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Highly restricted pattern of connexin36 expression in chick somite development

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Abstract

The gap junction protein connexin36 (CX36) has been well studied in the mature central nervous system, but there has been little information regarding its possible roles in embryonic development. We report here the isolation of the full-length chick *CX36* coding sequence (predicted M_r 35.1 kDa) and its strikingly restricted pattern of gene expression in the mesoderm of the chick embryo. *In situ* hybridization experiments demonstrated *CX36* expression in somites by embryonic day 2. The transcripts first appeared dorsomedially within the somite and expanded ventrolaterally to form stripes in the middle of each somite. The *CX36* stripes fell within somitic territories enriched in *MYOD* and *FGF8* expression and impoverished in *PAX3* transcripts, establishing that *CX36* mRNA is expressed in the myotome. We compared the somitic expression pattern of *CX36* with those of three other connexins, *CX42*, *CX43*, and *CX45*. At embryonic day 4, *CX42* transcripts were localized to the myotome in a pattern resembling that of *CX36*. In contrast, *CX43* was enriched in the dermomyotome, and *CX45* was detected in both the myotome and the dermomyotome. Immunoblotting using Cx36 antibodies demonstrated bands of identical electrophoretic mobilities in trunk and retinal homogenates, and Cx36 immunostaining detected punctate immunoreactivity in the myotome. These results demonstrate that some connexins in the developing mesoderm are broadly expressed whereas others are highly localized, and suggest that CX36, CX42, and CX45 are involved in intercellular communication among developing muscle cells.

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Keywords

Gap junction; *FGF8*; *MYOD*; *PAX3*; Myotome

Introduction

Intercellular communication through gap junction channels may be important for defining cell compartments during embryonic development. Gap junctions are membrane specializations containing clusters of intercellular channels that allow transfer of ions and molecules up to 1 kDa between adjacent cells. Gap junction channels are made of oligomeric assemblies of homologous proteins named connexins (CX). Most cells in the adult animal are coupled through gap junctions. One exception is skeletal muscle, in which cells are initially coupled but become uncoupled as development and differentiation proceed (Rash and Staehelin, 1974; Keeter et al., 1975; Kalderon et al., 1977; Schmalbruch, 1982).

During development, trunk paraxial mesoderm becomes segmented into somites. Somites give rise to distinct cell populations, including the sclerotome, the dermomyotome, and the myotome, from which skeletal muscle cells originate. Experiments using injection of a gap junction permeant dye established the presence of several stage-dependent communication compartments within somites. Myotome cells are coupled as an ensemble, as are dermomyotome cells; sclerotome cells are subdivided into rostral and caudal compartments separated by a 3rd communication compartment made of cells from the intrasclerotomal fissure (Bagnall et al., 1992). The molecular basis of these compartments has not been extensively studied. In the mouse, *Cx43* transcripts have been detected in the dermatome and sclerotome (Ruangvoravat and Lo, 1992) and *Cx40* transcripts in myoblasts and myotubes (Dahl et al., 1995). In the rat, Cx43 protein has been detected in the dermatome (Yancey et al., 1992).

The present experiments explored the expression of connexins during somite development in the chicken embryo with special emphasis on CX36, the ortholog of mammalian Cx36 (Condorelli et al., 1998; Söhl et al.,1998) and fish Cx35 (O'Brien et al., 1996; 1998). Here, we report the cloning of chicken CX36, its expression pattern in somites during development, and the relation of its expression pattern to those of signaling molecules, transcription factors, and other connexins.

Materials and methods

Chick embryos

Fertilized White Leghorn chicken eggs obtained from Charles River SPAFAS (Connecticut, USA) and local suppliers were incubated at 99.5°F in a humidified incubator. Embryos were staged according to trunk morphology (Hamburger and Hamilton, 1951) and somite maturation (Ordahl, 1993).

Connexin36 cloning

A DNA fragment of chicken *CX36* was obtained by PCR using genomic DNA and a set of primers based on the mouse, human, skate and perch *connexin 36* sequences (accession numbers: AF016190, AF153047, U43290, and AF059183, respectively) using the CODEHOP program (Rose et al., 1998). Total cellular RNA was isolated from brain and retina by the method of Chomczynski and Sacchi (1987). Complementary DNAs spanning the full *CX36* coding sequence were obtained using the SMART RACE cDNA Amplification Kit (Clontech, BD Biosciences, Palo Alto, CA). The *CX36* sequence was deposited into the GeneBank database (accession number: AF458098).

Wholemount *in situ* hybridization

Wholemount *in situ* hybridization was performed as previously described (Agarwala and Ragsdale, 2002) using embryos from HH stage 10 until day 7 with probes for *CX36*, *CX42* (Beyer, 1990), *CX43* (Musil et al., 1990), *CX45* (Beyer, 1990), *FGF8* (kindly provided by Dr. Gail Martin), *MYOD* and *PAX3* (kindly provided by Dr. Martyn Goulding). Some stained embryos were embedded in gelatin and sectioned at 32–40 μm on an SM 2000R sliding microtome (Leica, Houston, TX). Sections were dried onto glass slides, dehydrated, and mounted with coverslips and Eukitt Mounting Medium (Electron Microscopy Sciences, Fort Washington, PA). Sections were studied with an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY), and images were captured with an AxioCam digital camera (Carl Zeiss, Thornwood, NY). Composite figures were assembled using Adobe Photoshop (Adobe Systems, San Jose, CA)

Protein determination

Protein concentrations were determined using the BioRad Protein Assay (BioRad, Hercules, CA) based on the Bradford dye-binding procedure (Bradford, 1976).

Immunoblotting

Homogenates from embryonic day 4 (E4) trunk, E12 retina, and E12 liver were prepared in 4 mM EDTA, 2 mM phenylmethylsul-fonylfluoride in phosphate buffered saline (PBS), pH 7.4. One hundred μg of protein were resolved on an 11% SDS-containing poly-acrylamide gel and transferred to Immobilon-P (Millipore, Billerica, MA). Membranes were blocked in 5% nonfat milk in Tris-buffered saline (TBS), pH 7.4, and incubated overnight in rabbit polyclonal anti-Cx36 antibodies (Zymed Laboratories, South San Francisco, CA). Membranes were rinsed in TBS and incubated in peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) for 1 h and rinsed in TBS. Binding of secondary antibody was detected using enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

Immunofluorescence

Chicken embryos at day 6 were fixed in 4% paraformaldehyde in PBS for 4 h at room temperature. They were transferred to 30% sucrose in PBS and left at 4°C until they sank. Twelve-μm cryostat sections were incubated in 0.2% Triton X-100 for 30 min at room temperature, followed by incubation in blocking solution (5 mM EDTA, 1% fish gelatin, 0.05% NP40, 1% essentially immunoglobulin- free bovine serum albumin, 1% normal goat serum in PBS). Sections were incubated overnight at 4°C with anti-Cx36 antibodies (Zymed Laboratories, South San Francisco, CA) and the mouse monoclonal anti-chicken pectoralis myosin antibody MF20 (NICHD/University of Iowa Developmental Studies Hybridoma Bank). Sections were rinsed four times with PBS and then incubated in Cy2-conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch, West Grove, PA) for 1.5 h at room temperature. Sections were rinsed four times with PBS, and coverslips were mounted with 2% n-propyl-gallate in PBS:glycerol (1:1).

Results

DNA sequence for a fragment of chicken *CX36* encoding the 1st extracellular loop through the 4th transmembrane domain was obtained by PCR from genomic template DNA. Full coding sequence was obtained by RACE using total cellular RNA from brain and retina with primers based on the DNA fragment obtained by PCR. Analysis of the chicken *CX36* sequence predicted a 304 amino-acid protein of a molecular mass of 35.1 kDa showing a high degree of sequence identity to *CX36* sequence from mammals and fishes (Fig. 1).

The expression pattern of *CX36* was studied by wholemount *in situ* hybridization. A strong and striking pattern of hybridization was detected in the somites of the chick embryo. The *CX36* probe hybridized to a narrow stripe in the middle of the somite (illustrated in Fig. 2A for E4). At this stage, *CX36* hybridization was also detected in pancreas and heart (Fig. 2A). The hybridizing region in somites initially formed a triangular area in a dorsomedial position, within which labeled cells formed a "mosaic" pattern (E2; from HH stage 13 and illustrated for HH stage 15 in Fig. 2B). As somite differentiation proceeded, the hybridizing region extended ventrolaterally and became more restricted to the middle of the somite. The striped pattern of expression of *CX36* was observed by wholemount *in situ* hybridization through embryonic day 7, the latest stage studied; at this stage, hybridization in the tail region was much stronger than in the trunk region. Sectioned embryos showed labeling in the myotomal region of the somite and in cells that had populated the limb bud (Fig. 2C).

We compared the expression pattern of *CX36* to that of signaling molecules and transcription factors known to be expressed in somites. Two-color *in situ* hybridization was performed using probes for *CX36* and *PAX3*, *FGF8*, or *MYOD* on wholemount embryos. The gene expression patterns for *CX36* and the transcription factor *PAX3* were strikingly different. *PAX3* was detected in the mesoderm before the onset of *CX36* expression. At embryonic day 4, *PAX3* was strongly expressed in the dermomyotome (Goulding et al., 1994; Williams and Ordahl, 1994), whereas *CX36* appeared to identify only the myotome (Fig. 3A, B, E). Very little overlap at the dorsomedial and ventrolateral regions was observed between the *PAX3*- and *CX36*-hybridizing domains (Fig. 3B). In contrast, substantial overlap of the gene expression patterns was observed in experiments using probes for *CX36* and the muscle-specific transcription factor *MYOD*, which is expressed in the myotome (Pownall and Emerson, 1992), although the area of *MYOD* expression was broader than that of *CX36* (Fig. 3C, F). At this developmental age, *FGF8* transcripts were found in the myotome in agreement with the findings of Stolte et al. (2002), and the expression domains of *CX36* and *FGF8* showed extensive overlap (Fig. 3D).

The expression pattern of *CX36* was also compared to that of three connexins, *CX42*, *CX43*, and *CX45*, previously reported to be expressed in somites based on mRNA, protein, and lacZ reporter expression experiments (Ruangvoravat and Lo, 1992; Yancey et al., 1992; Dahl et al., 1995; Krüger et al., 2000). *Connexin42* was concentrated in the heart and blood vessels from early stages of development, but by embryonic day 4, the wholemount *in situ* hybridization pattern of *CX42* in somites showed a striking similarity to that of *CX36*. Serial sectioning demonstrated extensive overlap between the *CX42* and *CX36* signals in the myotome (Fig. 4B) and strong *CX42* labeling of intersomitic vessels (Fig. 4A). *Connexin43*, in contrast, was widely expressed in the chicken embryo. At embryonic day 4, *CX43* transcripts in the somites were found within the dermomyotome and sclerotome. No overlap between the *CX36*- and *CX43*-hybridizing regions was observed (Fig. 4E). Similarly, no significant overlap between the *CX42*- and the *CX43*-rich territories was observed (Fig. 4C, D). *Connexin45* was also widely expressed during development (Fig. 4F). Two-color *in situ* hybridization with *PAX3* and *CX45* established that *CX45* transcripts were expressed in both the dermomyotome and the myotome (Fig. 4G, H).

By embryonic day 5, hybridization of the *CX36* probe was also observed in the limb buds (Fig. 5A), eye muscles, and the central nervous system (not illustrated). The *CX36*-hybridizing domain in the limb bud showed substantial overlap with that of the *PAX3* (Fig. 5B) and *MYOD* (Fig 5C). This result suggests that *CX36* is also expressed in limb muscles; overlap of *PAX3* and *MYOD* expression has been previously reported in the limb bud (Goulding et al., 1994).

Expression of Cx36-like protein in the trunk region was confirmed by immunoblotting. A band with an electrophoretic mobility indistinguishable from that detected in homogenates of 12th embryonic day retina was detected in homogenates from the embryonic day 4 trunk region (Fig. 6). The localization of this protein was studied by immunofluorescence using anti-Cx36 antibodies. Immunoreactive spots with a distribution mimicking the shape of the myotome were observed in transverse sections (not illustrated). In horizontal sections, double immunofluorescence experiments using a monoclonal antibody that recognizes sarcomeric myosin showed punctate Cx36-like immunoreactivity at the plasma membrane of myotomal cells that was more evident at the caudal and rostral limits of each myotome (Fig. 7).

Discussion

Expression of *CX36* has previously been detected in neural cells (Condorelli et al., 1998) and β-cells of the pancreas (Serre-Beinier et al., 2000). In this paper, we report that during development, *CX36* is expressed within the myotome, limb and eye muscles, the heart, and the pancreas. By embryonic day 4, *CX36* mRNA expression was restricted to the middle of the somite, while staining for CX36 protein was evident in horizontal sections at the rostral and caudal ends of the myotome.

The myotome is formed from myoblasts derived from the dermomyotome. The first terminally differentiated myotome cells begin to appear by somite stage X (Ordahl, 1993). The onset of *CX36* expression (as determined by wholemount *in situ* hybridization) would thus correlate with the appearance of terminally differentiated myotomal cells. At this early stage, the hybridization pattern of *CX36* had a triangular shape. This transient triangular domain is reminiscent of the shape of the developing myotome defined by other muscle markers (i.e., desmin and acetylcholinesterase) (Kaehn et al., 1988), and it would result from migration of mesenchymal myoblasts to the rostral margin of the somite to form the primary myotome (Kahane et al., 2002).

The nuclei of the earliest postmitotic myotomal progenitors become reorganized by E4 into a narrow stripe restricted to the middle part of the myotome length (Kahane et al., 1998). Similarly, *CX36* transcripts became restricted between E3 and E4 to the central region of the somite corresponding to the position of myotomal fiber nuclei. Thus, it is possible that *CX36* transcripts are always restricted to the nuclear domain. A restricted localization of transcripts has been previously shown for the initial stages of slow myosin heavy chain expression (Sacks et al., 2003).

The finding of a mosaic pattern of hybridization for any gene expressed in somites is unexpected. However, nuclear localization of transcripts during migration of mesenchymal myoblasts and redistribution of nuclei during elongation of myotomal cells may explain the mosaicism observed in the hybridization pattern of *CX36* at early stages of development. Because a role for gap junctions containing Cx43 has been previously proposed for neural crest cell migration (Huang et al., 1998), it is also plausible that *CX36* may be involved in the migration events associated with myotome formation.

In contrast to the restricted localization of *CX36* transcripts within the nuclear domain, immunoreactive Cx36-like protein was found between myotome cells and appeared concentrated at the sites of attachment of myotome cells to the rostral and caudal ends of somites. The fine punctae of Cx36-like protein observed in cross-sections and the linear-like pattern of staining observed in horizontal sections are reminiscent of the distribution of gap junctions during amphibian development as seen by transmission electron microscopy (Keeter et al., 1975; Blackshaw and Warner, 1976). In these embryos, gap junctions have been observed between muscle cells within a given somite and between muscle cells in adjacent somites

(Keeter et al., 1975; Blackshaw and Warner, 1976). The linear-like appearance of staining at the rostral and caudal ends of the myotome as observed in horizontal sections might result from the convergence of myotomal cells to their sites of attachment which produces the fusiform shape of the myotome.

Skeletal muscle cells are initially coupled through gap junctions, but become uncoupled and lose these structures after formation of muscle fibers and innervation (Rash and Staehelin, 1974; Keeter et al., 1975; Kalderon et al., 1977; Schmalbruch, 1982). We have found that *CX36*, *CX42*, and *CX45* are expressed in the myotome. Because rodent *Cx40* and Cx43 have been previously reported to be expressed in somites (Ruangvoravat and Lo, 1992; Yancey et al., 1992; Dahl et al., 1995), it is possible that their expression in somites is conserved across species. Thus, our data confirm and extend previously published results and localize *CX42* (ortholog of mouse *Cx40*) to the myotome, *CX43* to the dermomyotome and *CX45* to both the dermomyotome and the myotome. The extensive degree of overlap between the *CX36*- and *CX42*-expressing regions of the myotome suggests that both connexins could participate in the dye coupling observed by Bagnall et al. (1992) between myotome cells.

During myotome formation, expression of *PAX3* precedes the appearance of *MYOD* transcripts (Goulding et al., 1994; Williams and Ordahl, 1994). Similarly, we found that *PAX3* expression preceded expression of *CX36*. Myotomal expression of *FGF8*, which is detected in somite stages IX–XII at stage 12 and somite stages VII–XII at stage 13 (Stolte et al., 2002), also preceded expression of *CX36*. The transient expression of *FGF8* in the myotome defining a subpopulation of muscle precursor cells (Stolte et al., 2002) suggests that *FGF8* may be involved in regulation of *CX36* expression in the myotome.

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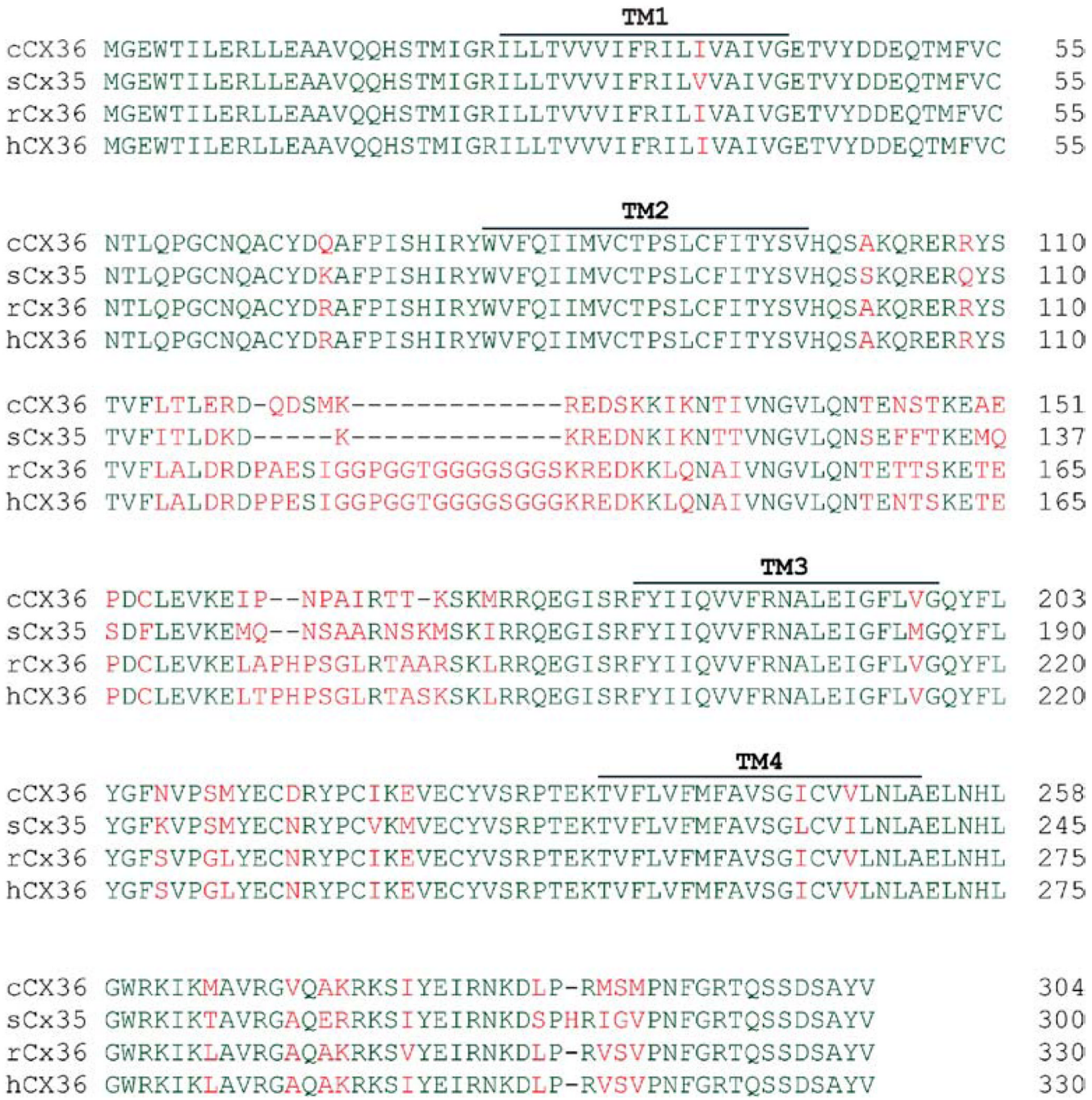

Fig. 1.

Alignment of the DNA sequences of connexin36 from chicken (cCX36), skate (sCx35), rat (rCx36), and human (hCX36). Amino acids that are identical in all sequences are indicated in *dark green*, while those that differ among the sequences are indicated in *red*. The transmembrane domains (TM) predicted by the HMM TOP server (http://www.enzim.hu/hmmtop/) are indicated with lines. There are 259, 271, and 276 identical amino acids between chicken CX36 and skate Cx35, rat Cx36, and human CX36, respectively

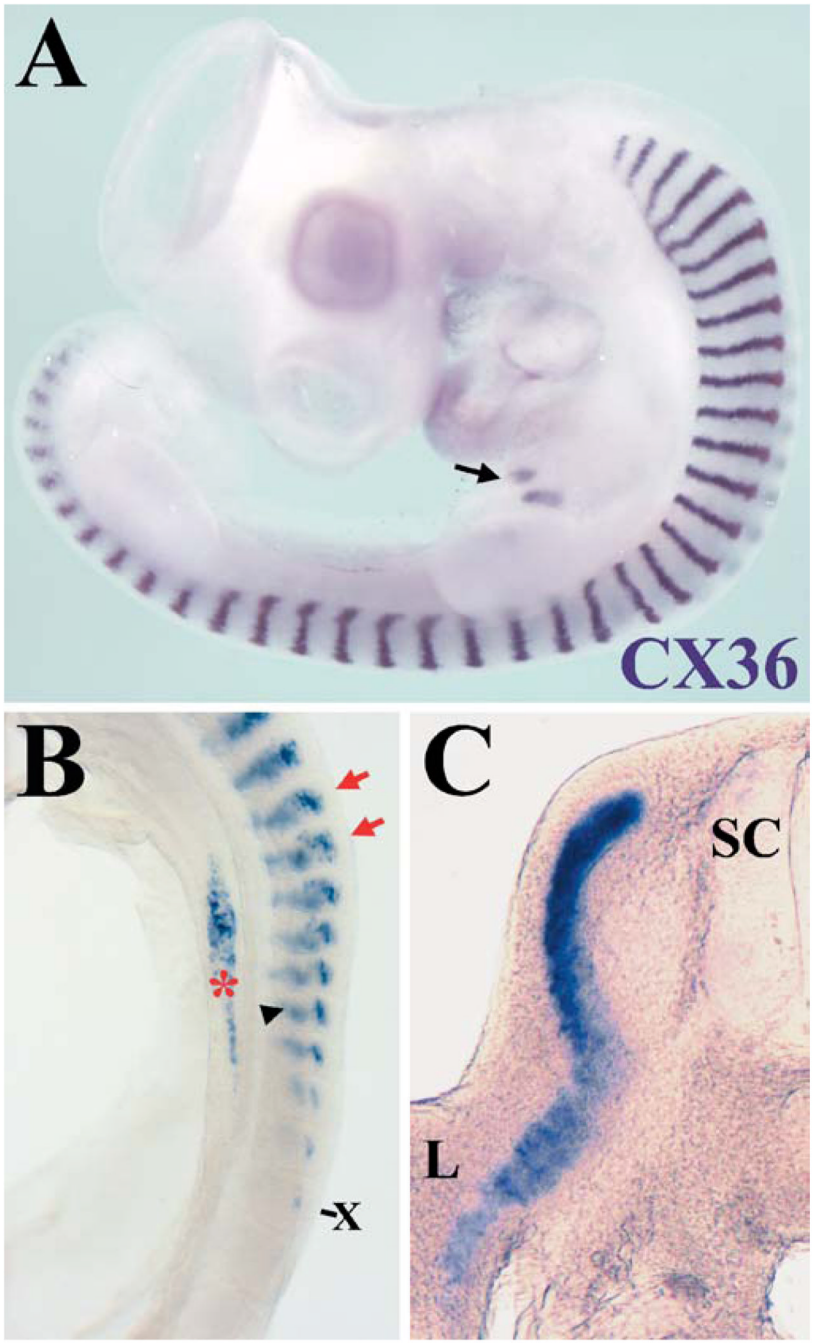

Fig. 2A–C.

Expression pattern of *CX36* in somites. **A** Photomicrograph of an embryonic day 4 (E4) chick (HH stage 24) demonstrates *CX36* expression in stripes in the middle of the somites, in the pancreas (*arrow*), and in the heart. **B** Lateral view of the trunk region of an E2 chicken (HH stage 15). Hybridization of *CX36* in the somites is first detected in the 10th-most distal somite (X). The mosaic pattern of *CX36* hybridization is indicated by the *red arrows*. Somitic hybridization in the contralateral side is seen out of focus (*black arrowhead*). Endoderm expression (*red asterisk*) can be detected by HH stage 14 and will later (HH stage 16–18) coalesce to mark the ventral and dorsal rudiments of the pancreas. **C** Transverse section through

the trunk region of an E5 chicken (HH stage 27) demonstrates hybridization of the *CX36* probe to the myotome and to cells that have populated the limb. (*SC* spinal cord, *L* limb)

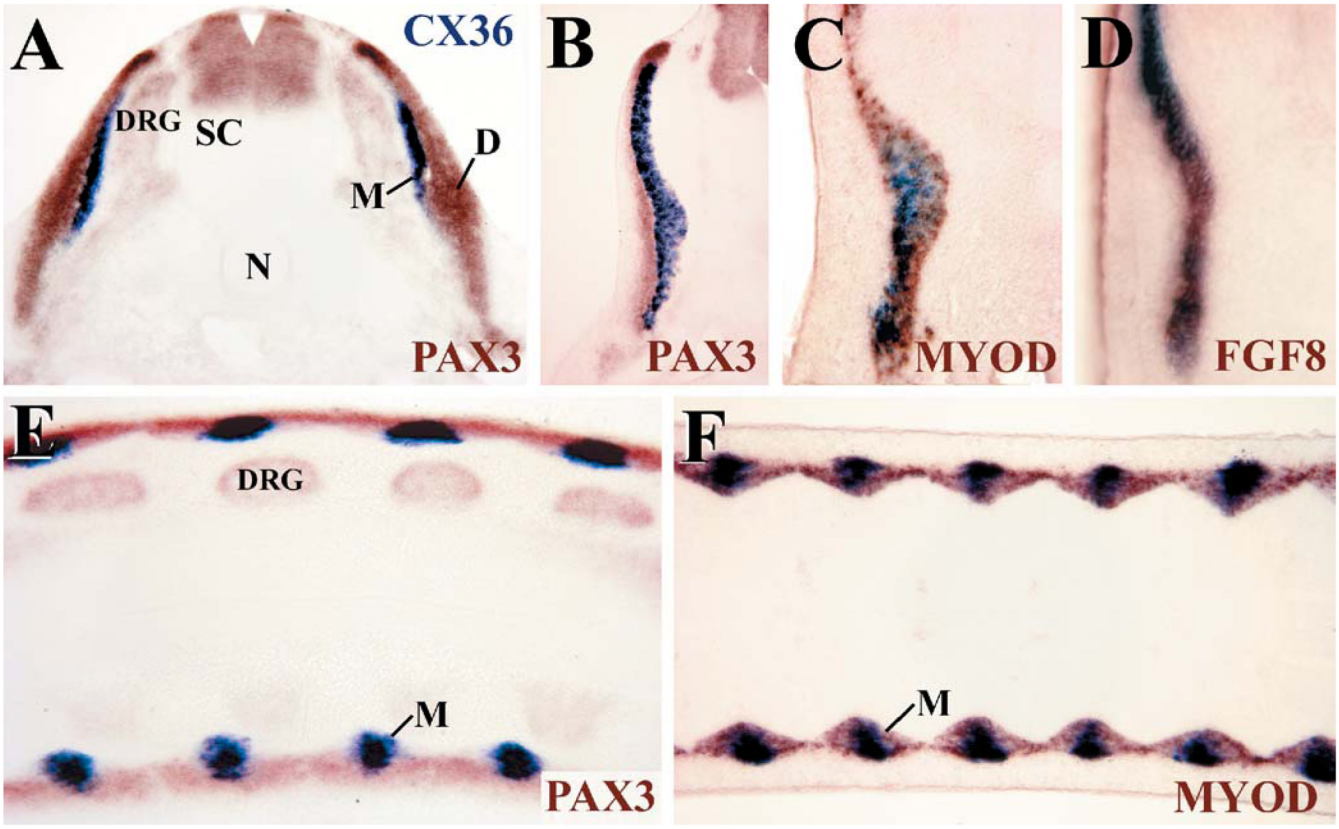

Fig. 3A–F.

Connexin36 is expressed in the myotome. Transverse (**A–D**) and horizontal (**E, F**) sections through somites from stage 24 embryos prepared for two-color *in situ* hybridization with probes to *PAX3* and *CX36* (**A, B, E**), *MYOD* and *CX36* (**C, F**), and *FGF8* and *CX36* (**D**). Hybridization of the *CX36* probe is marked by a *blue-purple* reaction product and that of the *PAX3*, *MYOD*, and *FGF8* probes appears in *brown*. (*D* dermomyotome, *DRG* dorsal root ganglion, *M* myotome, *N* notochord, *SC* spinal cord)

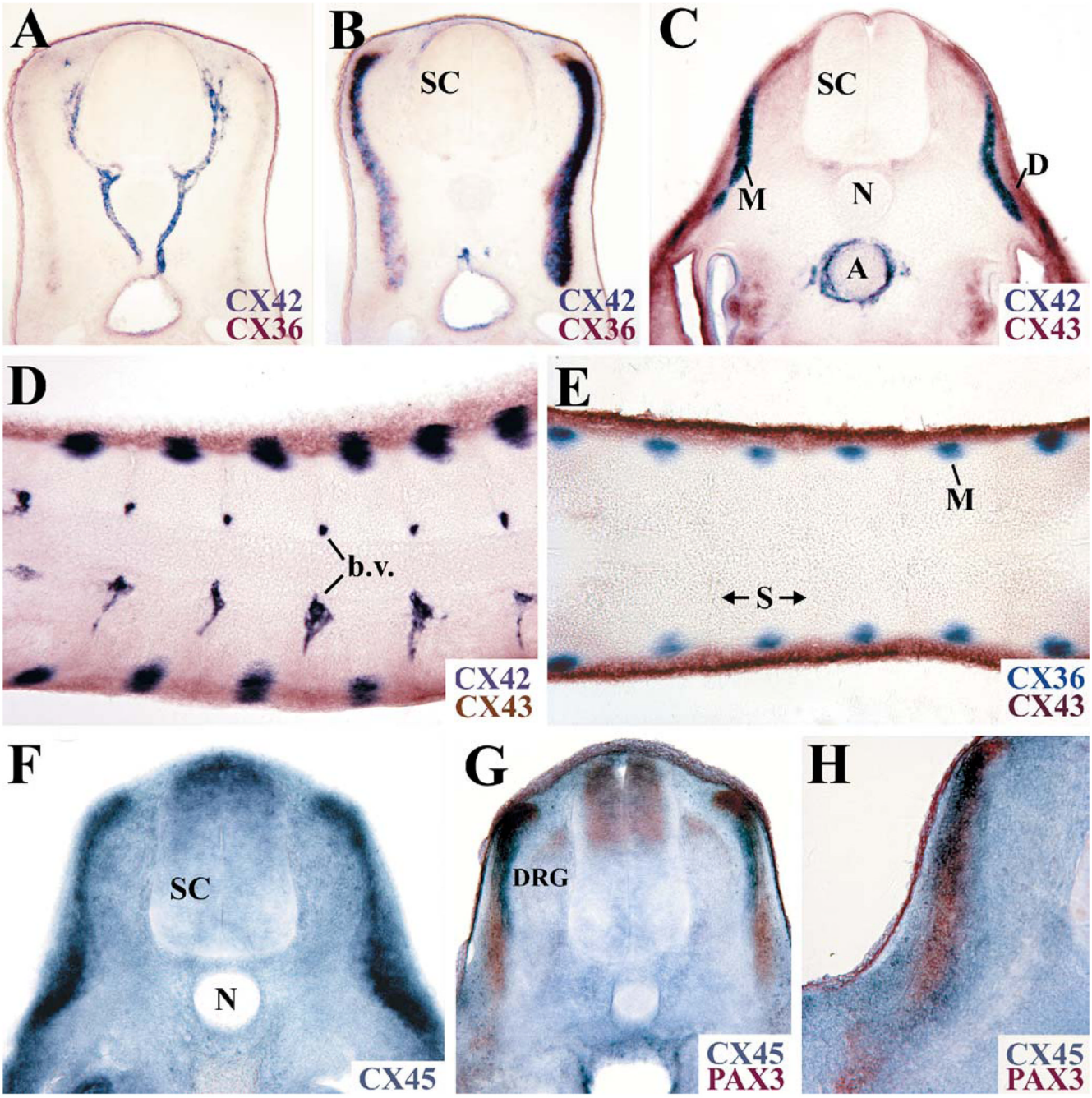

Fig. 4A–H.

Multiple connexins are expressed in developing somites. **A, B** Adjoining serial transverse sections from an embryo probed for *CX36* (*brown*) and *CX42* (*blue-purple*) expression. *Connexin42* and *CX36* are both expressed in the myotome (**B**) and *CX42* is enriched in the vasculature (**A**). **C, D** Transverse (**C**) and horizontal (**D**) sections from an embryo after two-color *in situ* hybridization with *CX42* (*blue-purple*) and *CX43* (*brown*) probes. **E** Horizontal section from an embryo hybridized with *CX36* (*blue-purple*) and *CX43* (*brown*) probes. **F** Transverse section from an embryo hybridized using a *CX45* probe. **G, H** Transverse sections from an embryo after *in situ* hybridization with *CX45* (*blue*) and *PAX3* (*brown*) probes. All tissues were obtained from HH stage 24 embryos. (*A* aorta, *b.v.* blood vessel, *D*

dermomyotome, *DRG* dorsal root ganglion, *M* myotome, *N* notochord, *S* somite, *SC* spinal cord)

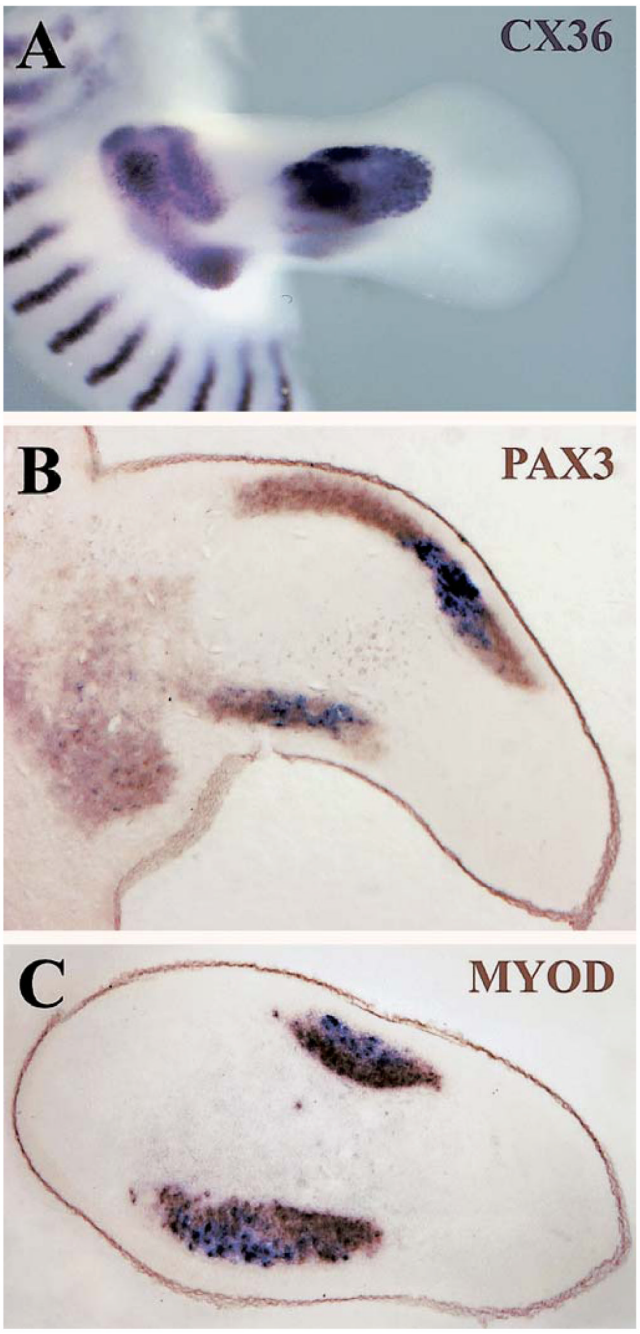

Fig. 5A–C.

Connexin36 is expressed in the limb buds. **A** Whole-mount *in situ* hybridization of an embryonic day 6 (HH stage 29) hindlimb probed for *CX36* expression shows hybridization in the dorsal and ventral regions of the proximal and distal parts of the limb bud. **B, C** Transverse sections of an embryonic day 5 forelimb after two-color *in situ* hybridization with probes to *CX36* and *PAX3* (**B**) and *CX36* and *MYOD* (**C**). Hybridization of the *CX36* probe is seen in *blue-purple* and that of *PAX3* and *MYOD* is shown in *brown*. Dorsal is oriented towards the top

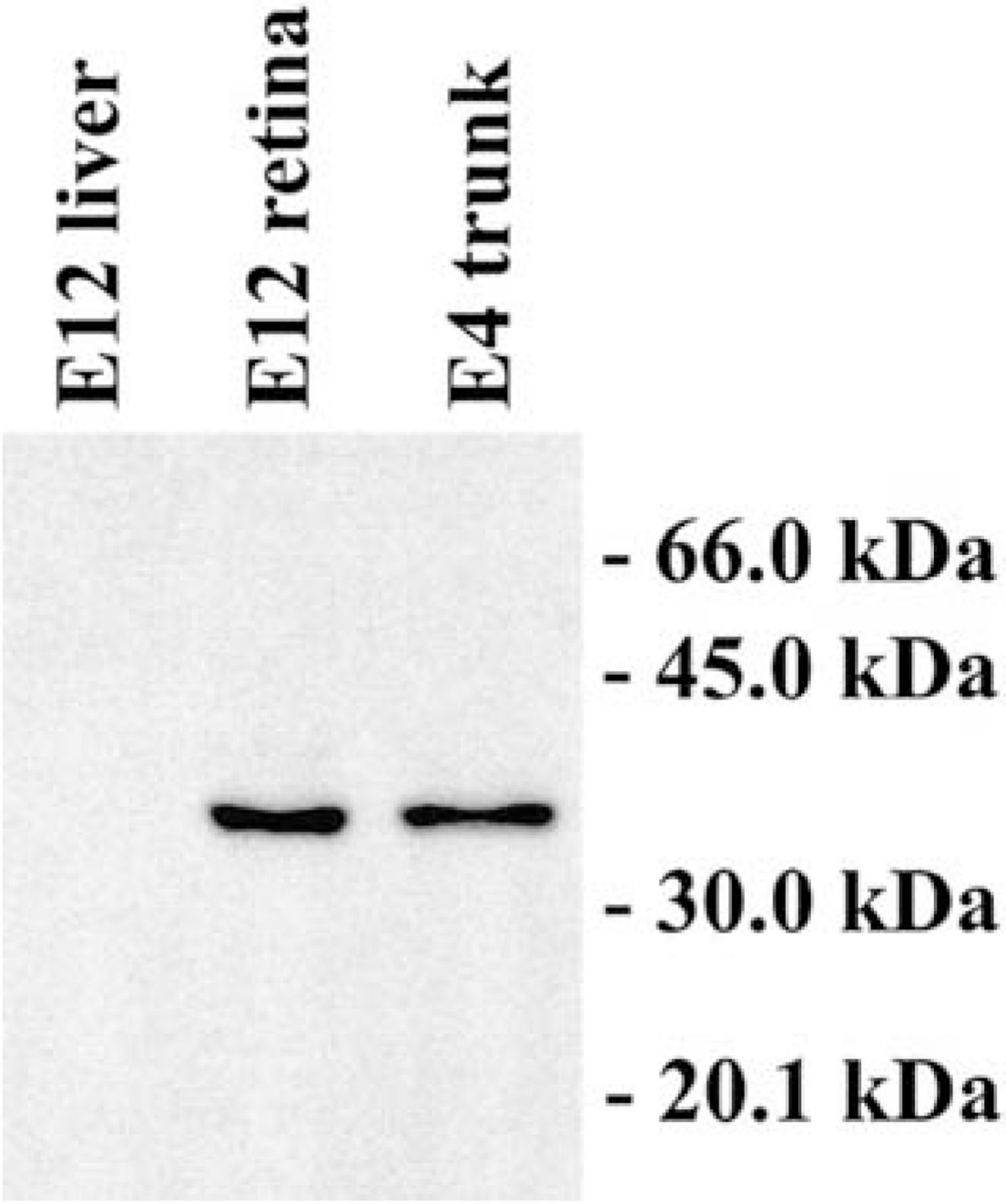

Fig. 6.
Immunoblot pattern of CX36. Homogenates of E12 liver, E12 retina, and E4 trunk were resolved by SDS-PAGE and subjected to immunoblotting using anti-Cx36 antibodies. The migration position of molecular mass standards is indicated

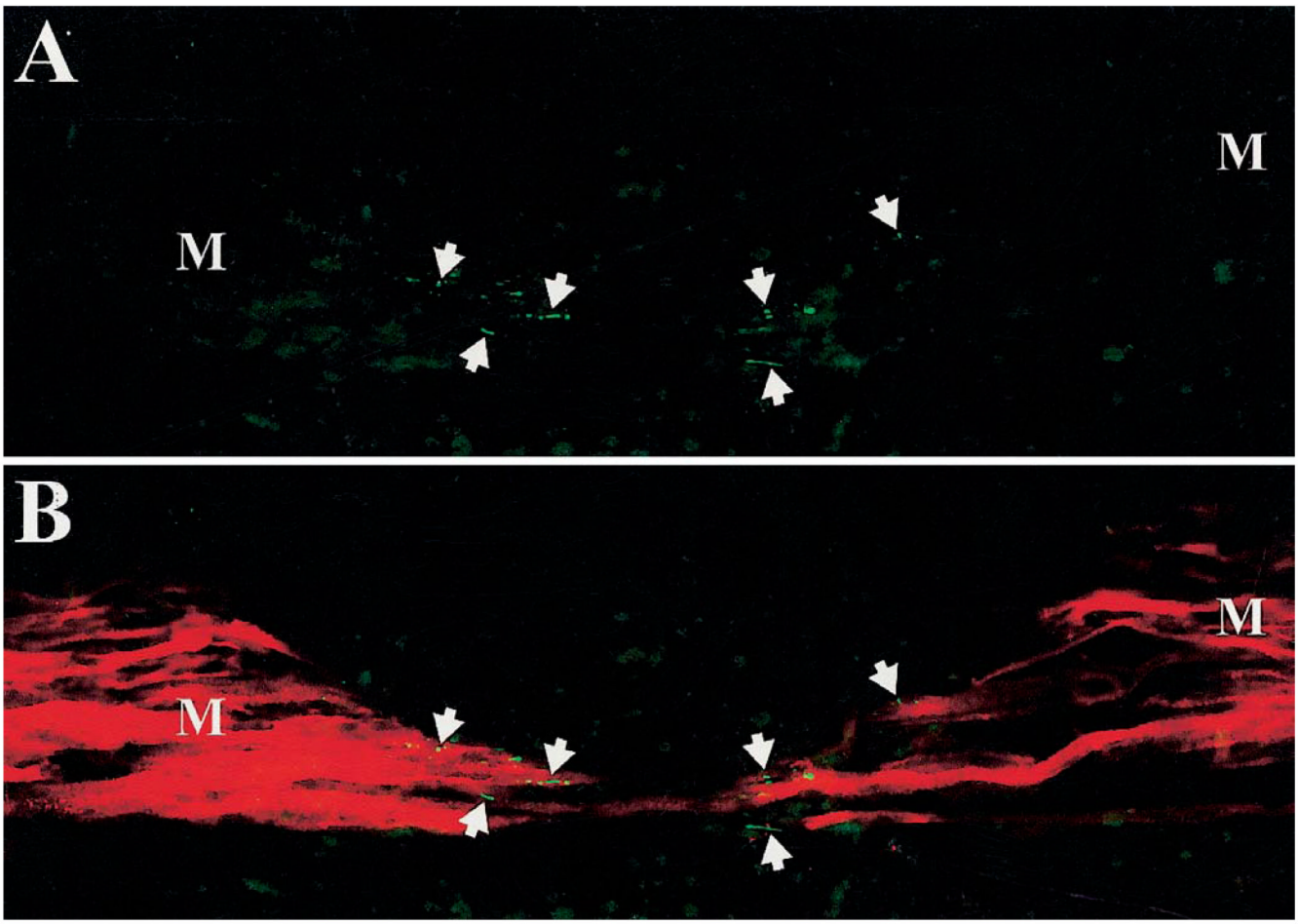

Fig. 7A, B.

Localization of Cx36-like immunoreactivity. **A, B** Photomicrographs of double immunofluorescence staining of a 12-μm horizontal section from an E6 chicken. Rabbit polyclonal anti-Cx36 antibodies and Cy2-conjugated goat anti-rabbit IgG antibodies (*green*) were used to detect Cx36-like protein, and a mouse monoclonal anti-chicken sarcomeric myosin (MF20) antibody and Cy3-conjugated goat anti-mouse IgG antibodies (*red*) were employed to label myotome cells. Connexin36-like staining (*arrows*) is shown in **A** (green channel alone), and its relationship to sarcomeric myosin is demonstrated in **B** (superposition of green and red channels). Punctate Cx36-like immunoreactivity (*arrows*) was observed between cells of the myotome (*M*). The lack of an extensive superposition between the red and green signals is due to the different cellular localizations of the proteins: Cx36-like immunoreactivity localizes to the plasma membrane, whereas MF20 immunoreactivity is present in the cytoplasm