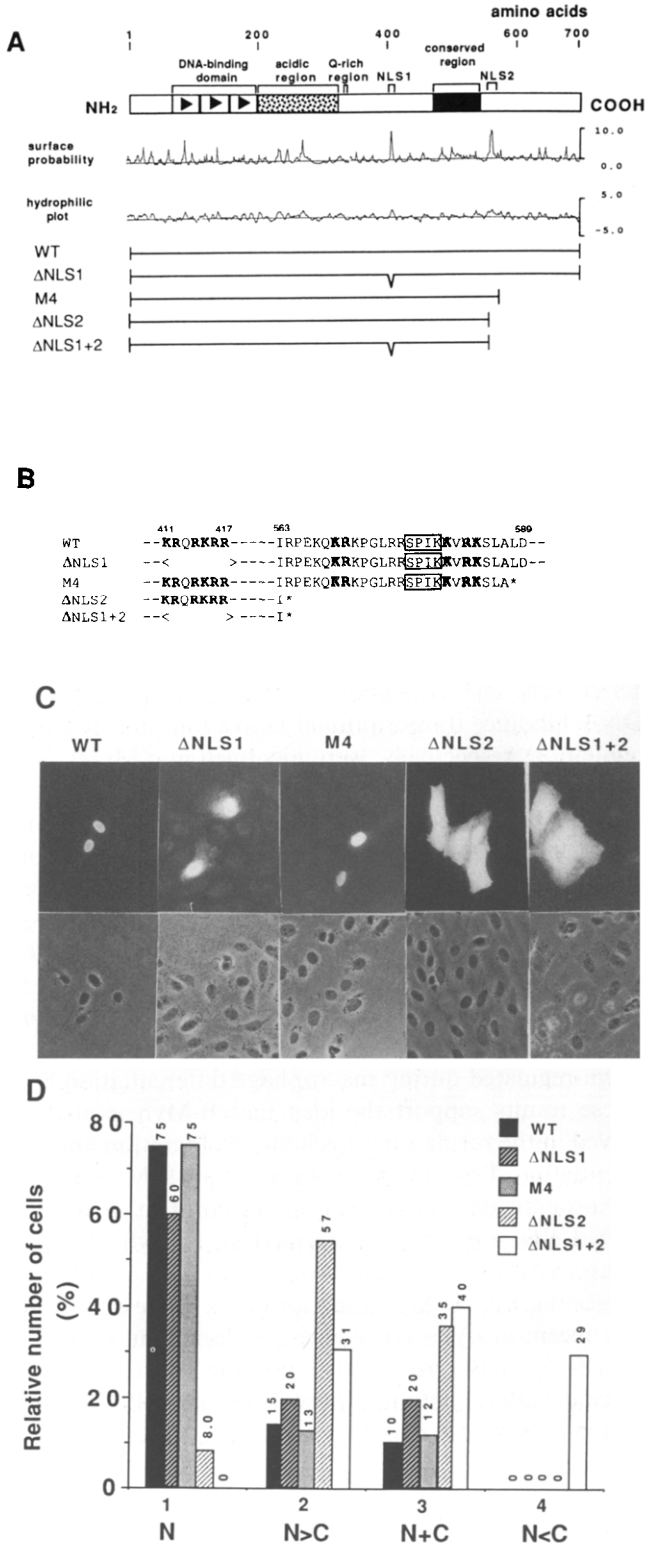

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Fig. 1. Role of two NLS's in the nuclear import of B-Myb. (A) Schematic representation of deletion mutants. On the top, the functional domains are shown. The surface probability and the hydrophilic plot of B-Myb are also indicated. Wild-type and four deletion mutants are schematically shown. (B) The amino acid sequence of the region around two NLS's in the various forms of B-Myb. The asterisk indicates the stop codon. The basic amino acids in NLS1 and the potential *cdc*2 kinase site within NLS2 are shown by bold letters and a box, respectively. The basic amino acids conserved in NLS2 and the NLS of *Xenopus* nucleoplasmin [24] are indicated by window letters. (C) A typical example of the subcellular distribution. CV-1 cells transfected with plasmids that express wild-type (WT) and mutant B-Myb proteins indicated above each column were stained with αB-MybAb and fluorescein-labeled second antibody (upper panel). Cells were also viewed by phase-contrast microscopy (lower panel). (D) Summary of the subcellular distribution. The transfected cells were scored by counting those in which immunofluorescence staining was exclusively nuclear (N), in which nuclear concentration was evident but where some cytoplasmic staining could be seen (N > C), in which immunofluorescence staining was observed almost equally in both nuclei and cytoplasm (N + C), and in which cytoplasmic staining was predominant (N < C). About 100 cells per each construct were scored, and the bar graphs give the precentage of cells in each category.

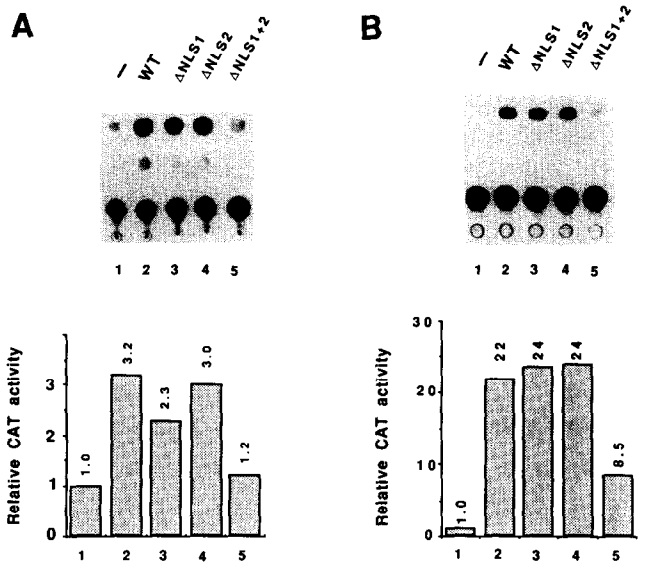

Fig. 2. Effects of the NLS deletion on the *trans*-activating capacity of B-Myb. (A) *trans*-Activation of pA10CAT6MBS-I by B-Myb mutants. A mixture of 6 μg of the pA10CAT6MBS-I reporter plasmid, 6 μg of the effector plasmid to express various B-Myb proteins shown above each lane or no protein (–), and 2 μg of the internal control plasmid pRSVβ-gal were transfected into CV-1 cells, and the CAT activity was assayed for 5 h. Relative CAT activity is indicated by a bar graph, below. (B) *trans*-Activation of p*myc*CAT by B-Myb mutants. The CAT co-transfection experiments with the p*myc*CAT reporter plasmid were done as described above. The CAT activity was assayed for 5 h.

an indirect immunofluorescence using polyclonal antibodies (αB-MybAb) (Fig. 1C). Both wild-type and M4, which lacked the region downstream from the C-terminal end of NLS2, were localized in the nuclei. As predicted, a deletion of either NLS1 or NLS2 partially abolished the nuclear localization of B-Myb. The population of cells in which ΔNLS1 and ΔNLS2 were detected predominantly in the nuclei were 60% and 8% of the transfected cells, respectively, indicating that NLS2 contributes more to the nuclear targeting of B-Myb than NLS1 (Fig. 1D). Moreover, B-Myb proteins lacking both NLS1 and NLS2 are located in both the nuclei and cytoplasm in most of the transfected cells.

To confirm the results obtained by the immunofluorescence studies, we examined the *trans*-activating capacities of the B-Myb mutants in the CAT co-transfection experiments (Fig. 2). As a reporter plasmid, both the artificial gene construct containing tandem repeats of Myb-binding site, pA10CAT6MBS-I, (Fig. 2A), and p*myc*CAT containing the human c-*myc* promoter (Fig. 2B) were used. A deletion of either NLS1 or NLS2 did not or only partly impaired the *trans*-activating capacity with either reporter plasmid. In contrast, the mutant lacking both NLS1 and NLS2 did not *trans*-activate pA10CAT6MBS-I. Furthermore, with p*myc*CAT, the *trans*-activating capacity of ΔNLS1 + 2 was about 1/3 of the wild-type.

By analyzing the mutant proteins expressed in the transfected cells, we also confirmed that a deletion of NLS1 and/or NLS2 did not decrease the protein stability (Fig. 3). These results indicate that NLS1 and NLS2 are essential for the efficient nuclear targeting of B-Myb.

3.2. *NLS2 belongs to a class of bipartite nuclear targeting sequences*

NLS2 is similar to the NLS of *Xenopus* nucleoplasmin in that both consist of two basic domains separated by about 10 intervening spacer amino acids [24]. In the case of nucleoplasmin, these two basic domains are interdependent, and both of them are required for efficient nuclear uptake. To analyze the role of each basic domain in NLS2, mutants lacking either the N-terminal (amino acids 564–573) or the C-terminal basic domain (amino acids 574–587) (M2 or M6) were generated (Fig. 4A), and their subcellular distributions were examined as described above. Since a deletion of NLS2 alone did not completely impair the nuclear targeting, these deletion mutations were combined with a NLS1 deletion to generate the mutants ΔNLS1 + M6 or ΔNLS1 + M2 (Fig. 4A). In about 60% of the transfected cells, ΔNLS1 and ΔNLS1 + M4 were localized predominantly in the nuclei, respectively (Fig. 4B,C). However, a removal of either the N- or C-terminal basic domain considerably abolished the nuclear entry of B-Myb, and the population of cells in which ΔNLS1 + M6 and ΔNLS1 + M2 were located exclusively in the nucleus were only 35% and 3% of the transfected cells, respectively. Thus, both basic domains in NLS2 are required for nuclear targeting, and the C-terminal domain appears to be more important. Furthermore, the transport defect of a mutation in one basic domain was amplified by a simultaneous mutation in the other. Therefore, these basic domains are also interdependent, like the NLS in *Xenopus* nucleoplasmin.

3.3. *Role of potential* cdc2 *kinase site within NLS2*

It has been pointed out that a group of proteins including the *Xenopus* nucleoplasmin, yeast transcription factor SWI5, and human B-Myb, contain a (putative) NLS

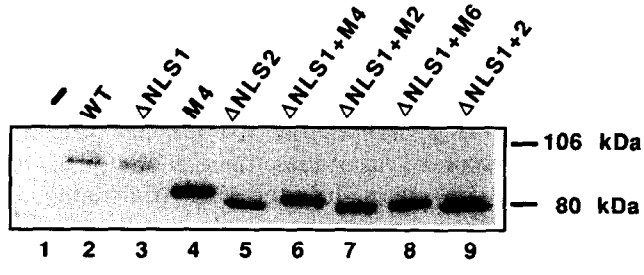

Fig. 3. Immunodetection of B-Myb mutant proteins. A mixture of 10 μg of plasmid DNA to express various forms of B-Myb indicated above each lane or no protein (–, lane 1) and 2 μg of the internal control plasmid pRSVβ-gal DNA was transfected into CV-1 cells. The B-Myb proteins were detected by Western blotting with αB-MybAb.

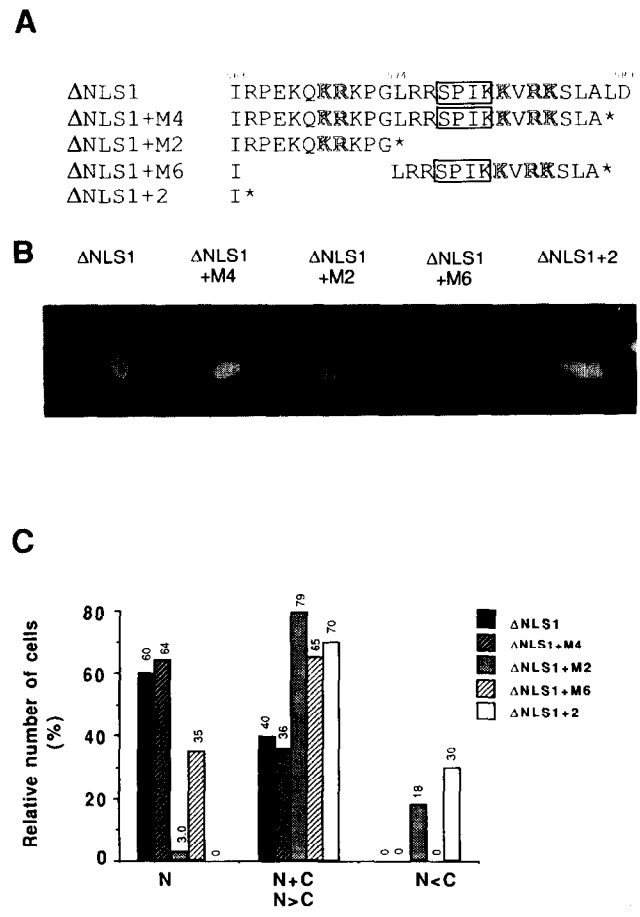

Fig. 4. Role of the N- or C-terminal basic domain in NLS2. (A) The amino acid sequence of the NLS2-containing region of the B-Myb deletion mutants. The amino acid sequence of the NLS2-containing region is shown as in Fig. 1B. (B) Typical immunofluorescence images of the NLS2 mutants. Subcellular distributions of various forms of B-Myb were examined as described in the legend to Fig. 1. A typical result for each construct is shown. (C) Summary of subcellular distribution. The results of the experiment described above are shown as in Fig. 2D, except that the cells corresponding to N > C and N + C are combined.

linked to the potential *cdc*2 kinase site [14,24]. Interestingly, the nuclear entry of SWI5 was demonstrated to be cell cycle regulated through the CDC28-dependent phosphorylation at the sites linked to the NLS [14]. The potential *cdc*2 phosphorylation site in B-Myb (^{577}SPIK) is within NLS2, just between the two basic domains. To clarify whether this site is critical for nuclear localization, like SWI5, point mutants of this site were generated (S/A, S/D, and S/E) (Fig. 5A), and their subcellular distributions were examined. All of these three mutant proteins were localized in nuclei, like the wild-type (data not shown). Therefore, to amplify the effect of these point mutations, they were combined with the NLS1 deletion mutation. The ΔNLS1 + S/A mutant protein, in which Ser-577 was replaced by alanine, was transported into the nucleus as effectively as the ΔNLS1 mutant (Fig. 5B,C). Furthermore, two mutants, ΔNLS1 + S/D and ΔNLS1 + S/E, also showed a subcellular distribution similar to ΔNLS1, indicating that the replacement of Ser-577 by aspartic or glutamic acid, of which the structure resembles the phosphorylated form, did not affect the nuclear entry.

To confirm these results, the *trans*-activating capacities of the Ser-577 mutants were examined in a CAT co-transfection experiment (Fig. 5E). None of the three mutants had significantly lower *trans*-activating capacities compared to the parental form ΔNLS1. In addition, the levels of these proteins expressed in transfected cells were similar to the ΔNLS1 (Fig. 5D). These results suggest that the potential *cdc*2 phosphorylatin site in NLS2 does not contribute to the regulation of nuclear entry.

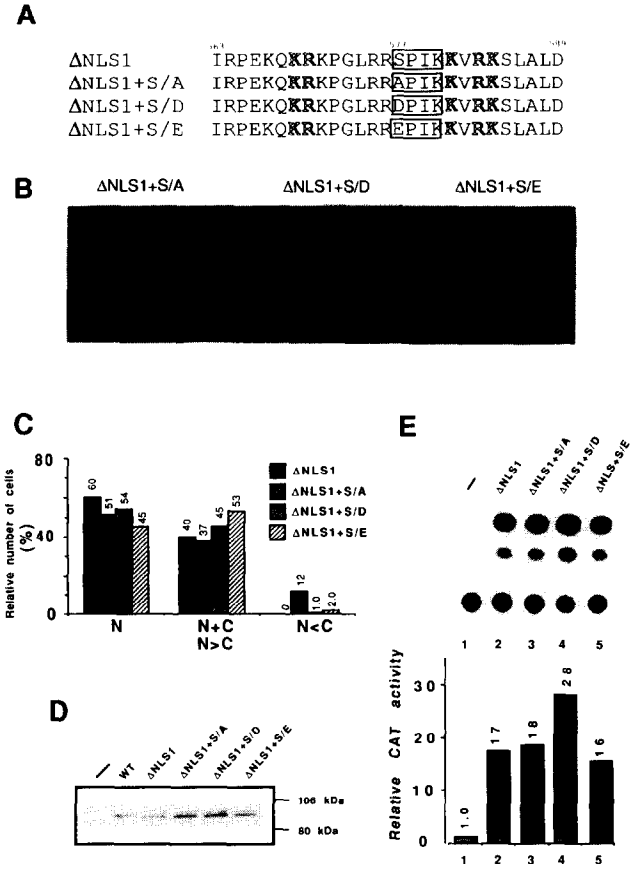

Fig. 5. Effects of the mutations of the potential *cdc*2 kinase site on the nuclear import of B-Myb. (A) The amino acid sequence of the potential *cdc*2 kinase site in various mutants. The amino acid sequence of the NLS2-containing region is shown as in Fig. 1B. The putative *cdc*2 kinase site, Ser-577, was changed to alanine (S/A), aspartic acid (S/D), and glutamine acid (S/E), respectively. (B) Typical immunofluorescence images. Subcellular distributions of the mutants were examined as described in the legends to Fig. 1C. (C) Summary of subcellular distribution. The results of immunofluorescence staining experiments are shown as in Fig. 4C. (D) Immunodetection of the mutants. The B-Myb mutant described above each lane were detected as described in the legend to Fig. 3. (E) *trans*-Activating capacities of the *cdc*2 kinase site mutants. The CAT co-transfection experiments by using p*myc*CAT were done, and the results are indicated as described in the legend to Fig. 2.

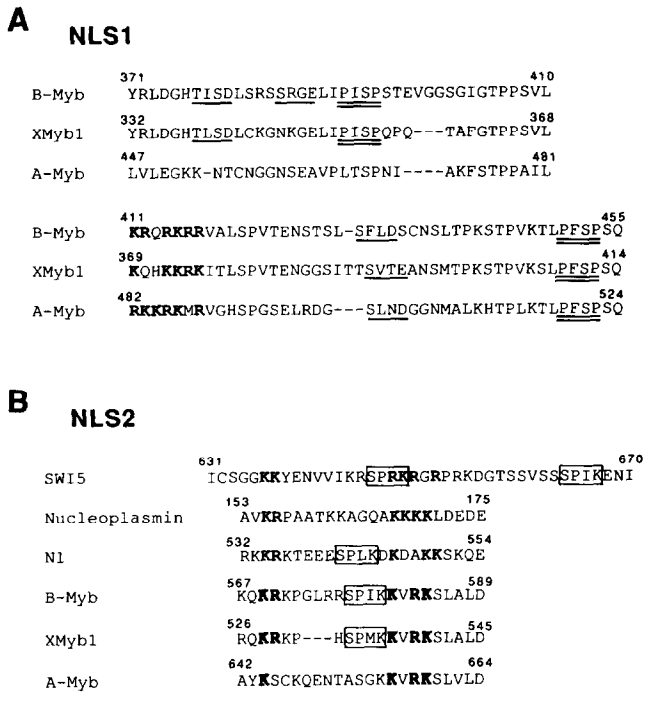

Fig. 6. Amino acid sequence of the region containing NLS in other members of the *myb* gene family. (A) NLS1-like motif. The amino acid sequence of the NLS1-containing region of B-Myb is aligned with those containing the putative NLS of the *Xenopus* Myb 1 (XMyb1) [2] and the human A-Myb (A-Myb) [21]. The potential phosphorylation sites by CK-II and MAP kinase are shown by an underline and a double underline, respectively. (B) NLS2-like motif. The typical bipartite NLS's of the SWI5 [14], *Xenopus* nucleoplasmin [24], and N1 [24] are indicated on the top, where the important basic residues are shown by bold letters. The amino acid sequences of NLS2 of B-Myb and the NLS2-like motif in the *Xenopus* Myb1 (XMyb1) [2] and the human A-Myb (A-Myb) [21] are shown below. The basic residues conserved between NLS2 and the NLS of nucleoplasmin are indicated by window letters. The potential phosphorylation sites of *cdc*2 kinase are boxed.

4. Discussion

We have demonstrated that the nuclear entry of B-Myb is controlled by two NLS's, NLS1 and NLS2. NLS1 contains one basic amino acid cluster, while the sequence of NLS2 is bipartite, comprising two interdependent clusters of basic amino acids separated by 8 intervening spacer amino acids. NLS1- and NLS2-like sequences are also found in the *Xenopus* B-Myb [2] and human A-Myb [21] (Fig. 6).

Only in about 60% and 8% of the transfected cells were the mutant proteins lacking either NLS1 (*Δ*NLS1) or NLS2 (*Δ*NLS2) normally transported into the nuclei (Fig. 1D). However, both *Δ*NLS1 and *Δ*NLS2 had the normal or considerably higher level *trans*-activating capacity (Fig. 2). This apparent discrepancy could be due to many B-Myb molecules being expressed in the transfected cells. If the number of B-Myb molecules is much higher than that of the CAT reporter plasmid, the partial defect of nuclear entry of B-Myb may not reflect the *trans*-activating capacity. Furthermore, in about 40% and 92% of the transfected cells, *Δ*NLS1 and *Δ*NLS2 are localized in both the nuclei and cytoplasm, respectively (Fig. 1D), and in these cells B-Myb localized in the nuclei *trans*-activates the CAT expression. The *Δ*NLS1 + 2 proteins are also located in both the nuclei and cytoplasm in most of the transfected cells (Fig. 1D). This may also explain why the mutants lacking both NLS1 and NLS2 (*Δ*NLS1 + 2) still have about 1/3 of the *trans*-activating capacity of the wild-type (Fig. 2). However, we cannot completely exclude the possibility that an unidentified NLS(s) other than NLS1 and NLS2 exists in B-Myb.

It is now believed that in mammalian cells, NLS is recognized by specific cytoplasmic proteins (receptor) and transported to the nuclear pore (for review, see [4]). The bipartite motif, which was originally found in *Xenopus* nucleoplasmin [24] and contains two clusters of basic residues separated by a spacer segment, appears to be a wide-spread type of NLS. It was proposed that the heat shock protein Hsp70 (or Hsc70) binds to and stabilizes a locally unfolded NLS, thus presenting the NLS to a second receptor in an unfolded conformation [4]. Therefore, it is interesting to know whether NLS2 in B-Myb also binds to Hsp70 and whether the two NLS's in B-Myb, NLS1 and NLS2, interact with the same or different cytoplasmic receptor(s).

The potential *cdc*2 kinase site adjoins NLS2. A similar sequence was also found in the *Xenopus* B-Myb (Fig. 6B). Similar linkages between NLS and putative phosphorylation sites were previously pointed out in the multiple nuclear proteins, including SWI5, p53, and nucleoplasmin. In fact, the importance of the phosphorylation sites linked to NLS was demonstrated in the case of SWI5 [14]. Three serine residues within or close to the NLS of SWI5 are phosphorylated by CDC28 kinase in a cell cycle-dependent manner, being phosphorylated when SWI5 is in the cytoplasm and dephosphorylated when it is in the nucleus. In constrast to the case of SWI5, a mutation of Ser-577 to alanine, aspartic acid, or glutamic acid did not affect the nuclear importation. However our results cannot exclude the possibility that the phosphorylation-dependent regulation of nuclear uptake is controlled by multiple phosphrylation sites, and mutation of only one site, such as Ser-577, is not sufficient to eliminate this regulation. In fact, in the case of SWI5, mutation of any one of three serine residues to alanine reduces but does not eliminate cell cycle-regulated nuclear entry. It should be noted that multiple potential phosphorylation sites by casein kinase II (CK-II), *cdc*2 kinase, and MAP kinase are located around NLS1, and some of them are conserved in the proteins encoded by other members of the *myb* gene family, human A-Myb and *Xenopus* B-Myb (Fig. 6A). Our results also do not exclude the possibility that Ser-577 controls the rate of transport, because in our immunofluorescence studies the nuclear accumulation of B-Myb was examined, but

not the rate of transport. In fact, the rate of nuclear transport of the SV40 T antigen was demonstrated to be determined by the CK-II site flanking the NLS [23]. Therefore, more precise work is required to clarify the role of the potential *cdc*2 kinase site in B-Myb.

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