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Two nuclear localization signals present in the basic–helix 1 domains of MyoD promote its active nuclear translocation and can function independently

MARIE VANDROMME*, JEAN-CLAUDE CAVADORE*, ANNE BONNIEU†, ANNE FROESCHLÉ†, NED LAMB*, AND ANNE FERNANDEZ*

*Cell Biology Unit, Centre de Recherches de Biochimie Macromoléculaire, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, BP 5051, 34033 Montpellier Cedex, France; and †Laboratoire de Differentiation Cellulaire et Croissance, Unité d'Endocrinologie Cellulaire, Institut National de la Recherche Agronomique, 34060 Montpellier Cedex, France

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ABSTRACT **MyoD, a member of the family of helix–loop–helix myogenic factors that plays a crucial role in skeletal muscle differentiation, is a nuclear phosphoprotein. Using microinjection of purified MyoD protein into rat fibroblasts, we show that the nuclear import of MyoD is a rapid and active process, being ATP and temperature dependent. Two nuclear localization signals (NLSs), one present in the basic region and the other in the helix 1 domain of MyoD protein, are demonstrated to be functional in promoting the active nuclear transport of MyoD. Synthetic peptides spanning these two NLSs and biochemically coupled to IgGs can promote the nuclear import of microinjected IgG conjugates in muscle and nonmuscle cells. Deletion analysis reveals that each sequence can function independently within the MyoD protein since concomittant deletion of both sequences is required to alter the nuclear import of this myogenic factor. In addition, the complete cytoplasmic retention of a β-galactosidase-MyoD fusion mutant protein, double deleted at these two NLSs, argues against the existence of another functional NLS motif in MyoD.**

MyoD belongs to the family of muscle-specific helix–loop–helix (HLH) proteins that includes Myf5, myogenin, MRF4/Myf6/herculin. Its expression is restricted to skeletal muscle cells wherein it acts as a transcriptional activator of muscle-specific gene expression during skeletal myogenesis (reviewed in refs. 1 and 2). Dimerization between myogenic factors and ubiquitous HLH proteins such as the products of E2A gene, E12 and E47, is essential for sequence-specific DNA binding and subsequent transcriptional activation (3). Immunofluorescence analysis has shown that MyoD has a nuclear localization in different cell lines (C2, azamyoblasts) (4), as well as in MyoD transfected cells (5), which is in agreement with its transcriptional activity.

Various studies on nuclear localization have led to the concept that transport across the nuclear envelope (which separates the cytoplasm from the nucleoplasm) is an active process mediated by one or several nuclear localization signal sequences (NLSs) present within either the protein itself or an associated cofactor (reviewed in refs. 6 and 7). Specialized transporter proteins known as NLS-binding proteins allow the transport of NLS-containing proteins to the nucleus. Initial evidence for the existence of NLSs arose from analysis of yeast MATα2 protein (8), *Xenopus* nucleoplasmin (9), and simian virus 40 large tumor antigen (SV40 T antigen) (10). Such NLSs have since been characterized in a growing number of nuclear proteins by two criteria: an NLS is sufficient to promote nuclear accumulation of an otherwise cytoplasmic protein when fused to it genetically or biochemically; in counterpart, deletion of an NLS(s) from a nuclear protein leads to its cytoplasmic retention. Although a strict consensus does not yet exist, it appears that most NLSs are short linear sequences [from 5 to 12 amino acids (aa)] generally containing several basic residues (arginine or/and lysine).

With a recent report suggesting that the localization of MyoD can be developmentally regulated in *Xenopus* (11), and our data showing the requirement for A kinase in MyoD nuclear import (12), the identification of MyoD NLSs represents a necessary and important step in understanding the regulation of MyoD localization. Here, we report that MyoD contains two independent functional NLSs, one localized in the basic region and the other in the helix 1 domain. Each of them is sufficient to translocate rabbit IgG to the nucleus of nonmuscle or muscle cells. Additionally, by deletion analysis, we showed that either sequence can function independently within the MyoD protein, since both NLSs must be deleted to alter the nuclear import of either MyoD or a β-galactosidase-MyoD fusion protein.

MATERIALS AND METHODS

Peptide Synthesis and Conjugation. Solid-phase synthesis of NLS peptides was performed as described (13). When necessary, a terminal cysteine was added to the MyoD peptide sequence in order to provide a disulfide bond with the carrier protein. Conjugation of peptides/protein was performed using the heterobifunctional crosslinking reagent sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane 1-carboxylate (Pierce, Interchim, Montluçon, France) following the manufacturer's instructions. Peptide-IgG conjugates were resuspended at 2 mg/ml in 100 mM Hepes (pH 7.2) for subsequent microinjection experiments. The approximate number of peptides coupled per mol of IgG was evaluated from the resulting shift in molecular mass of the IgG heavy chains analyzed by SDS/PAGE.

Mutagenesis. The MyoD cDNA was mutagenized in the Moloney sarcoma virus long terminal repeat expression vector pEMSV-scribe (a gift from H. Weintraub, Seattle, WA) (14) using the Promega *in vitro* mutagenesis kit (Coger, Paris) as described (12). Mutations were confirmed by sequencing using the United States Biochemical 2.0 sequenase kit (Amersham).

Purified MyoD Protein and Antibody. Production and purification of the full-length murine MyoD as well as affinity-purified anti-MyoD antibodies have been described elsewhere (12).

Microinjection Experiments. Rat embryonic fibroblasts REF52 were grown on glass coverslips as described (13). Peptide-IgG conjugates (1 mg/ml) were microinjected together with inert mouse IgG antibodies (1 mg/ml) as an

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Abbreviations: NLS, nuclear localization signal; SV40, simian virus 40; T antigen, large tumor antigen; HLH, helix–loop–helix.

injection marker into the cytoplasm of REF52 cells. At different times after injection, cells were fixed with 3.7% formalin before extraction with −20°C acetone as described. In the case of wild-type MyoD and deleted mutants, cells growing on plastic dishes were microinjected into the nucleus with pEMSV-MyoD (0.5 mg/ml) or with the purified protein (0.5–1 mg/ml) together with mouse inert IgG antibodies (1 mg/ml) to serve subsequently in identifying injected cells. Vizualization of MyoD localization and injected cells was done by immunofluorescence as described (12).

Heterodimerization Experiments. Two hundred to 500 ng of bacterially produced glutathione-*S*-transferase-E12 proteins bound to glutathione-Sepharose 4B (Pharmacia) was incubated with either *in vitro* translated MyoD labeled with [^{35}S]methionine (5 μl), bacterially produced MyoD (1 μg), or IgG-NLS conjugates (1 μg) in binding buffer [10 mM Hepes, pH 7.9/50 mM KCl/2.5 mM $MgCl_2$/10% glycerol/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride (PMSF)] overnight at 4°C. E12 proteins were collected by centrifugation and washed twice with RIPA buffer (10 mM Tris, pH 7.5/150 mM NaCl/1 mM EDTA/0.2% Nonidet P-40/1 mM PMSF). Proteins bound to E12 were separated by SDS/PAGE and analyzed by Western blotting to detect rabbit IgG in the case of NLS-IgG conjugates and by autoradiography of the gel in the case of the *in vitro* translated MyoD.

Fusion of MyoD to β-Galactosidase Using the pCH110 Vector and Transient Transfection Experiments. Wild-type and mutant forms of MyoD were genetically fused to β-galactosidase cDNA using the pCH110 expression vector (Promega). cDNAs were amplified by PCR using *Taq* polymerase. The sequence of the 5′ primer, 5′-CACGCCCAAGCTTATGGAGCTTCTATCGCCGCCA-3′, spanned the pCH110 vector sequence near the ATG and included a *Hin*dIII restriction site for subsequent cloning. The 3′ primer sequence, 5′-CGGCGGGTACCGCAAGCACCTGATTAAATCGCATT-3′, spanned the end of the coding sequence of MyoD and included a *Kpn* I site for subsequent cloning. After digestion with *Hin*dIII and *Kpn* I, the purified restriction fragment was ligated into the pCH110 expression vector after cutting with the same restriction enzymes. The resulting plasmid was used to transfect REF52 cells using *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate reagent (Boehringer) as described by the supplier. Forty-eight hours after transfection, localization of the expressed fusion protein was assessed by immunofluorescence using anti-β-galactosidase antibodies (Promega) and anti-MyoD antibodies.

RESULTS

Nuclear Import of MyoD is an Active Process. Nuclear import of MyoD was investigated through cytoplasmic injection of purified bacterially produced MyoD into rat embryo fibroblasts (REF52). The use of nonmuscle cells allows the study of nuclear localization of ectopic MyoD without interference from endogenous MyoD. Additionally, MyoD has been shown to activate myogenesis when expressed ectopically in nonmuscle cells (14, 15), showing that it can function independently of other muscle-specific factors. The purified MyoD protein was injected into the cytoplasm of REF52 fibroblasts and cells were fixed 15–60 min thereafter. As shown in Fig. 1, MyoD acquires a nuclear localization within 15 min of cytoplasmic injection (Fig. 1 *A* and *B*) and is predominantly nuclear after 30 min (Fig. 1 *C* and *D*). After 45 min, MyoD could be detected in only 50–60% of injected cells (Fig. 1 *E* and *F*) and by 1 h after injection, the protein was no longer detectable in injected cells (Fig. 1 *G* and *H*), showing that it is rapidly degraded, as expected from the short half-life of the protein described by Thayer *et al.* (16).

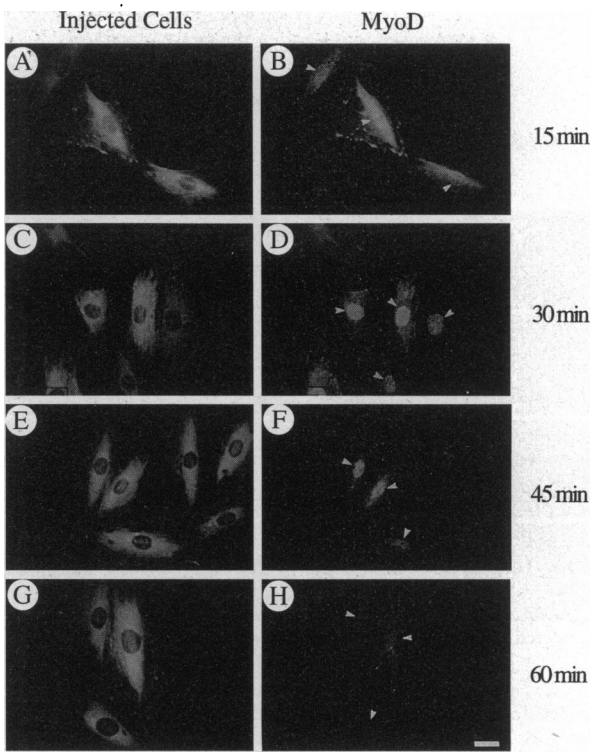

FIG. 1. Purified MyoD proteins isolated from a bacterial expression system were microinjected into rat embryo fibroblasts (REF52) to assess its intracellular localization and half-life. Injected REF52 cells were stained for the distribution of an inert antibody used to identify injected cells (*A, C, E,* and *G*) or for injected MyoD proteins with monospecific anti-MyoD antisera (*B, D, F,* and *H*). Cells were fixed 15 (*A* and *B*), 30 (*C* and *D*), 45 (*E* and *F*), or 60 (*G* and *H*) min after injection. Arrowheads in *B, D, F,* and *H* indicate the MyoD staining in the injected cells. (Bar = 10 μm.)

MyoD (318 aa: apparent molecular mass of 45–48 kDa) spontaneously forms homodimers and may form heterodimers with various partners such as E12 and E47 proteins inside the cell. Thus, its native size is predicted to be in the order of at least 80–90 kDa, making free diffusion through nuclear pores unlikely (17). To test whether nuclear import of MyoD was an active process, we assayed the effect of low temperature and depletion of ATP on nuclear import of MyoD. When MyoD was injected into cells kept at 4°C or into cells depleted of ATP by treatment with a mixture of 6 mM deoxyglucose and 10 mM sodium azide (18), we observed that nuclear import of MyoD was abolished (data not shown). Taken together, these results showed that the transport of MyoD, being energy and temperature dependent, is an active process.

Two MyoD Sequences in the Basic–Helix 1 Domains Function as NLSs. We further questioned whether MyoD contains functional NLSs that may direct its nuclear locale. By homology with a reported NLS consensus sequence (19), we identified the presence of six putative and partially overlapping NLSs in MyoD protein (Fig. 2 *Upper*). All of these sequences are present in the basic–helix 1 region of MyoD (aa 102–136). To test the activity of these putative NLSs, three peptides that mimic the MyoD NLS were synthesised. As shown in Fig. 2 *Upper*, the first peptide (CKRKTTNADRRKA; NLS234) covers the three putative amino-terminal NLSs (NLS2, NLS3, and NLS4) and only lacks the first amino acid of NLS1, because of the extensive overlapping of these four sequences. We chose to omit the first amino acid of NLS1 to have a natural cysteine at the amino-terminal position for subsequent coupling. The two other peptides (NLS5, CATMRERRRLSK; and NLS6 VNEAFETLKRC) contain the fifth and the sixth putative NLSs, respectively. Each peptide was biochemically coupled

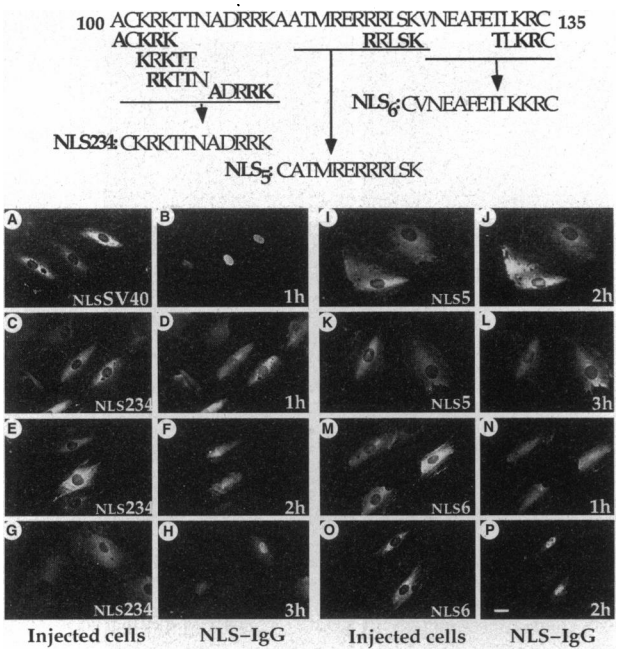

FIG. 2. (*Upper*) Shown from top to bottom is the amino acid sequence for murine MyoD spanning aa 100–135, the six putative NLSs with the amino acids that match the consensus sequence (RKTA)-K-K-(RQNTSG)-K (19) in boldface type, and the sequence of the three peptides synthesized spanning the NLSs detected in the MyoD sequence: NLS234, NLS5, and NLS6. (*Lower*) The three MyoD peptides as well as the SV40-NLS peptide (CPKKRKV) were conjugated to rabbit IgGs. The NLS-IgG conjugates were microinjected together with unconjugated mouse IgGs into the cytoplasm of REF52 cells. Cells were fixed at different times thereafter before staining for localization of the NLS-conjugated rabbit IgGs (*B, D, F, H, J, L, N,* and *P*) and for coinjected mouse IgGs to identify the injected cells (*A, C, E, G, I, K, M,* and *O*). (*A* and *B*) Injection of SV40-NLS-IgG conjugate into cells fixed 1 h afterward. (*C* and *D*, *E* and *F*, and *G* and *H*) Injection of NLS234-conjugated IgGs and fixation 1, 2, and 3 h afterward, respectively. (*I* and *J* and *K* and *L*) Injection of NLS5-conjugated IgGs and fixation 2 and 3 h afterward, respectively. (*M* and *N* and *O* and *P*) Injection of NLS6-conjugated IgGs and fixation 1 and 2 h afterward, respectively. (Bar = 10 μm.)

with rabbit IgG as detailed in *Materials and Methods*. Rabbit IgGs were chosen because of their inert activity in cells and large size, which prohibits passive diffusion through nuclear pores. As a positive control, we also coupled a peptide corresponding to the SV40 T antigen NLS (CPKKRKV) to rabbit IgG. In each case, the coupling ratio, estimated by the resulting shift in molecular mass of the IgG heavy chains observed by SDS/PAGE, was evaluated to 5–10 peptides per mol of IgG (data not shown). Each peptide-conjugate was microinjected into the cytoplasm of REF52 cells together with an inert mouse antibody for subsequent identification of injected cells. The localization of conjugates was assessed by immunofluorescence 1, 2, and 3 h after injection. As shown in Fig. 2 *Lower*, SV40 NLS-coupled IgGs were already totally nuclear 1 h after their cytoplasmic injection (Fig. 2 *Lower A* and *B*). NLS234-IgGs were also translocated to the nucleus but at a slower rate since they were detectable in the nucleus only 2 h after injection at a level of nuclear staining similar to the cytoplasmic staining (Fig. 2 *Lower E* and *F*), and 3 h following their injection, the NLS234-IgGs were mostly nuclear (Fig. 2 *Lower G* and *H*). NLS5 was ineffective in addressing IgGs to the nucleus since they remained totally cytoplasmic 2 h (Fig. 2 *Lower I* and *J*) and 3 h after their injection (Fig. 2 *Lower K* and *L*). In contrast, as shown in Fig. 2 *Lower*, NLS6 allowed efficient translocation of the IgGs into the nucleus; they became nuclear 1 h after their injection (Fig. 2 *Lower M* and *N*) and were totally nuclear after 2 h (Fig. 2 *Lower O* and *P*). Similar experiments in mouse C2 muscle cells gave identical results (data not shown).

To ensure that the activity of NLS6, located in helix 1, was not due to its association with another HLH protein inside the cell that would serve as a cotransporter to drive NLS6-IgG to the nucleus, we tested whether NLS6-IgG could heterodimerize with E12. In agreement with previous data from Davis *et al.* (20) showing that the integrity of the HLH region is necessary for heterodimerization, we found that NLS6-IgGs were unable to heterodimerize with E12 (data not shown). Taken together, these results show that among the three peptides containing putative NLSs in MyoD, two of them, NLS234 and NLS6, can function as true localization signals when coupled to a nonnuclear carrier protein, here rabbit IgGs.

Deletion of NLS234 and NLS6 Is Required To Impair the Ability of MyoD To Translocate to the Nucleus. The functionality of the two NLSs identified in MyoD was probed by deleting them from the wild-type MyoD and looking for the effect of such mutations on MyoD nuclear localization. Mutants of MyoD deleted at either NLS234 (aa 100–112), NLS6 (aa 130–135), or both were prepared by mutagenizing MyoD cDNA in the pEMSV eukaryote expression vector (Fig. 3), as described in *Materials and Methods*. Wild-type and deleted MyoD plasmids were injected directly into the nucleus of growing REF52 cells. Injected plasmids were allowed to express for 10–15 h before fixation of cells and analysis of MyoD expression and localization by immunofluorescence. As before, cells were coinjected with mouse IgGs for subsequent identification of injected cells. As shown in Fig. 3, in cells injected with the plasmid coding for wild-type MyoD, staining for expressed MyoD was found exclusively in the nucleus (Fig. 3*a*). A similar nuclear localization was observed in cells injected with either single deletion mutant, MyoDΔNLS234 or MyoDΔNLS6, although in both cases some cytoplasmic staining could also be observed, perhaps reflecting a diminished efficiency in nuclear import (Fig. 3 *b* and *c*). However, as revealed by the bright nuclear staining, each single mutant could efficiently translocate to the nucleus showing that NLS234 and NLS6 may act alone and independently in driving nuclear import. In contrast, anti-MyoD staining of cells injected with the MyoD double mutant (deleted for NLS234 and NLS6) showed a bright immunoreactivity in the cytoplasm (Fig. 3*d*). The nuclear staining also observed was

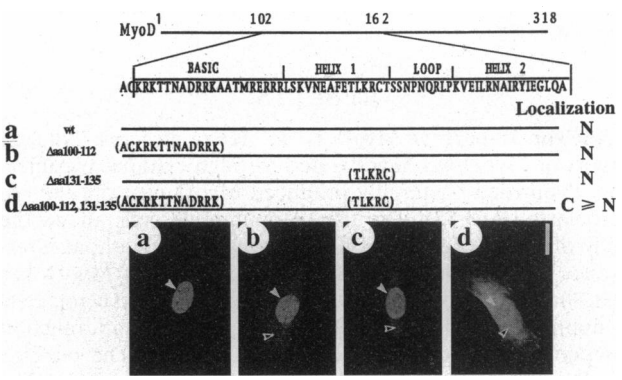

FIG. 3. Shown is the amino acid sequence of MyoD with the localization of the basic, helix 1, loop, and helix 2 domains in the molecule. Represented below are the single deletion performed at either NLS234 (*b*) or NLS6 (*c*) and the double deletion of NLS234 and NLS6 (*c*). Photomicrographs show the immunofluorescence staining for expressed MyoD proteins 15 h after injection of either wild-type MyoD coding plasmid (*a*) or the different deleted mutants represented on the left (*b–d*). Solid arrowheads indicate the distribution of staining in the nucleus; open arrowheads indicate the cytoplasmic compartment. (Bar = 10 μm.)

never detectably higher than the cytoplasmic staining and could reflect the passive diffusion of the double mutant MyoD protein. Indeed, one can expect that deletion of NLS6 present in the helix 1 region will interfere with dimerization, thus generating MyoD monomers, which could undergo passive nuclear diffusion (17). We confirmed that, in contrast to MyoDwt and MyoDΔNLS234, MyoDΔNLS6 and MyoDΔNLS234+ΔNLS6 were unable to dimerize with glutathione-*S*-transferase-E12 bacterially produced protein (data not shown).

To ensure that the partial cytoplasmic/nuclear staining of the double mutant protein was not due to the presence of an NLS other than the two we had mutated in MyoD, wild-type and deletion mutant MyoD were genetically coupled to β-galactosidase. After transfection of REF52 cells, the subcellular localization of the expressed β-galactosidase fusion protein was examined by double-immunofluorescence using monoclonal anti-β-galactosidase and polyclonal anti-MyoD antibodies. As a control, REF52 cells were transfected either with the parental pCH110 or the SV40 NLS-pCH110 vectors, which express a cytoplasmic (Fig. 4*C*) or a nuclear localized β-galactosidase (Fig. 4*A*), respectively. As shown in Fig. 4 *E* and *F*, MyoDwt-β galactosidase fusion protein was found exclusively in the nucleus, further demonstrating the capacity of MyoD to be actively translocated into the nucleus. Single deletion of either NLS234 (Fig. 4 *G* and *H*) or NLS6 (Fig. 4 *I* and *J*) led to a hybrid protein that was still actively transported into the nucleus, as revealed by the bright nuclear staining, confirming that each NLS can act independently. However, in both cases, a cytoplasmic staining could clearly be observed, showing that the capacity of MyoD to drive β-galactosidase into the nucleus was diminished in the absence of one of the two NLSs. Fusion of β-galactosidase to MyoD deleted for NLS234 and NLS6 gave rise to a protein that could not be imported into the nucleus. Only a cytoplasmic staining was observed in the double mutant transfected cells (Fig. 4 *K* and *L*). This result shows that the β-galactosidase fused to MyoD deleted at NLS234 and NLS6 fails to be actively transported into the nucleus and demonstrates that the nuclear staining observed in the case of the nonfused MyoD double mutant described below (see Fig. 3*d*) was the consequence of passive diffusion.

Together, these results show that NLS234 and NLS6 have to be deleted to impair the nuclear transport of MyoD and imply that each of these NLSs is efficient autonomously. Moreover, the failure of MyoD deleted at NLS234 and NLS6 to drive β-galactosidase to the nucleus argues against the presence of additional functional NLS motifs in MyoD.

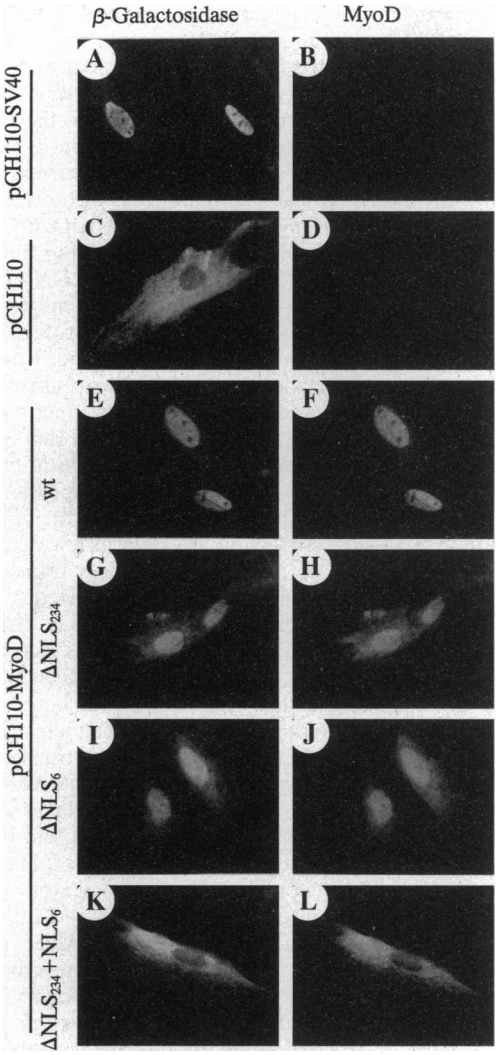

FIG. 4. MyoD wild-type and deletion mutants were genetically coupled to β-galactosidase using the pCH110 expression vector. The resulting plasmids called pCH110-MyoDwt, pCH110-MyoDΔNLS234, pCH110-MyoDΔNLS6, and pCH110-MyoDΔNLS234+ΔNLS6 and as control the parental pCH110 and pCH110-SV40 were transfected into REF52 cells. Localization of expressed proteins was assessed by immunofluorescence using anti-β-galactosidase (*A, C, E, G, I,* and *K*) and anti-MyoD (*B, D, F, H, J,* and *L*) antibodies. Shown are photomicrographs of pCH110-SV40 (*A* and *B*), pCH110 (*C* and *D*), pCH110-MyoDwt (*E* and *F*), pCH110-MyoDΔNLS234 (*G* and *H*), pCH110-MyoDΔNLS6 (*I* and *J*), and pCH110-MyoDΔNLS234+ΔNLS6 (*K* and *L*) transfected REF52 cells. (×800.)

DISCUSSION

We report here that the nuclear import of the myogenic factor MyoD is mediated by two NLS sequences present in the basic–helix 1 region of MyoD. Although the two peptides spanning the signals CKRKTTNADRRKA and VNEAFETLKRC were efficient in translocating biochemically coupled rabbit IgG to the nucleus, they functioned weakly when compared to the efficiency of the NLS of SV40 T antigen and required a high coupling ratio of peptide/IgG to drive the coupled protein into the nucleus (*ca.* 5–10 peptides per IgG). Nevertheless, we found that deletion of one single MyoD NLS did not significantly impair the ability of MyoD or MyoD fused to β-galactosidase to enter the nucleus, showing that each sequence can function efficiently in the context of the MyoD protein, even when present in a single copy. This observation suggests that the efficiency of the NLS in nuclear transport may be highly sensitive to the protein environment in which the NLS is located. Indeed, conformational structure of the NLS may be critical for the interaction with NLS binding proteins that participate in the nuclear import process (21). Such a structure may be partially lost in peptides, a hypothesis reinforced in light of the recently described crystal structure of MyoD showing the basic–helix 1 region presents a helical conformation (22). In contrast to the lack of effect of one single NLS deletion in MyoD, we found that the deletion of both NLS sequences resulted in a great loss of MyoD nuclear import, further demonstrating that the two NLSs we have characterized are individually sufficient to promote nuclear translocation of the MyoD protein. Moreover, since deletion of both NLSs abolished the ability of MyoD to drive fused β-galactosidase to the nucleus, it shows that no other region in MyoD than the two NLSs we identified has NLS capability in the cell systems we have used.

The existence of multiple karyophilic signals is a frequent phenomenon among nuclear proteins. Like MyoD, several nuclear proteins have been shown to contain two mutually independent NLSs that have to be deleted together to inhibit

nuclear import (23–25). Frequently, however, there appears to be a hierarchical function of each NLS when they are tested individually, revealing an additive role in nuclear import. The second NLS characterized in MyoD, TLKRC, appears to be more efficient, at least in translocating IgGs into the nucleus. However, this sequence, containing only two basic residues, diverges from other NLSs previously characterized. Importantly, the two NLSs we have characterized in mouse MyoD are conserved in the four myogenic factors (MyoD, myogenin, Myf5, MRF4) from different species. The finding that these two NLSs are mixed among the dimerization and DNA binding of MyoD is an unusual feature of NLSs and seems particular to the myogenic factor subclass in the HLH protein family. Indeed, in the case of the c-*myc* protooncogene, which like MyoD has a basic–HLH domain, of the two putative NLS domains identified (M1 and M2), only M1 is functional as a true NLS and is located outside the basic HLH region (26).

The presence of one of the two NLSs we identified in the dimerization domain of MyoD addresses the important question of the contribution of heterodimerization in the process of HLH protein nuclear import. Our data allow us to rule out that heterodimerization is a prerequisite for nuclear translocation of MyoD. Indeed, we showed that the NLS6 deleted MyoD, though unable to heterodimerize with E12, was efficiently translocated to the nucleus even when its size was increased by fusion with β-galactosidase, excluding passive diffusion. While this does not exclude that for wild-type MyoD heterodimerization may contribute to the process of MyoD nuclear import, it demonstrates that MyoD has the capability to translocate to the nucleus as a monomer and independently of its association with another HLH protein. This contrasts with the case of the Ah (dioxin) receptor, an example of a HLH protein that has to heterodimerize in the cytoplasm with another HLH protein, called Arnt (for Ah receptor nuclear translocator protein) prior to its translocation into the nucleus (27, 28). Similarly, Id protein is an example of a HLH protein for which nuclear translocation must be dependent upon prior heterodimerization with another HLH protein into the cytoplasm. Indeed, data from Jen *et al.* (29) described Id as a nuclear protein though it lacks a basic domain and a recognizable NLS sequence. Therefore, the nuclear localization of Id must result from its association with E12 or E47 in the cytoplasmic compartment, as recently described using a mutated HLH protein SCL/tal (30).

With recent data showing that in *Xenopus*, XMyoD is regulated through its intracellular localization in the course of muscle induction (11), it appears that control of MyoD subcellular localization may be implicated in its regulation. Therefore characterization of MyoD NLSs is important to determine if such a regulation takes place in other species and to allow further investigation of the regulation of MyoD nuclear translocation.

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