

Hereditary Pituitary Dwarfism in Mice Affects Skeletal and Cardiac Myosin Isozyme Transitions Differently

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ABSTRACT The dwarf mutation in mice interferes with the development of those anterior pituitary cells responsible for production of thyroid stimulating hormone, growth hormone, and prolactin. Myosin isozyme transitions in both cardiac and skeletal muscle were also found to be affected in this mutant. Electrophoresis of native myosins demonstrated that the fetal (V3) to adult (V1) ventricular cardiac isozyme transition was completely blocked in dwarf mice; in contrast, the neonatal to adult fast myosin transition in hind limb skeletal muscle was slowed but not totally inhibited. The persistence of neonatal myosin heavy chain for up to 55–75 d after birth in dwarf mice, as compared with 16 d in normal mice, was directly demonstrated by polypeptide and immunopolypeptide mapping. Morphological examination of 18–36-d-old dwarf skeletal muscles by optical and electron microscopy revealed a relative immaturity, but no signs of gross pathology were evident. Immunocytochemical analysis showed that the abnormal persistence of neonatal myosin occurs in most of the fibers. Multiple injections of thyroxine restored a normal isozyme complement to both cardiac and skeletal muscles within 11–15 d. Therefore, the effects of the dwarf mutation on myosin isozymes can be explained by the lack of thyroid hormone in these animals. Because the synthesis of growth hormone is not stimulated by thyroid hormone in dwarf mice as it would be in normal animals, these results demonstrate that thyroid hormone promotes myosin isozyme transitions independent of growth hormone production.

Six or more different isoforms of the myosin heavy chain can be found in mammalian cardiac and skeletal muscles. Although their structures are very homologous, these heavy chains are distinct polypeptides as shown by protein chemical, immunochemical, and molecular cloning approaches (5, 14, 16, 19, 20, 25, 29, 35, 41, 43, 44, 47). In both developing and adult muscles, transitions between different myosin isoforms take place within the same cardiac cells or skeletal muscle fibers (7, 8, 32, 34). The control of these transitions has in several cases been shown to be modulated by factors external to the muscle cell, although the nature of these controlling influences is known in general outline only (reviewed in reference 42). Some myosin transitions require a specific innervation or nerve-activity pattern (23, 33), whereas others can occur in the absence of innervation (8, 17). The hormonal status of the animal can also influence the type of myosin isozyme present.

Hypothyroidism has been shown to inhibit certain myosin isozyme transitions that normally take place during mam-

malian skeletal and cardiac muscle development (9, 13, 17, 30). However, thyroid hormone levels also contribute to the regulation of the postnatal increase in growth hormone (36), and growth hormone has effects on skeletal growth, at least some of which are mediated via the action of somatomedins (27). It is therefore not clear from previous results whether the inhibition of myosin transitions produced by hypothyroidism is a result of the lack of thyroid hormone, or whether it is a secondary effect due to lowered growth hormone levels.

To investigate this question, we have studied myosin isozyme transitions in a mouse model of pituitary dwarfism. The dwarf mutation in mice (39) interferes with the development of those anterior pituitary cells that normally produce thyroid stimulating hormone (TSH),¹ growth hormone (GH), and prolactin (4). As a result, these hormones are present at low levels in the sera of homozygous dwarf mice. In addition,

¹ Abbreviations used in this paper: GH, growth hormone; TSH, thyroid stimulating hormone.

synthesis of GH is undetectable in the pituitary at all ages (12, 37, 38), and exogenous thyroid hormone does not stimulate GH production (37). Dwarf mice are therefore a suitable model of the hypothyroid state that is not complicated by the stimulatory effects of thyroid hormone on GH production.

We have found that the normal developmental appearance of the adult cardiac myosin isozyme is inhibited in these animals. In contrast, accumulation of the adult skeletal muscle myosin forms is slowed but not completely blocked. Both of these developmental defects can be overcome by exogenous thyroxine. Since dwarf mice lack GH-producing somatotropes (12, 31, 38), these results demonstrate that thyroid hormone does not affect myosin isozyme transitions by stimulating growth hormone production.

MATERIALS AND METHODS

Mice of the Snell dwarf strain (4, 12) were used in this study. Optical and electron microscopy of fixed extensor digitorum longus muscles was performed using standard techniques (15) on dwarf and control mice of various ages, as follows: 18 d (five dwarfs and three controls), 24–25 d (six dwarfs and eight controls), and 31–36 d (three dwarfs and four controls). For electrophoresis of native myosin, muscle tissue from individual mice was extracted and prepared as described (7); the skeletal muscle extracts were made from the gastrocnemius muscles. Myosin was purified (5) using hind leg muscles pooled from several mice for a given age point. The myosins were denatured with SDS and cleaved with chymotrypsin as described previously (5, 43). The resulting polypeptides were separated on duplicate SDS-containing 10% polyacrylamide gels. One gel was stained with Coomassie Blue and the other was blotted onto a nitrocellulose sheet as described (6), except that 0.1% SDS was included in the buffer used for the electrophoretic transfer step and electrophoresis was carried out for 18–20 h. The nitrocellulose sheet was then treated and reacted with an antibody specific to rat neonatal myosin as described (7). Indirect immunofluorescence on gastrocnemius muscles was performed using rabbit antibodies to rat fast and neonatal myosins, prepared and characterized as described (7); a rhodamine-conjugated goat anti-rabbit immunoglobulin preparation (Nordic, Tilburg, The Netherlands) was used as a second antibody. For the hormone supplementation experiments, dwarf mice of ~5 g body wt were injected every 2–3 d with 1 μ g L-thyroxine (Sigma Chemical Co., St. Louis, MO) dissolved in 0.1 ml of 0.14 M NaCl, 10 mM sodium phosphate, pH 8.5. After 11–15 d of treatment, the native myosin isozymes were analyzed by electrophoresis.

RESULTS

Two ventricular cardiac myosin heavy chains, which combine to give rise to three bands in native electrophoresis, called V1, V2, and V3 (14, 20, 35), have been identified in all mammals examined (14, 20, 24, 35). In rats and mice, the V3 homodimer is predominant in fetal hearts but is replaced by the V1 homodimer within the first few weeks after birth (24, 35).

Fig. 1, lane *a* shows the analysis of a 10-d-old control mouse heart that contains one band corresponding to the V1 form (Fig. 1, lane *a*). In the hearts of dwarf mice, one major band

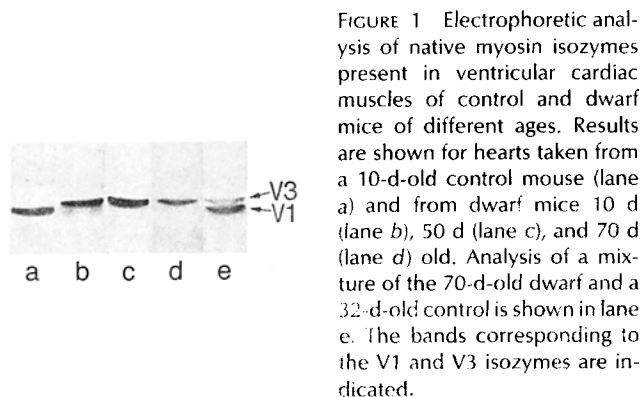


FIGURE 1 Electrophoretic analysis of native myosin isozymes present in ventricular cardiac muscles of control and dwarf mice of different ages. Results are shown for hearts taken from a 10-d-old control mouse (lane *a*) and from dwarf mice 10 d (lane *b*), 50 d (lane *c*), and 70 d (lane *d*) old. Analysis of a mixture of the 70-d-old dwarf and a 32-d-old control is shown in lane *e*. The bands corresponding to the V1 and V3 isozymes are indicated.

of ventricular myosin is observed by nondenaturing gel electrophoresis in all animals examined between the ages of 10 and 70 d; examples are shown for 10, 50, and 70 d (Fig. 1, lanes *b–d*). Co-migration with myosin from control adult hearts indicates that the dwarf isozyme is the V3 form (Fig. 1, lane *e*). The myosin light chain complement of control and dwarf hearts is the same, as determined by two-dimensional gel electrophoresis (not shown). Therefore, the V1 and V3 bands detected by nondenaturing electrophoresis probably reflect the presence of different heavy chain types, as expected from previous results (14, 20, 35). Since the limit of detection of this technique is ~5% (26), we cannot rule out the presence of V1 and V2 in dwarf mice at these levels. Nonetheless it is clear that the fetal V3 cardiac myosin isozyme persists in these dwarf mice up to at least 70 d of age, and the adult V1 form never appears during this time in quantities detectable by the native electrophoretic technique.

In newborn rats and mice, the major myosin isozyme present in developing skeletal muscle fibers has been referred to as neonatal myosin (44, 45). Protein chemical, immunological, and molecular cloning results have demonstrated that the heavy chain subunit of the rat neonatal isozyme is distinct from the adult heavy chain forms (5, 29, 44). After electrophoresis of native myosin, three bands are seen for both the neonatal and adult myosins (Fig. 2, lanes *a* and *d*), which correspond to the isoenzymes formed between the heavy chains and the two types of nonphosphorylatable light chains that can associate with both heavy chain forms (19). During normal mouse muscle development, adult fast myosin appears and eventually replaces the neonatal form (Fig. 2, lanes *a–d*). Based on this analysis, adult myosin begins to appear at ~7 d (Fig. 2, lane *b*), is predominant by 10 d (Fig. 2, lane *c*), and is the only form detectable throughout adulthood. The presence of neonatal myosin can be detected up to ~15 d after birth (not shown).

In skeletal muscles of dwarf mice analyzed by the same gel electrophoretic technique, bands that appear to correspond to adult myosin can be seen at 10 d (the earliest age examined) (Fig. 2, lane *e*). However, neonatal myosin is also present up to 5–6 wk of age; Fig. 2, lanes *f* and *g*, shows the results for 26 and 39 d of age. The multiple bands must reflect differences in the heavy chains since the myosin light chains present in

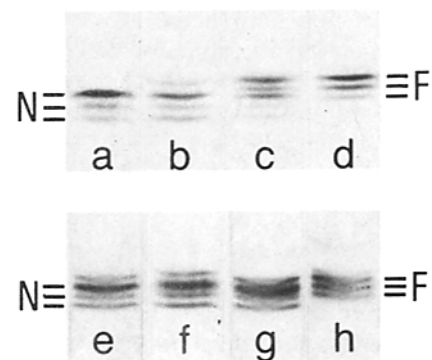


FIGURE 2 Electrophoretic analysis of native myosin isozymes present in control and dwarf skeletal muscles at different ages. The ages examined for the control mice are 4 d (lane *a*), 7 d (lane *b*), 10 d (lane *c*), and 26 d (lane *d*). For the dwarf mice, the ages were 10 d (lane *e*), 26 d (lane *f*), 39 d (lane *g*), and 70 d (lane *h*). The bands corresponding to neonatal (*N*) and adult fast (*F*) myosin isozymes are indicated.

control and dwarf mice at these ages are identical and correspond to the three adult fast-type light chains, as determined by two-dimensional gel electrophoresis (not shown). Neonatal myosin is undetectable only in 60–70-d-old dwarf mice (Fig. 2, lane *h*), the oldest animals that were examined by this technique. The transition from neonatal to adult myosin is therefore slower in dwarf mice since a mixture of these two myosins can be detected over a longer period than in control mice.

To demonstrate directly the presence of neonatal and adult fast myosin heavy chains, we used a polypeptide mapping approach (43, 44). Myosin was purified from control mice 4, 7, 16, 26, and 35 d old, and from dwarf mice 16, 26, 35, 55, and 75 d old. These myosins were subjected to partial digestion with chymotrypsin in the presence of SDS. The resulting polypeptide maps demonstrate that the cleavage patterns of mouse neonatal and adult myosin heavy chain types are different (compare Fig. 3, lanes *a* and *e*). Beginning at 16 d of age in normal mice, the cleavage pattern is principally that of the adult heavy chain (Fig. 3, lane *c*). In contrast, bands corresponding to neonatal heavy chain can be detected in the polypeptide pattern of dwarf muscle myosin up to 55 d (Fig. 3, lanes *f–i*). Adult myosin heavy chain cleavage products begin to be detected in the dwarf myosin at 26 d, and by 55 d the pattern is predominantly that of adult fast heavy chain (Fig. 3, lanes *g–j*).

Since a major heavy chain component present in the cleavage pattern can mask minor heavy chain types, we used a more sensitive way to detect the presence of neonatal myosin heavy chain. We prepared blot transfers of the cleavage products and then reacted them with an antibody specific for neonatal myosin (7). These immunopolypeptide maps demonstrate that neonatal heavy chain cleavage products are present in control mice at 4 and 7 d of age but barely

detectable at 16 d (Fig. 3, lanes *a'–c'*). No polypeptides characteristic of neonatal heavy chain are detected at 26 or 35 d (Fig. 3, lanes *d'* and *e'*). In contrast, neonatal myosin heavy chain is clearly present in dwarf muscles up to at least 75 d of age (the oldest animals examined; Fig. 3, lanes *f'–j'*). As judged from the intensities of the antibody reactions, the 75-d-old dwarf mice appear to contain neonatal myosin in amounts similar to those of 16-d-old control animals (compare Fig. 3, lanes *c'* and *j'*). Thus, electrophoresis of native myosin, polypeptide mapping, and immunoblotting all indicate that neonatal heavy chain is present for an abnormally long period in dwarf mice but that the adult myosin heavy chain nonetheless appears.

We investigated whether the presence of neonatal myosin in dwarf mice older than 16 d was due to a small number of immature or pathological fibers in otherwise normal muscle, or whether it was a general phenomenon that affected most or all fibers. Electron microscopic examination of dwarf mouse muscle from 14 animals between 18 and 36 d old (see Materials and Methods) demonstrated that no gross pathological features were evident. The dwarf muscles were more immature than their age-matched controls in that some central nuclei and apparently migrating nuclei were seen and the sarcoplasmic reticulum was less extensive. However, no myoblasts or myotubes were evident in dwarf muscles, the myofibrils were densely packed and well aligned, and the nerve-muscle junctions had well-developed secondary folds. Dense accumulations of glycogen were present in the I-band region of dwarf muscles, whereas control muscles had relatively few glycogen granules (Fig. 4, *A* and *B*). These accumulations were particularly evident at 18 d of age and are similar to the levels normally found in fetal mouse muscle. Although the fibers in dwarf muscles were about one-half to one-third the diameter of control muscle fibers between days 18 and 31

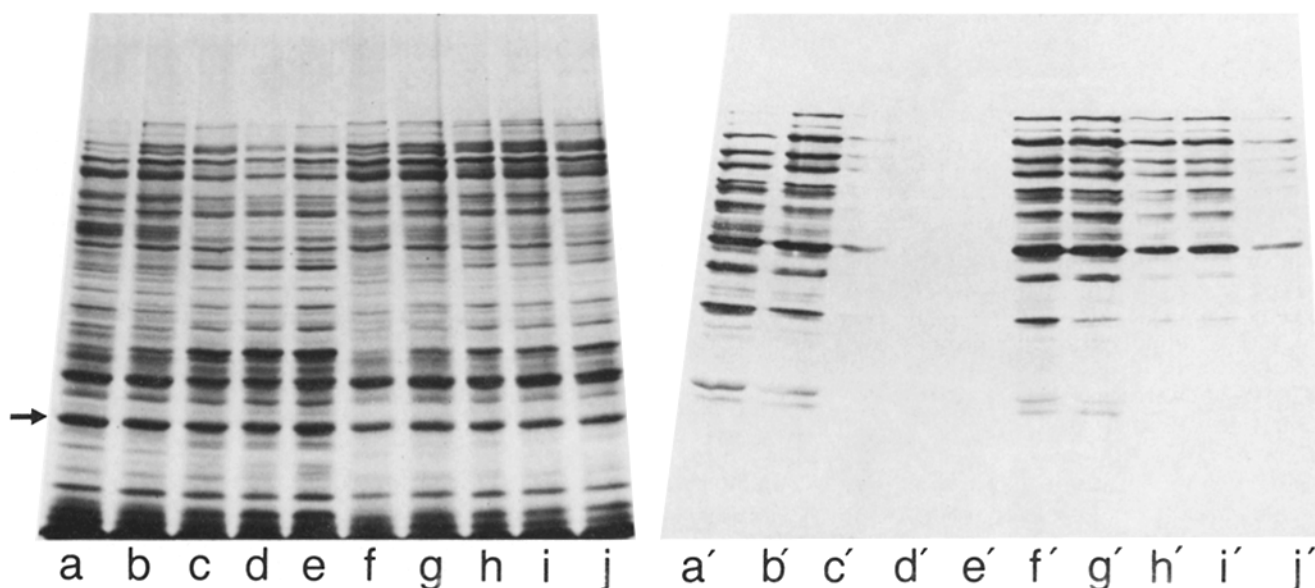


FIGURE 3 Polypeptide and immunopolypeptide mapping of myosin heavy chains present in skeletal muscles of control and dwarf mice of different ages. Lanes *a–j* are Coomassie Blue-stained proteins. The antibody reaction was detected by indirect immunoperoxidase staining; this is shown in lanes *a'–j'*. The myosins used were from control mice 4 d (lanes *a* and *a'*), 7 d (lanes *b* and *b'*), 16 d (lanes *c* and *c'*), 26 d (lanes *d* and *d'*) and 35 d (lanes *e* and *e'*) old. Dwarf mouse myosins were from animals 16 d (lanes *f* and *f'*), 26 d (lanes *g* and *g'*), 35 d (lanes *h* and *h'*), 55 d (lanes *i* and *i'*), and 75 d (lanes *j* and *j'*) old. The position of the LC1 myosin light chain is indicated by the arrow to the left of the figure; all polypeptides above this subunit should be derived from the myosin heavy chain.

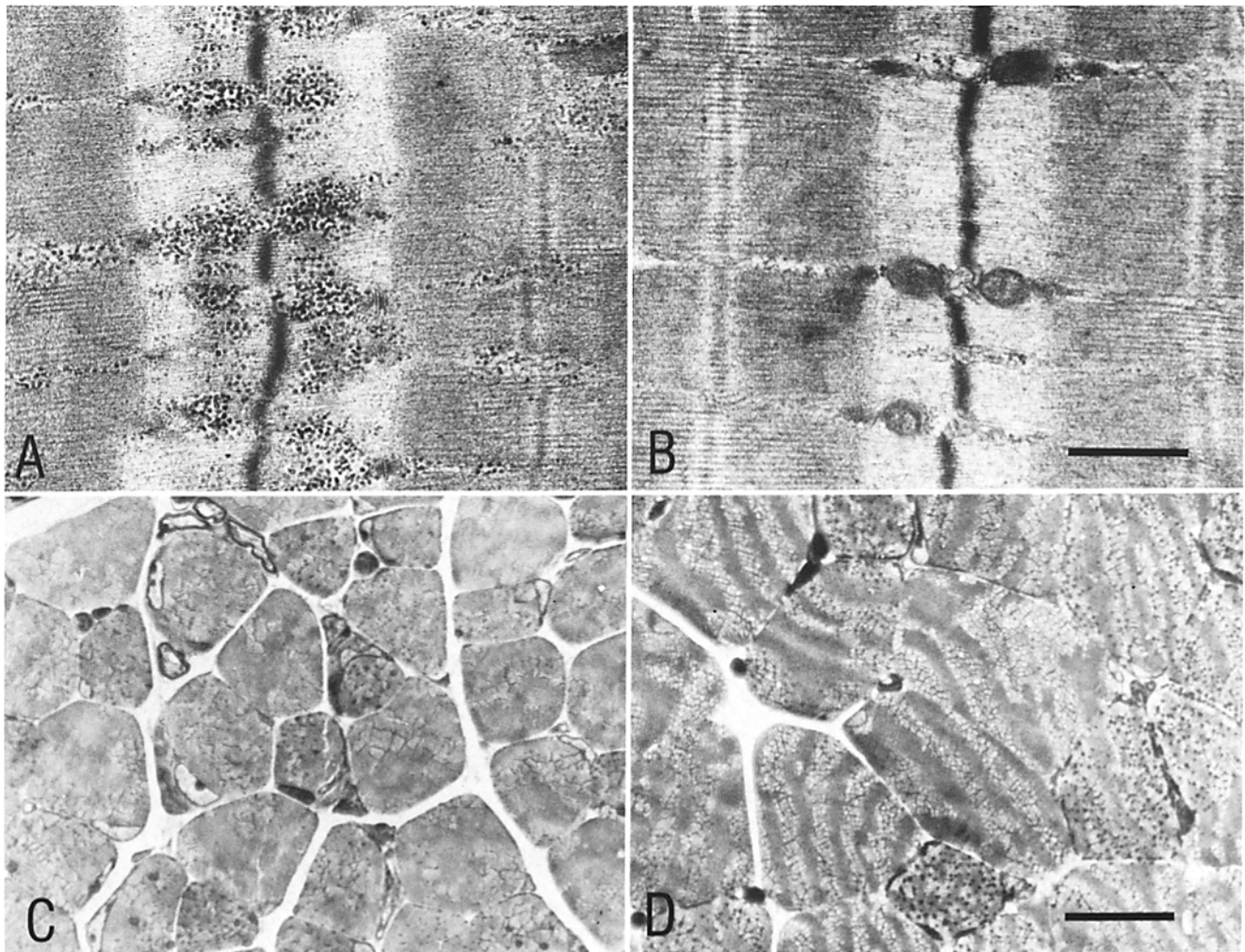


FIGURE 4 Morphological appearance of dwarf and control mouse skeletal muscle. Electron micrographs of longitudinal sections prepared from animals 18 d old are shown for dwarf (A) and control (B) mouse extensor digitorum longus muscle. Bar in B, 1 μ m. Transverse sections prepared from 31-d-old dwarf (C) and control (D) mice were stained with toluidine blue and photographed in the optical microscope. Bar in D, 50 μ m.

(Fig. 4, C and D), we found no signs of muscle fiber necrosis or regeneration. Thus, the dwarf muscles present a morphology that is less mature than that of control muscles, particularly with respect to fiber diameter and glycogen content. However, no obvious signs of muscle pathology are observed at up to 36 d of age.

We used immunocytochemistry to determine the myosin types present in individual fibers of the gastrocnemius muscles of 5-wk-old dwarf mice. At these ages, most of the fibers of control muscles are strongly stained with antibody to adult fast myosin (Fig. 5A), whereas neonatal antibody reacts only weakly with the smallest-diameter fibers (Fig. 5B). In dwarf muscles, most fibers show some reactivity with the fast antibody and many fibers are strongly stained (Fig. 5C). In contrast to control muscles however, almost all fibers are stained with the neonatal antibody (Fig. 5D). Thus, the persistence of neonatal myosin in dwarf muscles, demonstrated by biochemical techniques, reflects its presence in most muscle fibers, and not just the presence of a small population of fibers containing this myosin.

Since dwarf mice are deficient in TSH (4, 31) and are hypothyroid from birth (10), we have investigated whether

exogenous thyroid hormone is sufficient to overcome the block in the cardiac isozyme transition, and whether it would affect the skeletal muscle isozyme complement. Animals 3–4 wk old (~5 g) were injected intraperitoneally with 1 μ g thyroxine every 2–3 d; this amount produces a hyperthyroid state through most of the interval between injections, judging from previous studies of thyroid hormone supplementation in dwarf mice (10). After 11–15 d of treatment, an essentially complete transition from the V3 to V1 cardiac isozyme was observed, as determined by electrophoresis of native myosin (Fig. 6, lanes a–c).

Thyroxine injections also affect the myosin isozyme content of dwarf skeletal muscles. In the dwarf mice injected for 11–15 d, electrophoresis of native myosin shows that the neonatal isozymes have almost completely disappeared (Fig. 6, lane e), whereas in uninjected dwarfs of the same age the neonatal myosin bands are clearly present (Fig. 6, lane d). The electrophoretic pattern of the myosin from the injected mice now resembles that of control animals of a similar age (Fig. 6, lane f). Predominantly adult myosin isozymes were found in the skeletal and cardiac muscles in all of 13 animals injected for 11–15 d with thyroxine.

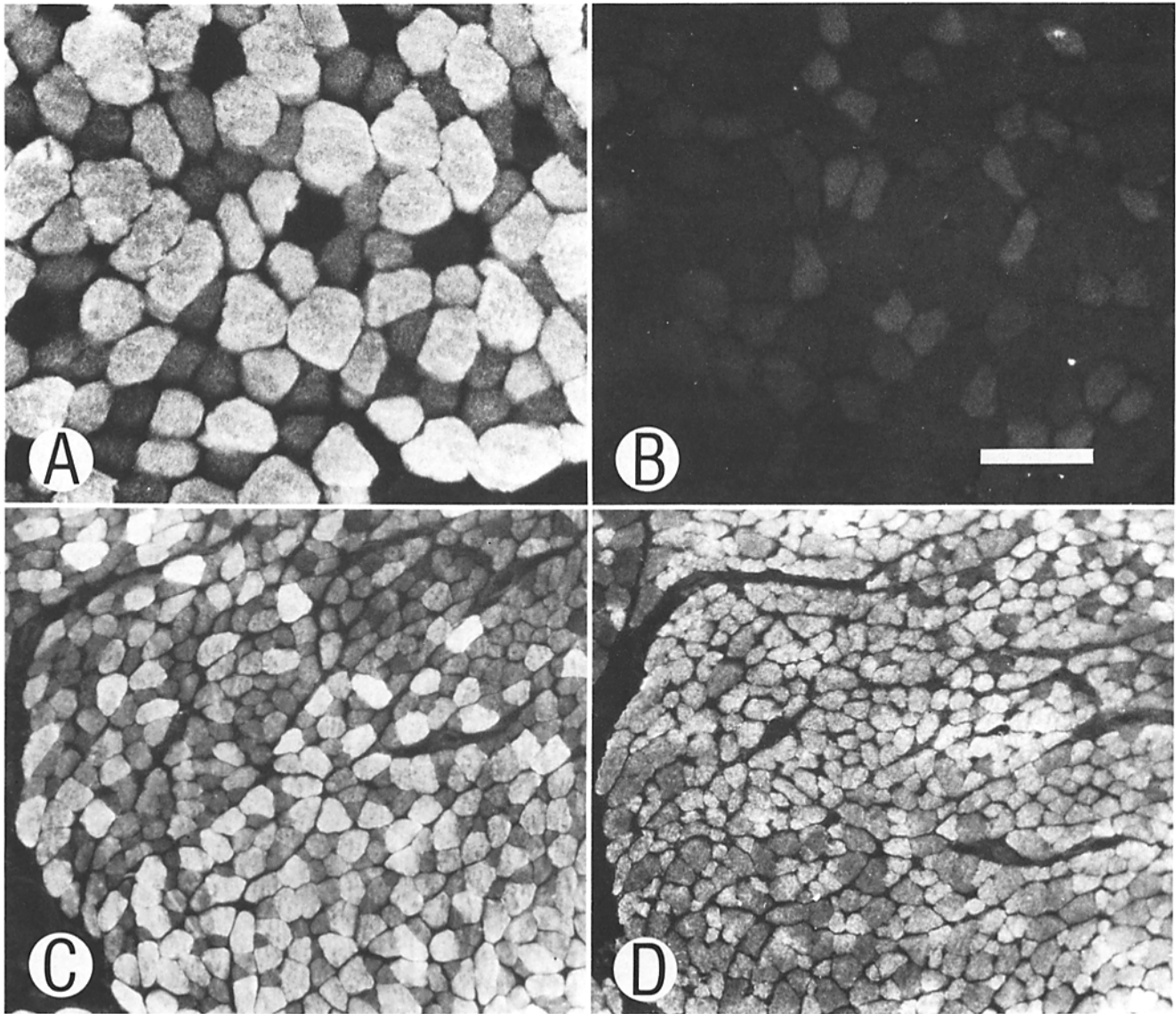


FIGURE 5 Indirect immunofluorescence on serial sections of skeletal muscle using antibodies to myosin. The gastrocnemius muscle was taken from 36-d-old control (A and B) and dwarf (C and D) mice, and serial sections were reacted with antibodies to either adult fast (A and C) or neonatal (B and D) myosins. The same region of the section is shown in A and B and in C and D, and therefore the myosin types present in individual fibers can be determined. Fibers that did not react with either antibody in the two sets of photos were observed in serial sections to be stained with antibody to slow myosin (not shown). Bar, 100 μm . $\times 167$.

DISCUSSION

In the mouse model of hereditary pituitary dwarfism studied here, the dwarf phenotype is associated with an abnormal presence of fetal V3 myosin in the heart and an abnormally slow transition from neonatal to adult myosin isozymes in hind limb skeletal muscles. Thus, this dwarf syndrome affects the two striated muscles differently, since the skeletal muscle isozyme transition is only slowed, whereas the cardiac transition is apparently completely blocked. In the dwarf skeletal muscles, no gross pathology was apparent, nor was there any evidence of degeneration or regeneration. The fibers do show general signs of immaturity consistent with the overall retardation of development expected to be associated with pituitary dwarfism. Immunocytochemistry demonstrated that the abnormal persistence of neonatal myosin in these mutant mice occurs in most of the muscle fibers rather than being localized

to a discrete fiber population.

The effects of pituitary dwarfism on myosin transitions must be secondary to the primary action of the dwarf mutation, which is known to affect the development of hormone-producing cells in the anterior pituitary, including the thyrotropes responsible for TSH production (4, 31, 46). The fact that an apparently normal isozyme complement can be restored to both types of striated muscle by injections of thyroxine suggests that it is the lack of thyroid hormone in dwarf mice that is responsible for the persistence of these fetal and neonatal myosin forms. For certain other phenotypic effects of the dwarf mutation, supplementation by one hormone is not sufficient to provoke a corrective response. For example, the morphology of the thyroid gland is not affected by thyroxine and only slightly modified by injection of TSH. However, administration of both TSH and GH results in considerable improvement in the structure of the gland (1-3). Like-

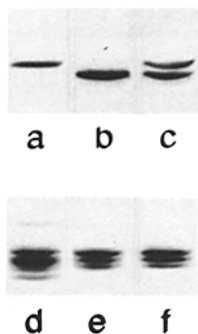


FIGURE 6 Electrophoretic analysis of skeletal and cardiac myosin isoforms in thyroxine-treated dwarf mice. In the results shown here, dwarf mice were injected with thyroxine every 2–3 d for 11 d beginning at 27 d of age. The cardiac isoforms are shown for a 38-d-old noninjected dwarf (lane a) and an injected dwarf mouse (lane b). The two extracts shown in lanes a and b were mixed and analyzed in lane c. The skeletal muscle isoforms present in a 38-d-old noninjected dwarf littermate are shown in lane d. The isoforms present in the injected dwarf are shown in lane e, those in a 38-d-old control mouse in lane f.

wise, growth stimulation in dwarf mice has been reported to be more effective when both thyroid hormone and GH are used (2).

Previous studies of the effect of hypothyroidism on myosin isozyme transitions in developing animals have depended on inducing the hypothyroid state by treatment with antithyroid drugs (9, 13, 17, 30). However, in normal animals the postnatal increase of GH is dependent on increases in thyroid hormone levels (36). Thus, when the stimulatory effects of thyroid hormone supplementation are investigated in drug-treated animals, the addition of exogenous thyroid hormone has direct effects as well as extensive indirect effects as a result of the stimulation of GH production (11, 18, 28, 36). It is therefore difficult to distinguish the effects of thyroxine from those of growth hormone in these situations. However, using the dwarf mouse as a model of the hypothyroid state in which GH is not produced in the pituitary either endogenously (12, 31, 37, 38) or in response to thyroxine treatment (37), we have been able to demonstrate that the addition of exogenous thyroxine has an effect on myosin isozyme transitions that is independent of GH production.

Although these results suggest that thyroid hormone-induced GH production does not play a role in controlling myosin isozyme transitions, it cannot be determined whether thyroid hormone acts directly on muscle tissue to exert its effects on myosin metabolism. The role of interactions of thyroid hormone with other hormonal systems or with tissues other than muscle remains to be studied. For example, another possibility would involve a retarded development of the nervous system, which is a well-known consequence of hypothyroidism (22). Although the appearance of adult fast myosin does not require continued innervation (8, 17), underdeveloped motor nerves might negatively influence muscle fiber development.

The effects of hypothyroidism on the myosin isozyme transitions in dwarf mice are apparently different for the two muscle tissues: the cardiac isozyme transition seems to be completely blocked whereas the neonatal to adult fast myosin transition in skeletal muscles clearly takes place, although at a slower rate than in normal mice. As compared with results of previous studies on rats in which both transitions were blocked by chemically induced hypothyroidism (9, 13, 17, 30), this result in dwarf mice was unexpected. The effects of hypothyroidism in dwarf mice could be explained if different levels of thyroid hormone were required to promote the myosin transitions in the two muscle tissues. Alternatively, the neonatal to adult fast myosin transition in skeletal muscle

might be under the control of a mechanism that is independent of but facilitated by thyroid hormone. In this case, the thyroxine-induced acceleration of this transition could be explained by the ability of thyroid hormone to generally stimulate metabolic rate.

Dwarf mice represent the first description of a genetically determined condition in which cardiac and skeletal myosin isozyme transitions are also affected. This model should allow detailed study of the endocrinological control of adult myosin appearance since certain myosin transitions can be completely and rapidly induced by exogenous thyroxine without the complicating effects of interaction of this hormone with pituitary somatotropes. The potential myopathic changes due to chronic pituitary hormone abnormalities (21) can be easily studied in this animal model. Conversely, the dwarf phenotype may have beneficial effects on certain muscle diseases: it was reported that the muscles of doubly homozygous dwarf-dystrophic mice do not undergo the myopathic changes characteristic of dystrophic mouse muscle (40). A case of a human pituitary dwarf simultaneously affected by Duchenne muscular dystrophy was also described (48); this patient has not shown the usual degenerative changes associated with this disease. Dwarf mice may therefore prove useful in testing certain ideas about possible effects of pituitary hormones, or even growth in general, on the progress of muscle disease.

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