Localization of Dystrophin Gene Transcripts during Mouse Embryogenesis

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Abstract. The spatial and temporal expression of the dystrophin gene has been examined during mouse embryogenesis, using in situ hybridization on tissue sections with a probe from the 5' end of the dystrophin coding sequence. In striated muscle, dystrophin transcripts are detectable from about 9 d in the heart and slightly later in skeletal muscle. However, there is an important difference between the two types of muscle: the heart is already functional as a contractile organ before the appearance of dystrophin transcripts, whereas this is not the case in skeletal muscle, where dystrophin and myosin heavy chain transcripts are first detectable at the same time. In the heart, dystrophin transcripts accumulate initially in the outflow tract and, at later stages, in both the atria and ventricles. In skeletal muscle, the gene is expressed in all myocytes irrespective of fiber type. In smooth muscle dystrophin transcripts are first detectable from 11 d post coitum in blood vessels, and subsequently in lung bronchi and in the digestive tract. The other major tissue where the dystrophin gene is expressed is the brain, where transcripts are clearly detectable in the cerebellum from 13 d. High-level expression of the gene is also seen in particular regions of the forebrain involved in the regulation of circadian rhythms, the endocrine system, and olfactory function, not previously identified in this context. The findings are discussed in the context of the pathology of Duchenne muscular dystrophy.

Deletions and other mutations in the human X-linked dystrophin gene are responsible for the myopathies of Duchenne and Becker (reviewed in Chamberlain and Caskey, 1990). It is therefore of medical importance as well as of biological interest to understand the function of dystrophin and when and where the gene is expressed. The full-length dystrophin messenger RNA is 14 kb long and is encoded by at least 75 exons distributed over 2.5 megabases of the human X chromosome (reviewed in Hoffman and Kunkel, 1989). Two transcripts of this size have been identified that are transcribed from two distinct 5' promoters, one of which is active in muscle and in glia, and the other in nonmuscle tissues, including liver and brain (Barnea et al., 1990). At least one other internal promoter is present encoding a 6.5-kb mRNA corresponding to the 3' half of the coding sequence, and found in a number of nonmuscle tissues, including liver and brain (Bar et al., 1990). Further dystrophin transcripts are generated from the mouse and human genes by alternative splicing of 3' exons (Feener et al., 1989; Bies et al., 1992). Some of these carboxy-terminal isoforms are expressed in different tissues and at different stages of development (Bies et al., 1992). In addition to the X-linked dystrophin gene, an autosomal gene encoding a dystrophin-related protein has been identified (Love et al., 1989).

The full-length 427-kD protein encoded by the X-linked dystrophin gene is present in skeletal muscle, localized to the sarcolemma (Ervasti et al., 1990), where it exists as a large oligomeric complex in association with a number of glycoproteins (Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991). The spectrin-like structure of the molecule and its localization suggest that dystrophin may be important in stabilizing the plasma membrane, acting as an anchor for cytoskeletal or sarcomeric structures. Antibody studies have suggested some concentration of the protein at myotendinous and neuromuscular junctions (e.g., Shimizu et al., 1989; Jasmin et al., 1990; Fardeau et al., 1990; Sealock et al., 1991; Yeadon et al., 1991) in addition to the presence of the dystrophin-related protein (Ohlendieck et al., 1991) at this site. Dystrophin protein is also present in cardiac and smooth muscle, although in the latter the plasma membrane is considerably less dystrophin immunoreactive (e.g., Hoffman et al., 1988; Byers et al., 1991). The dystrophin gene is also expressed in brain at a level ~10% that in muscle (Chamberlain et al., 1988). The protein is present in a number of regions in the brain (Jung et al., 1991). It is more abundant in neurons of the cerebral and cerebellar cortices than in other parts of the brain, and has been localized at the postsynaptic membrane, in the mouse (Lidov et al., 1990). Localization of dystrophin mRNA has also been reported for...
rodents in the hippocampus and cerebral cortex, and in Purkinje cells of the cerebellum (Gorecki et al., 1991). In addition to muscle and brain, low levels of transcripts from the X-linked dystrophin gene have been detected by polymerase chain reaction (PCR) analysis in other nonmuscle tissues (Chelly et al., 1988). The dystrophin-related autosomal gene is expressed at the neuromuscular junction, and at a higher level in smooth muscle and in some nonmuscle tissues such as the kidney (Love et al., 1991).

A few studies have been carried out on dystrophin gene expression in immature tissues. Dystrophin transcripts were detected by PCR analysis in RNA from skeletal muscle and brain in the mouse from embryonic day 14 (Chamberlain et al., 1991). In humans from 8 wk of fetal development in limb muscle, dystrophin protein is first observed at the myotendinous junction and is subsequently integrated into the sarcolemma (Wessels et al., 1991). Dystrophin is first detected in the sarcolemmal of human myotubes in tissue culture (Miranda et al., 1988). A recent study on human limbs using antibodies also reported dystrophin immunostaining from 9 wk gestation, with the presence of two distinct protein bands that vary in relative abundance as development proceeds (Clerk et al., 1992). This is in keeping with an earlier report on mouse muscle (Dickson et al., 1988). In rats, by immunohistochemical and immunoblot analysis dystrophin protein is detected from embryonic day 19 in the plasma membrane of the extensor digitorum longus limb muscle, and from embryonic day 15 as a weak diffuse cytoplasmic labeling (Hagiwara et al., 1989). In the rat heart, dystrophin protein was not detected until just before birth, whereas dystrophin mRNA was detectable by PCR analysis from embryonic day 10 (Tanaka et al., 1991). In the rat brain, dystrophin protein has been detected from 18 d of gestation (Jung et al., 1991); earlier stages were not examined.

We have carried out a systematic analysis of dystrophin gene expression during mouse embryonic and fetal development, using in situ hybridization on tissue sections, with a probe that recognizes transcripts from the 5' region of the gene, i.e., the 14-kb transcripts expressed in brain and muscle. This approach permits us to define when and where dystrophin transcripts first appear during mammalian development. We note that during the formation of skeletal muscle, transcripts accumulate with those of myosin, but in the heart, myosin mRNAs and the initiation of contraction precede the first detectable dystrophin mRNAs by at least 24 h. Dystrophin transcripts accumulate in smooth muscle and in the brain in the cerebellum and hippocampus, and also in previously unidentified locations in the forebrain, among which are the tissues that will give rise to parts of the pineal and pituitary glands. These observations are discussed in an embryological context and in terms of the function of the gene product and its dysfunction in disease.

**Materials and Methods**

**Preparation and Prehybridization of Tissue Sections**

The protocol that was used to fix and embed mouse embryos is described in detail in Lyons et al. (1990a). Briefly, embryos were fixed in 4% parafor-

1. Abbreviations used in this paper: MHC, myosin heavy chain; MHCa, mouse atrial myosin heavy chain; MLCIV, mouse ventricular myosin light chain 1; PCR, polymerase chain reaction.

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morphology, probably at a more advanced stage of differentiation (Sassoon et al., 1988), are present. The myogenin labeling of this region is less intense (Fig. 1 D), but this may be due to the plane of section. The level of dystrophin mRNAs in skeletal muscle gradually increases with development. The distribution of grains when examined at higher magnification is not uniform with the dystrophin probe (Fig. 2, C and D), in contrast to other muscle sequences that show more uniform distribution along the fibers (results not shown; Lyons et al., 1990a and 1991c). This is evident at 14.5-15.5 d (Fig. 2, B-D) and at later stages. By 17.5 d, the intensity of the signal with the dystrophin probe has reached its maximum level. Transcripts are detected in all muscles of the hind limb, including the soleus, a slow muscle, and in fast muscles such as the extensor digitorum longus (Fig. 2, E and F). Dystrophin is expressed not only in developing skeletal muscle, but also in cardiac and smooth muscle (Fig. 2, A and B).

**Cardiac Muscle**

The heart is formed from lateral mesoderm. It is initially present as a cardiac tube from ~7 d in the mouse embryo. By ~8 d it has begun to contract, and by 9.5 d atrial and ventricular compartments can be clearly distinguished. Dystrophin transcripts are first detectable by in situ hybridization in an 18-somite embryo (~9 d), and are still only just visible above background in a 21-24-somite embryo (Fig. 3, A and B). At a slightly later stage in a 10-d-old embryo, transcripts have begun to accumulate (Fig. 3, C and D). Thus, in the heart a functional sarcomere is present well before dystrophin gene expression, in contrast to the situation in the myotome where dystrophin transcripts begin to accumulate at about the same time as those for MHC. At later stages dystrophin continues to be expressed in both atrial and ventricular compartments of the heart at a time when myosin isoforms are beginning to be restricted. Thus, at 11.5 d MHCα transcripts are already beginning to accumulate preferentially in the atria (Fig. 4 C), and MLCIV in the ventricle (Fig. 4 D) (Lyons et al., 1990b), whereas dystrophin shows a similar distribution in both compartments (Fig. 4 B). The intensity of labeling with the dystrophin probe is much lower, and this despite the fact that the probe is considerably longer than the myosin probes, indicating the relatively low level of expression of this gene. Subsequently, dystrophin transcripts continue to accumulate in the heart reaching a maximum level between 14.5 and 15.5 d (see Fig. 2, A and B), and then progressively declining to give a lower, but still detectable, signal at 16 d and postnatally (results not shown).

**Smooth Muscle**

Smooth muscle is mainly present in the embryo in the walls of blood vessels, and of the digestive and respiratory sys-
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Figure 2. Expression of dystrophin is developing skeletal muscle. Phase contrast (A) and dark field (B) photomicrographs of a parasagittal section of a 14.5-d mouse embryo hybridized with the dystrophin probe showing labeling of intercostal (im) and diaphragm (di) skeletal muscles, of cardiac muscle (h) and of smooth muscle in the lung (lu). Bar, 360 μm. Phase contrast (C) and dark field (D) photomicrographs of a parasagittal section showing trunk musculature of a 15.5-d embryo. Bar, 45 μm. Dark field photomicrographs showing adjacent serial transverse sections of a 17.5-d embryo hindlimb hybridized with the dystrophin probe (E) and the myogenin probe (F). f, fibula; t, tibia; s, soleus; edl, extensor digitorum longus. Bar, 180 μm.

tems. Both the digestive and respiratory tubes are formed from embryonic endoderm. Smooth muscle surrounding the endodermal layer is derived from adjacent mesenchyme.

By 10 d of embryonic development the lung buds form, in the region of the pharynx, as extensions of the laryngotracheal groove, which itself is a prolongation of the foregut. At this stage the trachea separates from the esophagus. By 11 d the bronchi are formed and shortly afterwards begin to develop branches. At 12 d dystrophin transcripts become detectable in the walls of the bronchi and bronchioli (Fig. 5, A and B), and the gene continues to be expressed in the smooth muscle of the lung at later stages (Fig. 2, A and B, and results not shown).

Beginning at 11 d post coitum, the primitive gut forms the esophagus, stomach, small intestine, and large intestine (Rugh, 1990; Theiler, 1989). Posterior to the pharynx the digestive tube constricts from 11 d to form the esophagus, stomach, small intestine, and large intestine. Dystrophin
transcripts are only detected relatively late, at 14.5 d in the walls of the esophagus and very weakly in the smooth muscle of the stomach and intestine. It is only at 15.5 d that the latter labels more strongly with the dystrophin probe (Fig. 5, E and F).

Angiogenesis in the mouse begins at ~8 d with the appearance of the dorsal and ventral aortae; by 9 d the circulation is established. Most of the major blood vessels appear by 11 d. Initial detection of dystrophin transcripts in the smooth muscle of blood vessels is difficult; the signal is weak, at least in part because of the thinness of the layer of muscle tissue. We first detect a signal in blood vessels of the embryo at 11.5 d in the walls of the dorsal aorta, and in the extraembryonic circulatory system from 12.5 d (results not shown). As the embryo develops an increasing number of blood vessels are labeled with the dystrophin probe. At 15.5 d, for example, a clear signal is detected, which is only slightly weaker than that in adjacent skeletal muscle (Fig. 5, C and D). Unlike the latter, the signal in smooth muscle is uniform rather than punctate.

**Dystrophin Expression in the Nervous System**

The essential features of the brain begin to be established relatively early during embryogenesis (from ~8 d) and can be identified in large part from 11 d. By 14 d the embryonic brain is typically that of a mammal (Rugh, 1990). From 13 d dystrophin transcripts are detectable in the hindbrain in the metencephalon or cerebellum (Fig. 6, A and B), which continues to give a low signal during subsequent development. The pons, adjacent to the cerebellum, also gives a very low signal. At this stage dystrophin transcripts are also present in the forebrain, localized to the roof of the diencephalon, in the epithalamus (Fig. 6, C and D), and by 14 d in the epiphysis or the pineal body (Fig. 6, E and F), which will form the pineal gland. Another structure that is partially derived from the diencephalon is the pituitary gland. This forms from brain neurectoderm and from oral plate ectoderm. Part of the base of the diencephalon detaches by 12 d to form the infundibular process, which is the neural part of the pituitary. The glandular part of the pituitary develops from Rathke’s pocket, derived from the oral plate. It begins to function as a secretory organ between 17 and 18 d of development. By 14.5 d the dystrophin probe gives a relatively strong signal over Rathke’s pocket (Fig. 7, C and D). Dystrophin transcripts are also detectable from 13.5 d at a very low level in a limited area over the central region of the diencephalon, the thalamus (results not shown), and in the hypothalamus at the base of the diencephalon (Fig. 7, A and B). In Fig. 7 B, a strong signal is also seen in tissue underlying the olfactory epithelium on either side of the palate.

Dystrophin transcripts are also detectable in parts of the peripheral nervous system. A very weak signal is seen over

**Figure 3.** Dystrophin expression in the heart. Phase contrast (A) and dark field (B) photomicrographs of a 21-24-somite embryo (~9.5 d) hybridized with the dystrophin probe. Bar, 180 μm. Phase contrast (C) and dark field (D) photomicrographs of a 27-somite embryo hybridized with the same probe. a, atria; v, ventricle. Bar, 90 μm.
the spinal ganglia from 13 d (results not shown). This is also the case for a population of cells in the spinal cord from 14.5 d (results not shown). The presence of dystrophin in the spinal cord of 18-wk human fetuses has already been noted (Hoffman et al., 1988).

Discussion

The most evident lesion in patients with Duchenne or Becker muscular dystrophies is in skeletal muscle. We show here that transcription of the dystrophin gene is detectable when skeletal myogenesis is initiated in the embryo. This process is asynchronous, with α-actin, for example, preceding myosin heavy chain by at least 1 d (Lyons et al., 1990a) in the first skeletal muscle, the myotome. Dystrophin is not among the earliest muscle markers, but the transcripts begin to accumulate at the same time as those of the embryonic MHC. We would suggest that the activation of the dystrophin gene at this early stage of myogenesis reflects the importance of the protein in functional skeletal muscle. The question of when the protein first accumulates is open at present. In the rat (Hagiwara et al., 1989), low levels of protein were detected in limb skeletal muscle at embryonic day 15, which was the earliest time point examined. In this species primary fibers are present at this stage, and the protein is not yet concentrated at the plasma membrane, as it is later.

In the mouse, dystrophin transcripts are accumulated in all muscle masses, throughout development. There is no apparent distinction between fiber types. The distribution of the transcripts within muscle fibers, however, ceases to be uniform from about the time when innervation takes place (Ontell and Kozeka, 1984). A higher concentration of the X-linked dystrophin protein has been reported at the neuromuscular junction (Byers et al., 1991), and the possibility that dystrophin transcripts are being transcribed at a higher level of subsynaptic nuclei requires further investigation.

As in the case of other muscle genes, the dystrophin gene is transcribed throughout skeletal muscle formation and maturation. Different myogenic factors of the MyoD family, involved in the transcriptional activation of many muscle genes, are present at different developmental stages. Initially, when the dystrophin gene is activated in the myotome, myf-5 and myf-6 are probably present, whereas in the limb, MyoD and myogenin are the main myogenic regulatory proteins, to be replaced later by myf-6 (see Buckingham, 1992). If this family plays a role in regulating dystrophin transcription in skeletal muscle, there is no absolute requirement for a single factor. Recent results show that a CCArGG box present in the proximal promoter is important for preferential expression of the gene in muscle, thus pointing to the probable role of proteins other than those of the MyoD family (Gilgenkrantz et al., 1992).

Figure 4. Comparison of dystrophin and myosin transcripts in the heart. Phase contrast (A) and dark field (B-D) photomicrographs of adjacent parasagittal sections of an 11.5-d embryo hybridized with the dystrophin probe (A and B), a probe specific for MHCα transcripts (C), and for MLC1V transcripts (D). a, atria; v, ventricle; j, arterioventricular junction. Bar, 180 μm.
In the heart, distinct from skeletal muscle, dystrophin transcripts are not detectable initially when myosin is present and the heart is already contracting. It is noteworthy that malfunction of the myocardium is less progressive and debilitating in Duchenne patients than malfunction of skeletal muscle (see Moser, 1984). Dystrophin is expressed uniformly in both ventricular and atrial compartments. The fact that at certain specialized locations, such as the outflow tract, transcripts are more abundant may reflect the greater contractile strain placed on the sarcolemma at these sites. Myosin gene expression in this region also has distinct features (Lyons et al., 1990b). The conducting system that is associated with this part of the heart has been noted to be particularly affected in Duchenne patients (Moser, 1984). It
was noted by Bies et al. (1992) that human cardiac Purkinje fibers express dystrophin-spliced forms more similar to those in brain than in muscle. In rat hearts (Tanaka et al., 1991), dystrophin mRNA was detected from embryonic day 10, which correlates with our observations on the mouse, given that the latter species develops more rapidly and that the in situ hybridization technique is less sensitive than PCR. However, the protein was not detectable until embryonic day 19. This may reflect the fact that the mAb used was against a COOH peptide, and this is a region where differential splicing has been shown to occur (Feener et al., 1989). The 5' probe used in our experiments would not distinguish between different sequences resulting from alternative splicing, which has been shown by PCR analysis to occur in developing murine striated muscle and brain (Bies et al., 1992). Alternatively, translational control of dystrophin gene expres-
Figure 7. Expression of dystrophin in the olfactory apparatus, and in Rathke's pocket. Phase contrast (A) and dark field (B) photomicrographs of a frontal section of a 13.5-d mouse embryo in the head region hybridized with the dystrophin probe. Bar, 180 μm. Phase contrast (C) and dark field (D) photomicrographs of a parasagittal section of a 14.5-d mouse embryo in the head region hybridized with the dystrophin probe. Bar, 180 μm. p, palate; oe, olfactory epithelium; hy, hypothalamus; sc, sphenoid cartilage; rp, Rathke's pocket.

Expression may be important. In skeletal muscle, for example, this is clearly the case for the myogenic regulatory factor, myogenin (Cusella de Angelis et al., 1992).

In smooth muscle the question of probe specificity is particularly critical, since the dystrophin-related autosomal gene is expressed at a particularly high level in this tissue (Love et al., 1991), where we detect a clear signal. Sequence is not available for the equivalent 5' region of the dystrophin-related gene; however, in situ hybridization conditions are relatively stringent, and the fact that we see no signal in the kidney where the dystrophin related protein is expressed at a high level (Love et al., 1991) strongly suggests that cross-hybridization with this sequence is not a problem. The presence of dystrophin protein has been reported in smooth muscle (Hoffman et al., 1988; Byers et al., 1991). We show here that the gene is activated as smooth muscle forms in the foregut and its prolongations, in the lung, for example. In the hindgut, transcripts are detectable relatively later in the stomach and intestine. Perturbations in smooth muscle function are not a major feature of the pathology of Duchenne dystrophy. A contributory factor to respiratory failure, which is frequently a cause of death in Duchenne patients, may be the lack of dystrophin in the walls of the bronchi and bronchioli.

About one-third of patients with Duchenne muscular dystrophy suffer from some degree of mental impairment (see Moser, 1984). Transcription of the dystrophin gene to give a 14-kb transcript in the brain (Chamberlain et al., 1988) is probably initiated from both upstream promoters (Barnea et al., 1990; Bies et al., 1992). Use of the internal promoter, which generates a 6.5-kb mRNA (Bar et al., 1990), and different splicing mechanisms also contribute to the complexity of dystrophin gene transcripts present in this tissue (Bies et al., 1992). It is not yet known whether different isoforms are confined to specific structures in the brain, however, in the adult, accumulation of dystrophin protein (Lidov et al., 1990; Jung et al., 1991) and transcripts (Gorecki et al., 1991) in the Purkinje cells of the cerebellum has been demonstrated. The cerebellum is involved in the coordination of movements; older mdx mice have been reported to have some lack of coordination and tremors (Bulfield et al., 1984). In keeping with these results we detect dystrophin transcripts in the cerebellum shortly after its formation during embryogenesis. On the other hand, in the cerebellar cortex, where both protein and transcripts were detected in the adult (see Lidov et al., 1990; Jung et al., 1991; Gorecki et al., 1991), we did not detect a clear signal above background in the immature tissue. This may be partly due to the presence of other isoforms not recognized by our probe; however Gorecki et al. (1991) detected a signal in the cortex, and in the hippocampus using oligonucleotides derived from the 5'
region of the gene, in adult rodents. The hippocampus was also negative in the prenatal brain in our experiments. A clear signal is present however in another region of the forebrain, in structures forming part of the diencephalon. Label is seen in the epithalamus. The habenular structures of this region are important in assuring the contact between the olfactory centers and the brain. The epithysis, which will form the pineal gland, also expresses the dystrophin gene at a high level. The pineal gland plays a role in the establishment of circadian rhythms, via perception of dark/light cycles. Cells of the gland remain multipotent and can, for example, form skeletal muscle when cultured in vitro (Watanabe et al., 1988).

Another striking site of dystrophin gene expression in the embryo is in the precursor cells that will form the glandular component of the pituitary. In this case, dystrophin may be playing a role in conjunction with the cytoskeleton in the secretory processes essential for function of the gland. It is perhaps significant that a clear signal is also seen in the hypothalamus, which is a region of the diencephalon that affects pituitary function. Given the high level of dystrophin transcription in Rathke's pocket, it is probable that pituitary function is perturbed when the gene is not expressed correctly, during formation of the gland.

In the light of the new localization of dystrophin gene expression described here, we would predict that Duchenne patients may suffer from endocrine malfunction, from perturbations in circadian and seasonal rhythms, and from olfactory deficiencies.

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