DNA Dendrimers Localize MyoD mRNA in Presomitic Tissues of the Chick Embryo

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Abstract. MyoD expression is thought to be induced in somites in response to factors released by surrounding tissues; however, reverse transcription-PCR and cell culture analyses indicate that myogenic cells are present in the embryo before somite formation. Fluorescently labeled DNA dendrimers were used to identify MyoD expressing cells in presomitic tissues in vivo. Subpopulations of MyoD positive cells were found in the segmental plate, epiblast, mesoderm, and hypoblast. Directly after laying, the epiblast of the two layered embryo contained ~20 MyoD positive cells. These results demonstrate that dendrimers are precise and sensitive reagents for localizing low levels of mRNA in tissue sections and whole embryos, and that cells with myogenic potential are present in the embryo before the initiation of gastrulation.

Key words: myogenesis • epiblast • segmental plate • in situ hybridization • muscle transcription factor

Introduction

Reverse transcription PCR (RT-PCR)1 can reveal the presence of messenger RNA not detectable by in situ hybridization. This raises the question of whether messages present in low abundance are functionally significant, an issue particularly relevant to the study of the MyoD family of transcription factors that regulate skeletal muscle development (Weintraub et al., 1991; Rudnicki and Jaenisch, 1995; Molkentin and Olson, 1996). A widely held view states that the expression of MyoD in the somites of avian embryos and Myf5 in the mouse is initiated by factors secreted by the neural tube, notochord, and ectoderm. mRNA for these factors is not detected by in situ hybridization until after somites pinch off from the segmental plate mesoderm and an intact segmental plate will not form muscle in vitro unless it is cocultured with the neural tube and/or notochord (for review see Cossu et al., 1996; George-Weinstein et al., 1998, 1999; Buckingham and Tjabksh, 1999). However, MyoD has been detected by RT-PCR in the segmental plate and the epiblast that gives rise to the mesoderm during gastrulation (George-Weinstein et al., 1996a,b). Furthermore, both the segmental plate and epiblast give rise to an abundance of skeletal muscle when isolated from surrounding tissues, dissociated into a single cell suspension, and cultured in serum-free medium (George-Weinstein et al., 1994, 1996a, 1997). These and other experiments (Krenn et al., 1988; Choi et al., 1989; Holtzer et al., 1990; Chen and Solursh, 1991; von Kirschhofer et al., 1994) suggest that myogenic cells are present in early embryos, but are repressed from differentiating in vivo until after somite formation. Inductive factors secreted by the neural tube and notochord may upregulate the expression of MyoD in cells that already have low levels of message. Thus, in this case, low abundance mRNA appears to be an indicator of developmental potential.

Whereas RT-PCR can detect low levels of mRNA, it does not reveal how many cells contain MyoD or where they are located within the embryo. To identify those cells that express MyoD before somite formation, we have performed in situ hybridizations with increasingly younger tissues using the recently developed and sensitive probes, fluorescently labeled 3DNA dendrimers. Dendrimers are highly branched, multilayered structures synthesized by sequential hybridizations of partially complimentary heteroduplexes called DNA monomers (Fig. 1; Nilsen et al., 1997; Vogelbacker et al., 1997; Wang et al., 1998). Greater
than 500 molecules of fluorochrome, \( ^{32}\text{P} \), digoxigenin, or biotin can be incorporated into the dendrimer. Many of the single-stranded outer arms are cross-linked with an oligonucleotide sequence specific for a particular mRNA or DNA sequence. Since dendrimers produce a 100- to 1,000-fold increase in signal compared with single-stranded oligonucleotide probes in Northern and Southern blots and can be tagged with a fluorochrome, they were predicted to be precise and sensitive probes for mRNA in single cells in tissue sections. In situ hybridizations with Cy3 dendrimers revealed that subpopulations of MyoD positive cells are present throughout the segmental plate, epiblast, mesoderm, and hypoblast.

Materials and Methods

Synthesis of DNA Dendrimers

Core DNA dendrimers were synthesized as described previously (Nilsen et al., 1997; Vogelbacker et al., 1997; Wang et al., 1998). A assembly proceeds by sequential hybridization of seven single-stranded DNA 116mers. Each 116-mer is designed to partially hybridize via a central region of 50 nucleotides, yielding a heteroduplex monomer with a double-stranded waist region and four single-stranded arms (Fig. 1 A). These initiator dendrimers are hybridized to monomers to produce the one-layer growing structure (Fig. 1 B). Dendritic assembly was continued by the subsequent addition of monomers to the growing one-layer structure to yield a four-layer dendrimer (Fig. 1 C). At each hybridization, the structure is covalently cross-linked with \( 4,5',8 \)-trimethyl psoralen (trioxysalen; 1/10 vol/vol of psoralen-saturated ethanol), followed by a 10-min exposure to UV light (in a Simms Instruments ultraviolet reaction chamber 3000). The four-layer core dendrimer was purified from denaturing sucrose gradients (10–50% sucrose, 50% formamide, 50 mM tris-HCl, 10 mM EDTA, pH 8.0) at 40°C to 35°C in w-27.

Antisense oligonucleotides for specific mRNAs plus 7–30 bases complementary to the dendrimer were either psoralen cross-linked or ligated to at least 20 of the outer surface dendrimer arms. Fluorescent dendrimers were prepared by hybridizing and cross-linking a Cy3labeled oligonucleotide to at least one half of the arms on the outer surface of the dendrimer. Each dendrimer contained from 250–500 Cy3 molecules.

Dendrimers contained the following cDNA sequences for antisense mRNA: chicken MyoD (Dechesne et al., 1994), 5'-TTC TCA AGA GCA AAT ACT CAC CAT TTG TGT AGT AAG AGC TGC TG-3'; chicken embryonic fast myosin (Freyer and Robbins, 1983), 5'-CAC GAG GGT CTC ACG GTC CTC CCT CCA CGT GTC GAG GGT CTT CTC CAT CCT CTC TCC AAG G-3'; and chicken glyceraldehyde-3-phosphate dehydrogenase (Dugaiczyk et al., 1983), 5'-ATC AAG TCC ACA ACA CGG TTG CTG TAT CAC TCA TG TGG CAG GA A-3'. Dendrimers lacking a specific recognition sequence were used as a negative control for background fluorescence.

In Situ Hybridization

The in situ hybridization protocol was modified from that of Sassoon and Rosenthal (1993) and Raap et al. (1994). White Leghorn chick embryos (Truslow Farms) were staged according to the method of Hamburger and Hamilton (1951). Stage 16 (28 pairs of somites), stages 13-14 (17–22 pairs of somites), and stage 4 embryos were fixed in 4% formaldehyde, embedded in paraffin, sectioned transversely at 10 μm, and applied to 3-well teflon printed slides (Electron Microscopy Sciences) coated with 0.2% gelatin. After rinsing in 60% formamide, nuclei were labeled with bis-benzamide (Electron Microscopy Sciences). The in situ hybridization protocol was modified from that of Sassoon and Rosenthal (1993) and Raap et al. (1994). White Leghorn chick embryos (Truslow Farms) were staged according to the method of Hamburger and Hamilton (1951). Stage 16 (28 pairs of somites), stages 13–14 (17–22 pairs of somites), and stage 4 embryos were fixed in 4% formaldehyde, embedded in paraffin, sectioned transversely at 10 μm, and applied to 3-well teflon printed slides (Electron Microscopy Sciences) coated with 0.2% gelatin. After rinsing in 60% formamide, nuclei were labeled with bis-benzamide (Sigma Chemical Co.) in 0.01 M HCl. 30 μl of hybridization buffer containing 60% deionized formamide, 2× SSC buffer, 50 mM sodium phosphate, 5% dextran sulfate (Sigma Chemical Co.), 15 μg yeast RNA, 15 μg salmon sperm DNA (Boehringer), and 18 ng of Cy3labeled dendrimers was applied to each section. Sections were incubated at 80°C for 10 min then at 37°C overnight. After rinsing in 60% formamide, nuclei were labeled with bis-benzamide (Sigma Chemical Co.; 1 ng/ml deionized water). Sections were mounted in Gelmount (Fisher Scientific) and observed with a Nikon Eclipse E800 epifluorescence microscope (Optical Aparatus). Photomicrographs of differential interference contrast (DIC) images, bis-benzamide-labeled nuclei, and Cy3 dendrimers were produced with the Optronics DEI 750 video camera and Image-Pro Plus image analysis software (Phase 3 Imaging Systems). Results were consistent in sections from 9 stages 13–14 embryos and 5 stage 4 embryos.

In situ hybridizations also were performed on whole, unsectioned embryos. Hamburger and Hamilton (1951) stage 1 embryos were further divided into stages X–XII by the method of Eyal-Giladi and Korchav (1976). Stages X–XII and stage 2 embryos were fixed and permeabilized with Triton X-100 and pepsin as described above. Each embryo was applied to a one-well teflon printed slide (Electron Microscopy Sciences), incubated with 100 μl of hybridization buffer, and processed as described above.
Consistent results for MyoD localization were obtained in 4 stage X, 5 stage XI, 3 stage XII, and 5 stage 2 embryos.

**Immunofluorescence Localization**

M yosin protein was localized in tissue sections with the M F20 mAb to myosin heavy chain (Bader et al., 1982) obtained from the D developmental Studies Hybridoma Bank. Sections were deparaffinized, rehydrated, permeabilized in 0.5% Triton X-100, and incubated in primary then secondary antibodies diluted in 10% goat serum in PBS. The secondary antibody was affinity-purified, goat anti-mouse IgG F(ab)2 fragments conjugated with rhodamine (The Jackson Laboratory). Nuclei were counterstained with bis-benzamide.

**Reverse Transcription–Polymerase Chain Reaction**

RT-PCR was carried out as described previously (George-Weinstein et al., 1996a,b). RNA was extracted from Eyal-Giladi and Korchav (1976) stages X—XI embryos and Hamburger and Hamilton (1951) stage 2 embryos. Stage 39 (day 13) pectoralis muscle was included as a positive control for MyoD expression. Primer pairs for MyoD were: nucleotides 620–639, 5’-CGT GAG CAG GAG GAT GCA TA-3’; and nucleotides 864–883, 5’-GGG ACA TGT GGA GTT GTC TG-3’ (Lin et al., 1989). Primer pairs for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: nucleotides 880—899, 5’-CAT GCT CCC TGA GGT GTA TG-3’; and nucleotides 990–1009. 5’-AGG ATC AAG TCC ACA ACA CG-3’ (Dugaiczyk et al., 1993). Reaction products were separated on 6% polyacrylamide gels and 32P-incorporation visualized by autoradiography.

**Results**

**Localization of MyoD and Myosin mRNA in the Somites**

Validation of the use of dendrimers as probes for mRNA in tissue sections was carried out, in part, by comparing the expression of myosin mRNA and protein in the stage 16 embryo (28 somites). M yosin dendrimers and the M F20 antibody to myosin heavy chain protein localized to the myotome of the somite (Fig. 2, C and D). A few myosin dendrimers also were found in the sclerotome and dermatome (Fig. 2 C). The expression of MyoD was similar to, but more extensive than, myosin. MyoD dendrimers bound most abundantly to the dorsal—medial portion of the dermatome and myotome closest to the neural tube (Fig. 2 H). Some dendrimers were observed within the chordogenic sclerotome and neural tube (Fig. 2 H). By contrast, dendrimers with a recognition sequence for the enzyme GAPDH bound to cells throughout the section (Fig. 2 F), whereas only one to three dendrimers lacking a specific recognition sequence were randomly distributed throughout each section (Fig. 2 E).

The labeling pattern of MyoD dendrimers in the less mature, wedge-shaped somites of the stage 14 embryo was similar to that seen in the older embryo. Fluorescence was most abundant in the dorsal—medial portion of the dermomyotome (Fig. 3 B). These results are consistent with previous in situ hybridizations using conventional oligonucleotide probes (Sassoon et al., 1989; Charles de la Brousse and Emerson, 1990; Ott et al., 1991; Pownall and Emerson, 1992). A few cells of the sclerotome also were fluorescent (Fig. 3 B), supporting the hypothesis that muscle precursors are present in myogenic and chordogenic regions of the somite (George-Weinstein et al., 1998). MyoD dendrimers also were found in low abundance in the neural tube (Fig. 3 B). This is consistent with the finding that transgenic mice containing lacZ targeted into the M yf5 locus express M yf5 in the neural tube (Tajbakhsh and Buckingham, 1995). Furthermore, some cells of the murine neural tube can differentiate into muscle in vitro (Tajbakhsh et al., 1994), and glial-like cells from chick neural tube explants contain MyoD protein (our unpublished observation).

The less mature, epithelial somites of the stage 14 embryo contained MyoD positive cells (Fig. 3 F). D endrimers were concentrated in the ventral region of these somites and those that had just pinched off from the segmental plate (not shown). This could reflect an inductive effect of the notochord on adjacent mesoderm cells via the secretion of Sonic Hedgehog (shh), although mRNA for patched, sh’s receptor, was not detected in the segmental plate by conventional in situ hybridization (Borycki et al., 1998).

Dendrimers to embryonic fast myosin produced a similar pattern to MyoD in the wedge-shaped somite, but were less abundant (Fig. 3 D). Fluorescence was most intense in the dorsal—medial portion of the dermomyotome. A few cells in the sclerotome and neural tube also were positive (Fig. 3 D). Since the expression of myosin is downstream of MyoD (Weintraub, 1993; Rudnicki and Jaenisch, 1995; Molkentin and Olson, 1996), MyoD mRNA may be translated into protein in these relatively immature somites. Dendrimers to GAPDH produced intense fluorescence throughout the somite (Fig. 3 G). Only one to three dendrimers lacking a specific recognition sequence were randomly distributed throughout each section (Fig. 3 C).

**Localization of MyoD mRNA in the Segmental Plate Mesoderm**

Since dendrimers correctly detected MyoD mRNA in the dermomyotome, they were tested for their ability to bind to tissues that give rise to skeletal muscle in vitro, and that contain MyoD mRNA detectable by RT-PCR, but not by conventional in situ hybridization.

The pattern of MyoD expression in the segmental plate was similar to that seen with immature somites. MyoD positive cells were present throughout the segmental plate; however, fluorescence was slightly more abundant in the ventral portion of this tissue (Fig. 3 J). MyoD dendrimers also were found in the neural tube (Fig. 3 J). Only a few myosin dendrimers were present in the segmental plate (Fig. 3 H). One to three dendrimers lacking a specific recognition sequence bound to the entire section (not shown).

**Localization of MyoD mRNA in Gastrulating Embryos**

The same low level of background seen in older embryos was observed in sections through the stage 4 embryo (Fig. 4, C and I). During this stage of development, cells from the dorsal epiblast layer ingress into the primitive streak to form the mesoderm and endoderm (Rosenquist, 1971; Fontaine and Le Douarin, 1977; Bellairs, 1986; Stern and Canning, 1990). MyoD positive cells were a mixture of intensely (>6 dendrimers) and weakly (one to two dendrimers) labeled cells. Consistent with the results obtained with RT-PCR (George-Weinstein et al., 1996a), MyoD dendrimers were found in cells throughout the epiblast, mesoderm, and hypoblast (Fig. 4, D, G, and H). Cells

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with a strong signal within the epiblast or hypoblast were adjacent to fluorescent cells in the mesoderm (Fig. 4, G and H).

The number of labeled cells varied in different regions of the embryo. Staining was strongest in the rostral end of the streak near Hensen’s node (Fig. 4 D), a structure that produces a variety of cytokines (Mitrani et al., 1990a,b; Cooke and Wong, 1991; Kisbert et al., 1995; Stern et al., 1995). Some fluorescence also was observed in more posterior regions of the streak (Fig. 4 E). Cells from the epi-

Figure 2. Localization of MyoD and myosin mRNAs in the somite of the stage 16 embryo. A and B, Low magnification DIC images of rostral and caudal sections, respectively. The area outlined in A is shown at higher magnification in C and D. E and F, High magnification images of the areas outlined in B. The fluorescence photomicrographs are merged images of bis-benzamide-labeled nuclei in blue and Cy3-labeled dendrimers in red. The dendrimers bound within the cytoplasm. Rostral somites with fully developed dermatomes (d), myotomes (m), and sclerotomes (s) were hybridized with dendrimers to myosin mRNA (C) and the MF20 antibody to myosin protein (D). Both probes localized to the myotome. Only 1–3 dendrimers lacking a specific recognition sequence bound to each section (E), whereas dendrimers to GAPDH produced fluorescence throughout the somite and neural tube (nt; F). The length of the dermatome and myotome is shown as a composite in G and H. MyoD dendrimers were concentrated in the dorsal-medial portion of these tissues (H). A few dendrimers were found in the sclerotome and neural tube. Bar: (A and B) 54 μm; (C–H) 9 μm.
Figure 3. Localization of MyoD and myosin mRNAs in the somites and segmental plate mesoderm of the stage 14 embryo. Photomicrographs in A, E, and I are DIC images of the merged images of bis-benzamide-labeled nuclei and Cy3-labeled dendrimers in B, F, and J, respectively. MyoD dendrimers were concentrated in the dorsal–medial portion of the dermomyotome (dm; B). A few cells of the sclerotome (sc) and neural tube (nt) also were labeled. The pattern of labeling with myosin dendrimers was similar to, but less abundant than, MyoD (D). GAPDH dendrimers produced intense fluorescence throughout the dermomyotome and sclerotome (G), whereas dendrimers lacking a specific recognition sequence did not bind to the somite (C). A subpopulation of cells in the epithelial somite (s; F) and segmental plate (sp; J) contained MyoD dendrimers. A few myosin dendrimers were found in the segmental plate (H). Bar, 9 µm.
blast become mesenchymal and switch from E- to N-cadherin as they ingress into the streak to form the mesoderm (Edelman et al., 1983; Hatta and Takeichi, 1986). Since the epiblast epithelium needs to be dissociated and its cells must downregulate E-cadherin and upregulate N-cadherin in order to form muscle in vitro (Geoge-Weinstein et al., 1997), it is not surprising to see expression of MyoD in the primitive streak. Finding MyoD positive cells throughout
the entire epiblast is consistent with the fact that cells from all regions of this tissue can form muscle in culture (George-Weinstein et al., 1996a).

**Localization of MyoD mRNA in Pregastrulating Embryos**

Stages X–XII embryos consist of an epiblast and an incompletely formed hypoblast (Eyal-Giladi and Kochav, 1976). MyoD mRNA was detected by RT-PCR in these embryos, as well as in the stage 2 embryo (Fig. 5). Dendrimers were used to localize MyoD in single cells of whole, unsectioned stage X embryos. Approximately 20 MyoD positive cells were located in the posterior epiblast (Fig. 6 C). Most cells were intensely labeled with >10 dendrimers. The number of MyoD positive cells increased in stages XI–XII embryos, extending more laterally in the posterior epiblast (Fig. 6 F). By stage 2, fluorescence also was observed in the anterior-lateral epiblast (Fig. 6 H). The central region of the epiblast was negative. Background from myosin dendrimers (Fig. 6, D and G) and dendrimers lacking a recognition sequence (not shown) was as low as in the older embryos (one to three dendrimers per section).

**Discussion**

This study demonstrates, first, that dendrimers are sensitive and precise reagents for detecting low abundance mRNA in tissue sections and whole embryos, and second, that the early chick embryo contains small numbers of cells with MyoD mRNA. Intensely labeled MyoD positive cells were detected in the epiblast at the time the egg is laid. As hypoblast formation progressed, more cells expressed MyoD, although they were less intensely fluorescent than the original population of MyoD positive cells. Whether the increase in labeled cells resulted from proliferation of the original population of positive cells, or the onset of MyoD expression in other cells, remains to be determined. From this stage on, the number of weakly labeled cells exceeded that of intensely labeled ones until the dermomyotome formed in the somite, the time when MyoD or Myf5 can be detected in the somite by in situ hybridization using conventional oligonucleotide probes (Sassoon et al., 1989; Ott et al., 1991; Pownall and Emerson, 1992).

We propose that the small number of intensely labeled MyoD positive cells in presomitic tissues are stably committed to the myogenic lineage, whereas the weakly fluorescent population may be programmed to follow other fates, depending on their location within the embryo. The evidence for a committed population of muscle precursors is that the number of epiblast cells from stages X–XII embryos that differentiate into muscle in culture (≈1%)...
is similar to the number of cells with a relatively high amount of MyoD within the embryo (George-Weinstein et al., 1996a, 1997; DeLuca et al., 1999). Over the next 24 h, a change occurs within the epiblast that enables >90% of cells to form muscle in culture (George-Weinstein et al., 1996a). This may reflect the increase in the weakly labeled MyoD positive cells in vivo, a release from inhibitory signals when the epiblast is isolated from the mesoderm (G eorge-Weinstein et al., 1996a), the ability of these older epiblast cells to switch from E- to N-cadherin, and cadherin-mediated cell–cell communication in vitro (George-Weinstein et al., 1997). Interestingly, even though >95% of stage 4 epiblast cells synthesize MyoD protein in vitro and most differentiate, a few neurons, chondroblasts, and notochord cells develop among the multitude of muscle cells (George-Weinstein et al., 1996a). This suggests that small numbers of cells are stably committed to a variety of lineages at early stages of development. However, the majority of epiblast cells appear to be uncommitted because, even though most will form muscle in culture, the epiblast does give rise to all tissues of the embryo (Rosenquist, 1971; Fontaine and LedOUar, 1977; Bellairs, 1986; Stern and Canning, 1990).

Cells with MyoD were located throughout the epiblast, mesoderm, and hypoblast. This resembles the ubiquitous expression of MyoD in the Xenopus embryo at the midblastula transition as determined by RT-PCR (Rupp and Weintraub, 1991). In theory, stably committed myogenic cells that are randomly distributed throughout the epiblast would eventually become incorporated into nonmuscle tissues as well as the somites. This would explain the presence of cells with myogenic potential in the central nervous system (Figs. 2 and 3; TAJbakhsh et al., 1994), bone marrow (Wakitani et al., 1995; Ferrari et al., 1998), and dorsal aorta (De aNgelis et al., 1999). A though committed to the myogenic lineage, they may remain undifferentiated in an environment that is not permissive for myogenesis.

In the embryo, committed precursors may be responsible for influencing surrounding uncommitted cells to follow the same pathway of differentiation as themselves (Gurdon, 1992; Horvitz and Hershkowitz, 1992; Schnabel, 1995). Both committed and uncommitted stem cells are present in the adult (BJornson et al., 1999; Pittenger et al., 1999). The adult bone marrow contains myogenic cells that can be recruited to regenerate skeletal muscle in vivo (Wakitani et al., 1995; Ferrari et al., 1998). It is not known whether these cells are pluripotent, stably committed myogenic precursors, or both. Given the sensitivity and precision of fluorescently labeled dendrimers, these reagents will be useful in determining the extent of heterogeneity in stem cell populations. Once identified and isolated, stably programmed cells might be used to seed populations of pluripotent cells before implantation into diseased tissues.

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