

Homeodomain proteins Mox1 and Mox2 associate with Pax1 and Pax3 transcription factors

Despina Stamatakis^a, Maria-Christina Kastrinaki^b, Baljinder S. Mankoo^{c,1}, Vassilis Pachnis^c,
Domna Karagogeos^{a,d,*}

^aUniversity of Crete Medical School, Heraklion, Crete, Greece

^bUniversity of Crete Department of Biology, Heraklion, Crete, Greece

^cDivision of Developmental Neurobiology, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

^dInstitute of Molecular Biology and Biotechnology, P.O. Box 1527, 711 10 Heraklion, Crete, Greece

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Abstract Mox1 and Mox2 homeobox genes have been shown to be critical in axial skeleton and in limb muscle development respectively. Pax1 and Pax3 gene products are also implicated in these processes. Mox and Pax expression patterns are highly overlapping both spatially and temporally during embryonic development. We show here for the first time that Mox proteins physically interact with Pax1 and Pax3 using the yeast two-hybrid protein interaction assay as well as in vitro biochemical assays. There is a strong preference of Mox1 to associate with Pax1 rather than Pax3 and of Mox2 to associate with Pax3 rather than Pax1. The observed interactions are mediated through the homeodomain of Mox. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Homeobox; Mox1; Mox2; Pax1; Pax3; Protein–protein interaction

1. Introduction

Homeoproteins have been recognized as critical regulators of morphogenetic processes [1]. The development of the axial skeleton and musculature require the concerted function of various homeoproteins and other factors. The Mox genes – Mox1 and Mox2 – constitute a subfamily of non-clustered, diverged, antennapedia-like homeobox-containing genes which are expressed in a wide range of mesodermal structures [2–5]. Mice homozygous for a null mutation in the Mox1 locus have vertebral abnormalities manifested mainly as hemi-vertebrae, tail kinks and craniovertebral fusions (B.S. Mankoo and V. Pachnis, unpublished observations). On the other hand, mice homozygous for a null mutation of Mox2 have a developmental defect in the limb musculature, characterized by an overall reduction in muscle mass and elimination of specific muscles. Mox2 is essential for the normal regulation of myogenic genes in limb myoblasts, as demonstrated by the down-regulation of Pax3 and Myf5 in Mox2-deficient limb buds [6]. Rat Mox2 (Gax) is implicated in myocyte growth

arrest [7,8], while chick Mox2 is associated with non-proliferating myoblasts in the limb (Rallis et al., in press).

Another family of transcription factors, the Pax genes, have been shown to participate in various developmental processes. The Pax genes – Pax1 to Pax9 – are characterized by the presence of the paired domain, a conserved amino acid motif with DNA binding activity [9]. Pax1 is expressed in the sclerotome and plays an important role during axial skeletal development [10]. Homozygous mutant mice of a defined Pax1 null allele exhibit morphological abnormalities of vertebrae and intervertebral disks [11]. In the absence of Pax1 and Pax9, which is also expressed in the sclerotome, the sclerotomal cell population is reduced by a decrease in cell proliferation, followed by an abnormally high rate of apoptosis that later contributes to the substantial loss of sclerotome size [12].

Pax3 is expressed in the dermomyotome and has been shown to function upstream of MyoD during skeletal myogenesis [13–15]. Mice carrying a null mutation for Pax3 (splotch mutants) show severe defects in skeletal muscle development [16]. In myoblast cultures, Pax3 induces cell proliferation and inhibits differentiation while in limb buds, Pax3 is associated with proliferating myoblasts [17,18].

Mox genes and members of the Pax gene family are implicated in common developmental processes as mentioned above. Their expression patterns are highly overlapping both spatially and temporally during embryonic development. Mox1 and Mox2 are highly expressed in the developing somites, especially in the sclerotome where they are co-expressed with Pax1. Mox2 and Pax3 are also expressed in migrating myoblasts ([6,13,18], Rallis et al., in press). We decided to test the hypothesis that Mox proteins physically interact with Pax1 and Pax3. Interactions of the Mox with the Pax family are shown for the first time in this report, both by the yeast two-hybrid protein interaction assay and by in vitro biochemical assays. The physiological implications of these interactions are discussed.

2. Materials and methods

2.1. In vitro interactions

Full-length Mox1 and Mox2 and their deletions (Fig. 2) were cloned in the pGEX vector (Amersham) in frame with glutathione S-transferase (GST), then produced in *Escherichia coli* and purified on glutathione Sepharose beads according to [19]. The Pax3 deletion construct Pax3dC encodes amino acids 1–230 and Pax3dHD encodes amino acids 1–306 out of 479 amino acids of the full-length protein.

*Corresponding author. Fax: (30)-81-39 45 30.
E-mail: karagoge@imbb.forth.gr

¹ Present address: Randall Centre for Molecular Mechanisms of Cell Function, GKT School of Biomedical Sciences, New Hunt's House, King's College London, Guy's Campus, London SE1 1UL, UK.

Full-length Pax1 and Pax3 were cloned in the pCMX-Flag vector [20] in frame with the Flag epitope. Transient transfections of COS1 cells were performed using the calcium phosphate method according to [21]. Forty hours post-transfection of COS1 cells, total extracts were prepared by lysing the cells in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml aprotinin at 4°C. The cell extracts were clarified from the insoluble material by 30 min centrifugation at 10000 rpm and were precleared by incubation with glutathione Sepharose beads at 4°C for 1 h. GST fusion proteins loaded on glutathione Sepharose beads were incubated in the presence of the precleared cell extracts for 3 h at 4°C, then washed five times with lysis buffer. Samples were boiled for 5 min in Laemmli sample buffer and electrophoresed on 10% SDS polyacrylamide gel. Western blot analysis was performed as described in [22] except the bands were developed with the ECL detection kit (Amersham). The anti-Flag M2 antibody (Sigma) was used to detect Flag-Pax1 and Flag-Pax3. Whenever ³⁵S-labeled proteins were used they were prepared with the TNT system (Promega) according to the manufacturer's instructions. Glutathione Sepharose beads loaded with GST fusion proteins were equilibrated in washing buffer (150 mM KCl, 20 mM HEPES pH 7.9, 5 mM MgCl₂, 0.1% Nonidet-P40, 1 mM PMSF, 1 mM dithiothreitol). Then they were combined with 2 µl ³⁵S-labeled reticulocyte lysate in a final volume of 200 µl of washing buffer, 0.2% (w/v) bovine serum albumin (interaction buffer) on a rotator for 4 h at 4°C. The beads were then washed five times with washing buffer, the bound proteins were eluted by boiling in Laemmli sample buffer and subjected to SDS-PAGE. Bound proteins were visualized by autoradiography.

2.2. Yeast two-hybrid assay

Bait plasmids were constructed by cloning the Pax1 (from the 230th nucleotide of its coding sequence, that is from the third helix of the paired domain, to the stop codon) and Pax3 full-length coding sequences in frame with the GAL4 DNA binding domain of the pAS1-CYH2 cloning vector (Clontech). Prey plasmids were constructed by cloning Mox1 and Mox2 coding sequences (full-length and deletions; Fig. 1) in frame with the VP16 transactivation domain of the pVP16 cloning vector described in [23].

Yeast strain Y187 was used and the genotype is MAT α , ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4 Δ , met⁻, gal80 Δ , URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ [24]. Standard synthetic media were used; YPD medium contained 2% glucose. SD minimal medium contained glucose as carbon source and a dropout solution that contains essential nutrients (amino acids and nucleotides) was used in yeast transformations to select for specific phenotypes. Standard procedure was used for yeast transformations.

2.3. LacZ assays

β -Galactosidase assays were performed on yeast cultures grown in the appropriate media and harvested during early log phase ($A_{600} < 1.0$). LacZ values (A_{420}) were normalized to A_{600} . Each plus (+) in Tables 1 and 2 represents β -galactosidase activity three-fold over the corresponding negative control (Pax1-VP16 or Pax3-VP16). Estimations were based on at least three independent transformants.

Table 1

Interactions between Pax1, Mox1 and Mox2 in the two-hybrid assay

Partners ^a	Binding ^b
Pax1-VP16	—
Pax1-Mox1	+++
Pax1-NMox1	—
Pax1-CMox1	+++
Pax1-Mox2	+
Pax1-NMox2	—
Pax1-CMox2	+++

^aMox1 and Mox2 constructs were in fusion with the VP16 transactivation domain, the Pax1 and Pax3 constructs were in fusion with the GAL4 DNA binding domain. See Fig. 1 for diagram of Mox protein and deletions.

^bBinding was estimated by measuring β -galactosidase activity in liquid cultures (see Section 2).

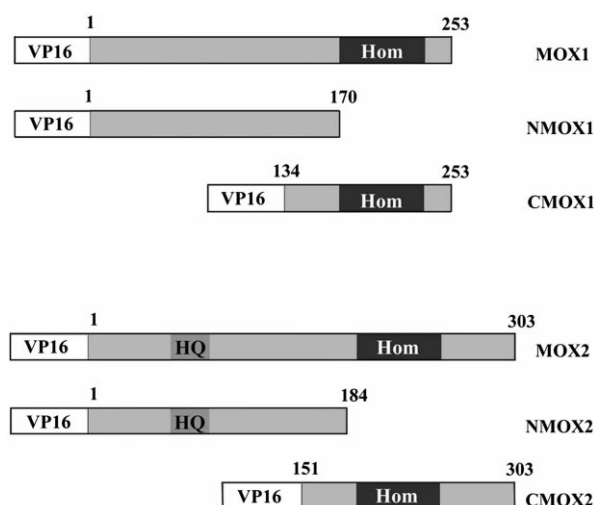


Fig. 1. Diagram of Mox1 and Mox2 proteins and their deletions used for the two-hybrid assay. Proteins were fused with the VP16 transactivation domain in their N-termini. Numbers refer to amino acids present in Mox proteins.

3. Results

3.1. Mox associate with Pax in the two-hybrid assay

We investigated whether Mox1 and Mox2 proteins interact with proteins of the Pax family, namely Pax1 and Pax3, using the two-hybrid assay. Full-length Mox proteins and their deletions used in this assay are shown in Fig. 1. β -Galactosidase activity is presented in Tables 1 and 2 (see also Section 2). We observed that Pax1 interacted with Mox1 and weakly with Mox2. In addition, the Mox deletions containing the homeodomain, CMox1 and CMox2, interacted with Pax1. The interactions of Pax1 with the Mox shown here were independent of the N-terminal 76 amino acids of Pax1 (see Section 2). A strong interaction was observed between Pax3 and Mox2, while Pax3 and Mox1 interacted weakly. The Mox deletion derivatives containing the homeodomain, CMox1 and CMox2, also interacted with Pax3. Given these observed interactions we further investigated whether these proteins associate in vitro.

3.2. Mox1-Pax1 and Mox2-Pax3 interact in vitro

To test whether Mox1 and Pax1 as well as Mox2 and Pax3 associate in vitro we expressed these proteins and their dele-

Table 2

Interactions between Pax3, Mox1 and Mox2 in the two-hybrid assay

Partners ^a	Binding ^b
Pax3-VP16	—
Pax3-Mox1	+
Pax3-NMox1	—
Pax3-CMox1	+++
Pax3-Mox2	+++
Pax3-NMox2	—
Pax3-CMox2	+++

^aMox1 and Mox2 constructs were in fusion with the VP16 transactivation domain, the Pax1 and Pax3 constructs were in fusion with the GAL4 DNA binding domain. See Fig. 1 for diagram of Mox protein and deletions.

^bBinding was estimated by measuring β -galactosidase activity in liquid cultures (see Section 2).

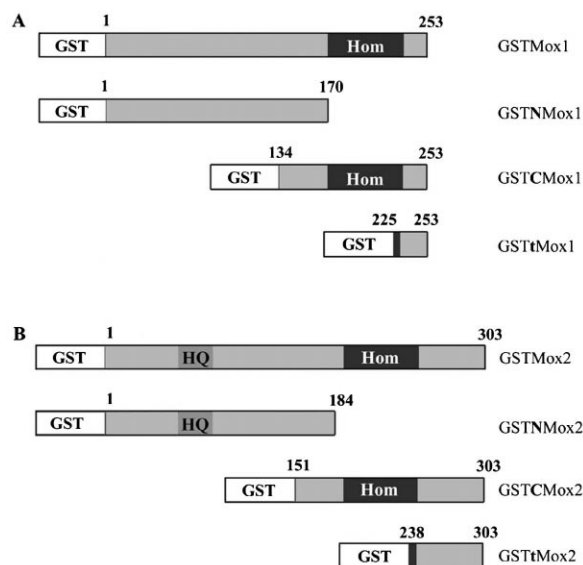


Fig. 2. Diagram of GSTMox1 and GSTMox2 proteins and their deletions used for the in vitro interaction assays. Proteins were fused with GST in their N-termini. Numbers refer to amino acids present in Mox proteins.

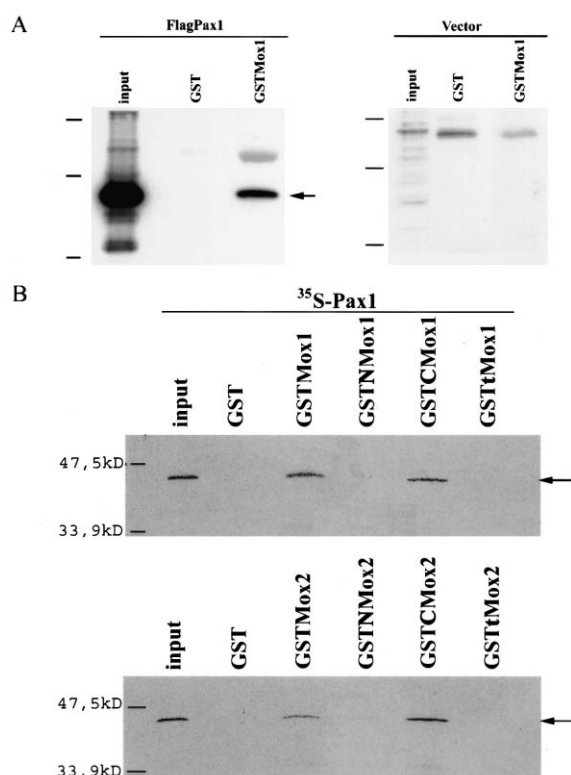


Fig. 3. Pax1 interacts with Mox1 and Mox2 in vitro. A: GST interaction assays were performed using GST, GSTMox1 (4 µg) and cell lysates prepared from COS1 cells transfected with a control plasmid or one expressing Flag-tagged Pax1. Immobilized proteins (arrow) were resolved by SDS-PAGE and visualized by Western blot analysis using an anti-Flag antibody. Input, 1% of the input protein extract. In A, dashes indicate molecular mass markers: 81, 49.9, 36.2 kDa. B: Pax1 interacts with Mox1 and Mox2 through the Mox homeodomain. GST interaction assays were performed with 4 µg GST, GSTMox1, GSTMox2 and their N- and C-terminal deletions (see Fig. 2 for diagrams) and ³⁵S-labeled Pax1. Immobilized proteins (arrow) were resolved by SDS-PAGE and visualized by autoradiography. Input, 20% (1 µl) of ³⁵S-labeled Pax1.

tions in bacteria and tested them in biochemical assays. In parallel, we attempted to coimmunoprecipitate Mox2 and Pax3 in cell extracts from developing tissues. As these experiments failed, probably due to limiting amounts of Mox2 protein and/or the sensitivity of available reagents, we proceeded with the in vitro approach.

Recombinant full-length Mox1 and Mox2 proteins fused to GST were expressed in *E. coli*. Flag epitope-tagged Pax1 and Pax3 proteins were expressed in COS1 cells transfected with the respective plasmids. GSTMox1 and GSTMox2 fusion proteins were purified on glutathione Sepharose beads and then incubated with cell extracts of COS1 expressing FlagPax1 and FlagPax3 respectively. The interaction was detected with Western blot analysis using monoclonal anti-Flag antibody after SDS-PAGE of the proteins retained on the glutathione beads. As a negative control we used glutathione Sepharose beads loaded with GST alone as well as cell extracts from COS1 cells transfected with a control plasmid. Pax1 interacted with Mox1 (Fig. 3A) and Pax3 with Mox2 (Fig. 4A) in the in vitro interaction assay, in agreement to the two-hybrid assay results.

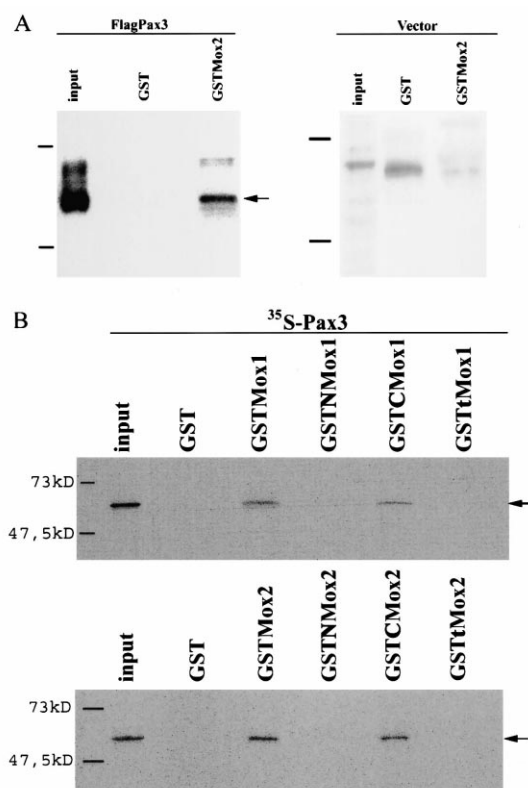


Fig. 4. Pax3 interacts with Mox1 and Mox2 in vitro. A: GST interaction assays were performed using GST or GSTMox2 (4 µg) and cell lysates prepared from COS1 cells transfected with a control plasmid or one expressing Flag-tagged Pax3. Immobilized proteins (arrow) were resolved by SDS-PAGE and visualized by Western blot analysis using an anti-Flag antibody. Input, 1% of the input protein extract. In A, dashes indicate molecular mass markers: 81, 49.9 kDa. B: Pax3 interacts with Mox1 and Mox2 through the Mox homeodomain. GST interaction assays were performed with 4 µg GST, GSTMox1, GSTMox2 and their N- and C-terminal deletions (see Fig. 2 for diagrams) and ³⁵S-labeled Pax3. Immobilized proteins (arrow) were resolved by SDS-PAGE and visualized by autoradiography. Input, 20% (1 µl) of ³⁵S-labeled Pax3.

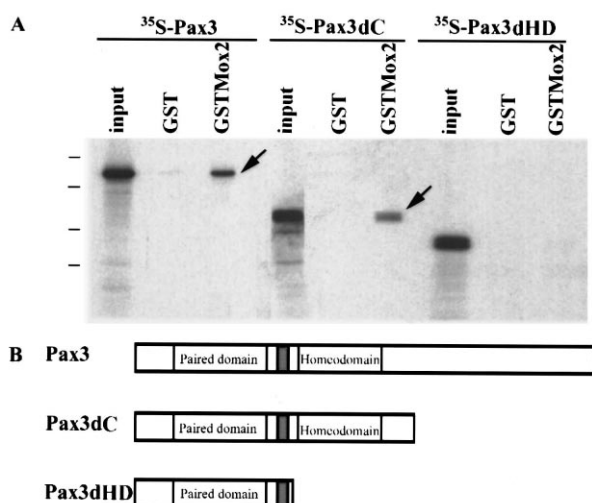


Fig. 5. Pax3 homeodomain is necessary for interaction with GSTMox2. A: Interaction assays were performed using 4 μ g GST or GSTMox2 and 2 μ l of the indicated 35 S-labeled Pax3 polypeptides. Input, 50% (1 μ l) of 35 S-labeled Pax3 polypeptides. Dashes indicate molecular mass markers: 81, 49.9, 36.2, 29.9 kDa. B: Pax3 and its deletions (Pax3dC–Pax3dHD) with the paired domain, homeodomain and octapeptide (gray box) indicated.

3.3. Interaction between Mox and Pax is mediated by the homeodomain of the Mox proteins

To map the regions of Mox1 and Mox2 required for the interaction with Pax1 and Pax3 respectively, we performed GST interaction assays using 35 S-labeled Pax1 and Pax3 and a series of truncated GSTMox1 and GSTMox2 proteins (Fig. 2). We found that 35 S-labeled Pax1 and Pax3 interacted only with the full-length Mox1 and Mox2 and with their C-terminal deletions that contain the homeodomain (Figs. 3B and 4B). Furthermore, in the same assay, GSTMox1 does not interact with 35 S-labeled Mox2 (data not shown), pointing to the specificity of the Mox–Pax interaction through the Mox homeodomains and not any homeodomain in general.

3.4. Pax3 homeodomain is necessary for the interaction with Mox2

To map the regions of Pax3 required for its interaction with Mox2 we generated a series of Pax3 truncated polypeptides (Fig. 5B) and tested their interaction with GSTMox2. We found that Pax3 and Pax3dC, both of which contain the homeodomain, interacted with GSTMox2 (Fig. 5A), whereas no such interaction was detected between GSTMox2 and Pax3dHD (Fig. 5A) or a shorter (amino acids 1–187) Pax3 deletion (data not shown).

4. Discussion

In this study we aimed to investigate whether Mox family

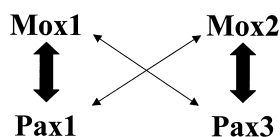


Fig. 6. A model for Mox–Pax protein–protein interactions. The thick arrows point to the preferred interactions while the thin ones point to weaker interactions.

proteins physically interact with Pax1 and Pax3. Indeed, such interactions were revealed using the yeast two-hybrid and *in vitro* biochemical assays. We show that Mox1 and Mox2 proteins are capable of interacting with Pax1 and Pax3. There is a strong preference of Mox1 to interact with Pax1 rather than Pax3 and of Mox2 to interact with Pax3 rather than Pax1 (Tables 1 and 2, Fig. 6). These interactions are mediated through the homeodomain of Mox. The weak interactions of both Mox1 protein with Pax3 and Mox2 with Pax1 could be due to the high degree of similarity between the Mox1 and Mox2 homeodomains (98%). The specificity of interactions is therefore determined by a different region of the Mox proteins. In the case of the Mox1–Pax1 pair, our two-hybrid results do not implicate the first two helices of the paired domain of Pax1 (which are necessary for its DNA binding activity) in the interaction with Mox1. In the case of the Mox2–Pax3 pair, the homeodomain of Pax3 is necessary for the interaction to occur. Interestingly, the paired domain of Pax3 has been shown to associate directly with the homeodomain protein Mx1, which antagonizes the myogenic activity of Pax3 in migrating limb muscle precursors so that premature differentiation is avoided [25].

These data were confirmed in two independent assays and are supported by *in vivo* expression data. The spatiotemporal expression patterns of the genes/proteins are highly overlapping. In particular, Mox1 is expressed in the sclerotome and dermomyotome of the developing somite; Mox2 is expressed in the sclerotome, in the migrating myoblasts that colonize the limbs and the premuscle mass of the limbs [2,5,6]. Pax1 is expressed in the sclerotome [10], Pax3 in the dermomyotome, the migrating myoblasts and the premuscle masses that will give rise to the limb musculature [13]. The expression patterns of the genes are consistent with the possibility of physical association of the gene products *in vivo*. The phenotypes of mutant mice are also consistent with this possibility although the observed defects (at least in the case of Pax3) are more widespread. Mox1 and Pax1 mutants have malformations in the axial skeleton, while Mox2 and Pax3 mutants show defects in the limb musculature. Considering the preference of Mox1 to interact with Pax1 and of Mox2 to interact with Pax3, the expression patterns and phenotypes of mutant mice, we hypothesize that only the Mox1–Pax1 and Mox2–Pax3 interactions occur in the developing embryo.

Mox-interacting proteins have not been identified up to now. Given the importance of Mox1 and Mox2 in the development of axial skeleton and limb musculature respectively, this information provides further insight into how these processes may be regulated. Mox2 and Pax3 are both implicated in the regulation of myoblast proliferation. Rat Mox2 (Gax) is implicated in growth arrest of myocardium and vascular smooth muscle cells [7,8] and chicken Mox2 is associated with non-proliferating skeletal myoblasts in the limb (Rallis et al., *in press*). On the other hand, Pax3 induces cell proliferation and inhibits differentiation in skeletal myoblast cultures [17]; and in the limb bud, Pax3 is associated with proliferating myoblasts [18]. It is possible that protein–protein interactions between Mox2 and Pax3 exist mainly in the myoblasts that colonize the limb; these interactions may be associated with the regulation of myoblast proliferation during their migration from the somite to the limb bud.

At present, there are no indications as to what the function of Mox1 may be at the cellular level. Its interacting partner,

Pax1 (as well as Pax9 which is thought to be redundant in function), is required to maintain a high rate of cell proliferation during a restricted phase of sclerotome development [12]. It would be interesting to investigate how the direct interaction of Mox1 with Pax1 may influence sclerotomal development. It should be noted that Pax1, unlike Pax3, does not possess a homeodomain, therefore it is not possible to make a direct comparison between Mox1–Pax1 and Mox2–Pax3 interactions.

Different gene families are involved in the control of the assignment of somite cells to their myogenic, chondrogenic and other lineages as well as in the regulation of the balance between proliferation, apoptosis and differentiation. The evidence presented here points to specific associations of Mox and Pax proteins. We propose that the Mox family of homeodomain proteins participates in the molecular signaling network regulating the diverse events of somite development through the physical interaction with the Pax1 and Pax3 members of the Pax family.

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