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Histochemical Characteristics of Soleus Muscle in hGH Transgenic Mice (42561)

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Abstract. Histochemical characteristics of soleus muscle were compared in human growth hormone (hGH) transgenic mice vs their nontransgenic littermates. Plasma of transgenic mice contained hGH (7.1 ± 0.7 and 6.7 ± 0.4 ng/ml, mean ± SE, at 5 and 11 months of age, respectively); hGH was not detectable in plasma of nontransgenic littermates. Body and soleus weights were greater (approximately 55 and 25%, respectively) and both type I and type IIA fibers were larger in transgenic animals. Most significantly, fiber type composition of the soleus muscle was different in hGH-transgenic animals, i.e., the percentage of type I fibers was significantly greater than in nontransgenic mice (77.2 ± 5.1% vs 58.4 ± 2.5%). It is generally believed that skeletal muscle fiber composition is determined predominantly by neural influences (1, 2). These data suggest hormonal factors, growth hormone, also affect the phenotype of skeletal muscle myosin.

It has become possible during this decade to introduce well-characterized, genetic information into the chromosomal complement of mammals (3, 4), *i.e.*, to engineer and produce transgenic mammals. Mouse lines have been established containing gene-constructs coding for a variety of substances, including human growth hormone (hGH) from several species (for a recent review, see (5)). Since the transgenes are heritable and expressed *in utero*, the gene products are apparently recognized "as self" by the immune system of the host animal (6). The transgenic animal thus represents a unique model system for studying the long-term effects of substances on various tissues and physiologic processes.

Previous studies in our laboratory have been concerned with factors that affect the metabolic enzyme machinery and fiber type composition of skeletal muscle (7, 8). It is generally believed that skeletal muscle fiber composition is predominantly under neural control (1, 2). There is, however, evidence that the functional demands of muscle (9–11) and hormonal factors are involved (12). Although the effects of exogenously administered growth hormone on growth rate have been known for more than 50 years (13), little is known regarding the effects of the hormone on skeletal muscle fiber type composition. In this study, we report the effects of hGH, produced endogenously in transgenic animals, on the histochemical characteristics of mouse soleus muscle.

Materials and Methods. *Animals.* Hybrid (B6C3F), transgenic, male mice were kindly provided by Dr. Thomas E. Wagner of the Edison Animal Biotechnology Center, Ohio University, Athens, Ohio. The second-generation transgenic animals are the offspring of mice produced by microinjecting recombinant DNA directly into the pronuclei of zygotes shortly after fertilization, using procedures essentially similar to those previously described (4). The chromosomal complement of these animals contains a fusion gene, consisting of the promoter sequence of the mouse metallothionein I gene ligated to the structural gene which codes for hGH; the gene is expressed in that the transgenic animals synthesize hGH-messenger RNA and hGH (5). The animals used in the present study were bred from matings of transgenic males with normal (nontransgenic) BCF1 females (Jackson Laboratories, Bar Harbor, ME). The 13 mice obtained were bled (10 AM, 350 μl, tail vein) at 5 and 11 months of age and aliquots (duplicate) of heparinized plasma were radioimmunoassayed for hGH using a double monoclonal antibody technique (Hybridtech, Inc., San Diego, CA). Plasma obtained from 6 mice (transgenic) contained hGH (7.1 ± 0.7 and 6.7 ± 0.4 ng/ml, mean ± SE, at 5 and 11 months of age, respectively). These data are similar to those obtained in our laboratory on other groups of transgenic animals with regard to (1) the absolute level of hGH, and (2) the relative constancy, in a given animal, of plasma hGH on both a short-term (daily) and a long-term (monthly) basis. hGH was not detected (<0.2 ng/ml) in plasma of the 7, non-transgenic lit-

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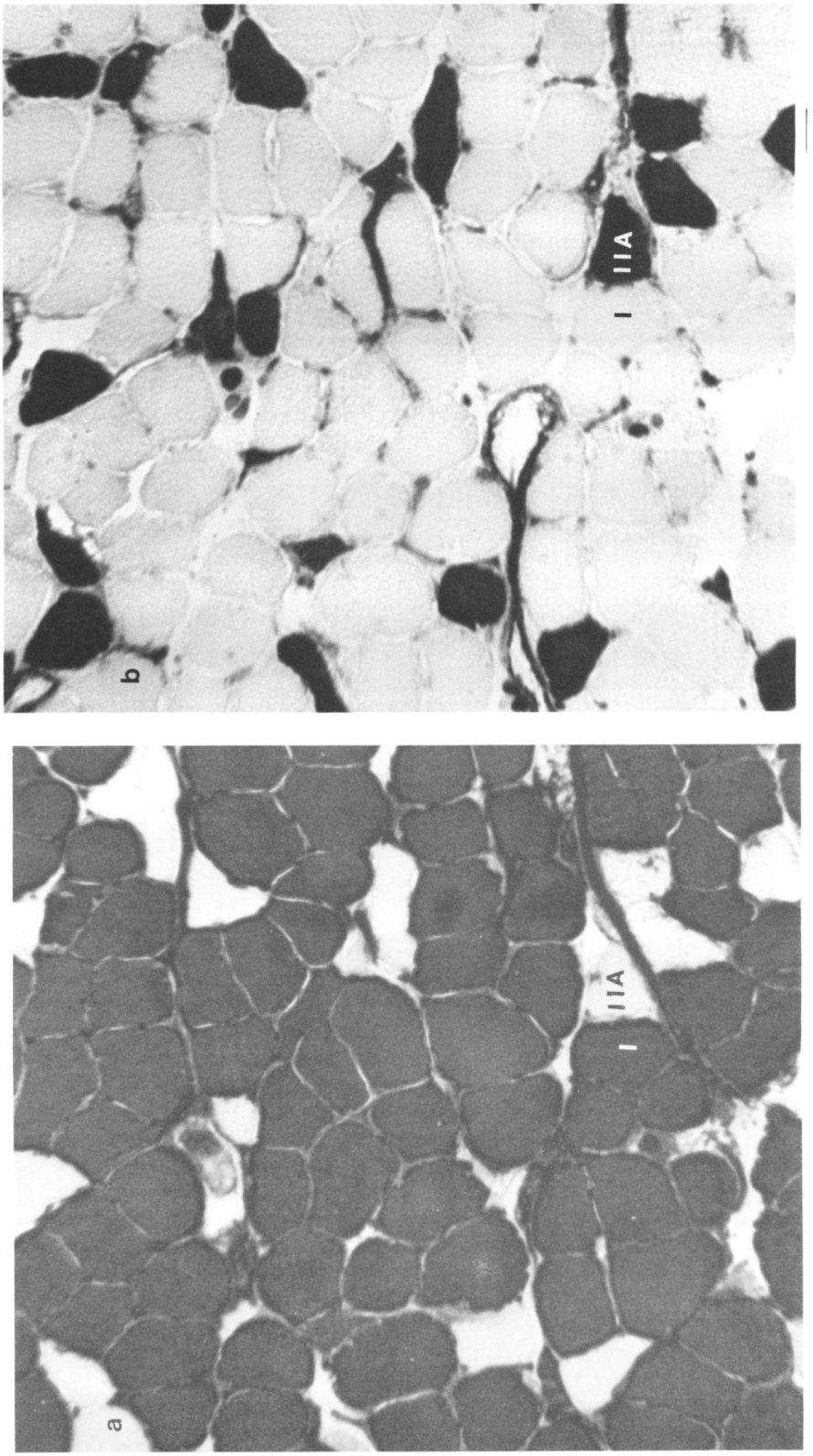

FIG. 1. (a–d) Serial cross sections of soleus muscle of transgenic (a and b) and control (c and d) mice. Cross sections were assayed for myofibrillar adenosine triphosphatase after acid preincubation at pH 4.35 (a and c) or after alkaline preincubation at pH 10.3 (b and d). IIA, type IIA. I, type I. Types IIB and IIC were extremely rare (see Results) and not evident in figure (×160).

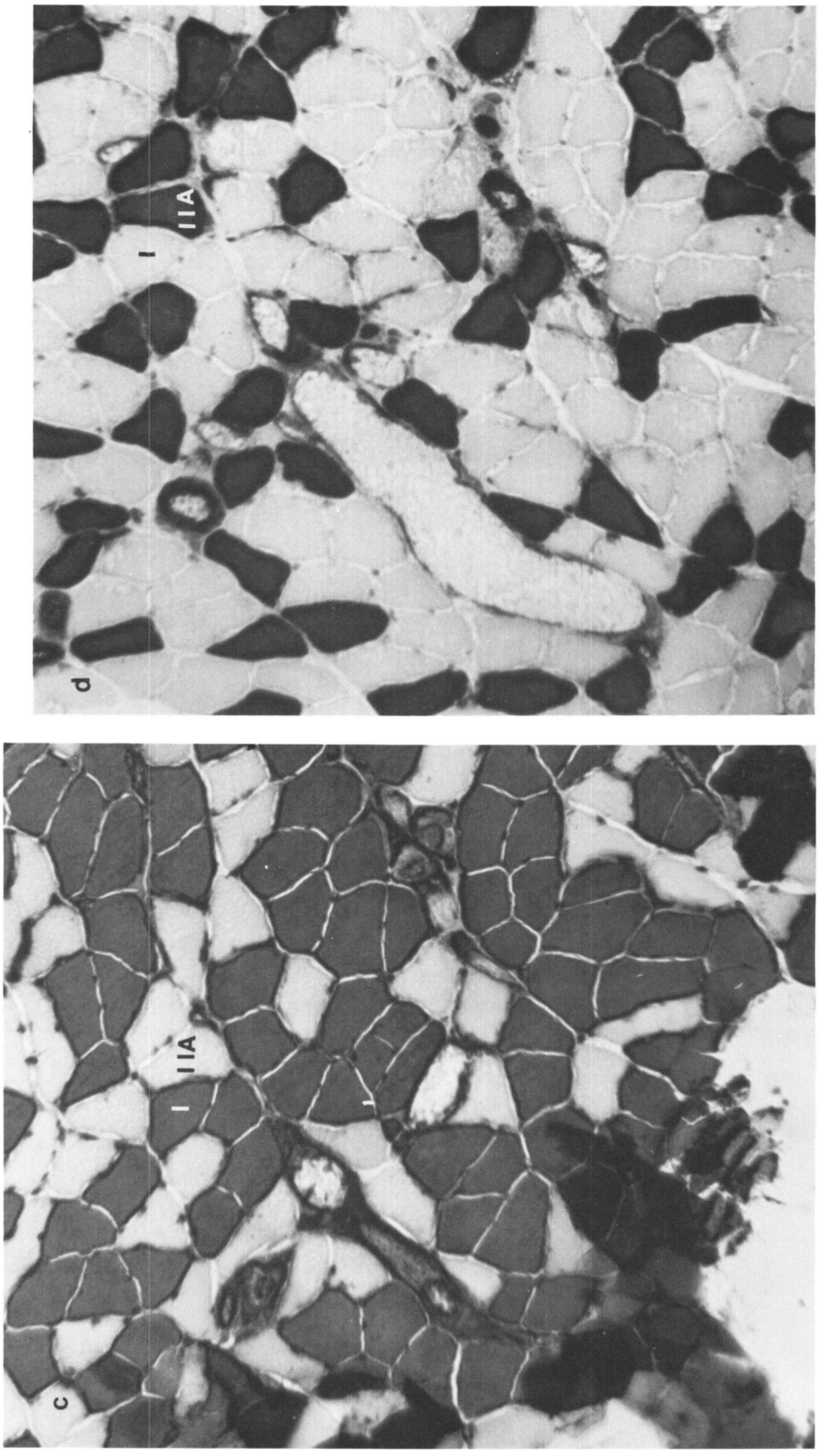

FIG. 1—*Continued.*

termates (controls). This pattern of inheritance is consistent with the findings of Palmiter *et al.* (6) which show that a metallothionein I/rat growth hormone fusion transgene is transmitted in a Mendelian manner to half the offspring during two generations.

Animal care. Mice were housed 2–4 per cage in a temperature controlled room (21°C) with a 12:12 hr 1:day cycle and maintained on Purina mouse chow and water *ad libitum.* Beginning at 5 months of age, they were also provided a standardized daily physical activity period. Animals walked (10- to 15-sec bouts with 1.5 min of nonlocomotory activity between efforts) on a motor driven treadmill (7.5 m/min, 45° incline) 5 days each week in an effort to provide sufficient activity to maintain "normal" muscle mass (i.e., to minimize hypokinetic-induced decreases). Physical activity of this intensity and daily duration would not be expected to result in "exercise-trained" animals (7, 14). Two of the transgenic mice would not perform physical activity and were excluded from the study.

Tissue preparation. At 11 months of age and 48 hr after the last physical activity session, the soleus muscle of one hindlimb was excised, trimmed of connective tissue, weighed wet, oriented, placed in embedding medium (O.C.T. Compound, Tissue-Tek II) and frozen in isopentane cooled to −160°C by liquid nitrogen. Serial cross sections (10 μm thick) of the mid-belly of each muscle were cut on a cryostat at −20°C, mounted on glass coverslips and air-dried for 60 min for histochemistry.

Histochemistry. Cross sections were assayed for myofibrillar adenosine triphosphatase activity essentially as described previously (8). A modification of the method of Brooke and Kaiser (15) was used to differentiate type I (slow-twitch) and type II (fast-twitch) fibers and fast-twitch subtypes (types IIA, IIB, and IIC). Acid preincubation was performed for 5 min (21°C) in a solution of 250 mM acetate, 150 mM Na barbital and 100 mM HCl, adjusted to pH 4.35. Alkaline preincubation, essentially as described by Guth and Samaha (16, 17), was conducted for verification of fiber types I (slow-twitch) and II (fast-twitch). Sections were preincubated for 15 min at 21°C in a medium containing 18 m*M* $CaCl_2$ and 100 m*M* Sigma 221 buffer (pH 10.3). Sections were incubated 45 and 15 min after acid and alkaline preincubation, respectively, at 21°C in a medium containing 18 m*M* $CaCl_2$ and 4.5 m*M* ATP in 100 m*M* Sigma buffer, adjusted to pH 9.4.

Entire cross sections were projected onto tracing paper with a Zeiss microscope (Standard 14 with drawing tube, Carl Zeiss, West Germany) and all muscle cells (*n,* 335 minimum–806 maximum) were typed (see below) and their perimeter was drawn at constant magnification. Type IIA fibers stained weakly at pH 4.35 and intensely at pH 10.3 (Fig. 1). The reverse was true for type I fibers (Fig. 1). Type IIB fibers stained intensely at pH 10.3 and moderately at pH 4.35 while type IIC fibers stained intensely in acid and alkali. Fiber area was determined by tracing the perimeter of 50 fibers of each type on a digitizing tablet with areas computed by the Zeiss Video-Plan 2 (Carl Zeiss, West Germany) and Kontron CP/M2.2, Version 5.42 software (Kontron Electronik, West Germany). Fiber type and area determinations were conducted by an individual blind to the source (transgenic or control mouse) of a given cross section.

Statistical procedures. Data were compared between groups by independent *t* test. Ratios and percentages were log-transformed prior to analyses.

Results. *Body and muscle weights.* Transgenic (T) mice were larger ($P < 0.001$) than control (C) mice at 5 (40.3 ± 1.5 vs 25.8 ± 1.0 g) and at 11 months (56.3 ± 4.3 vs 36.2 ± 2.2 g) of age and the increase in this variable was greater ($P < 0.001$) in the former group. Weight of the soleus muscle was greater ($P < 0.01$) for T than C mice (10.7 ± 0.8 vs 8.6 ± 0.4 mg) as was the ratio ($\times 10^3$) of body to soleus weight (5.3 ± 0.1 vs 4.2 ± 0.2).

Soleus muscle histochemistry. The percentage of type I fibers was greater ($P < 0.002$) in T than C mice and the converse was evident for type IIA fibers (Table I). The sum of the percentages of types IIB and IIC fibers was less than 2% in both groups. Type I and type IIA fibers were significantly larger (approximately 33 and 19%, respectively) in T than C mice (Table I). The small proportion of types IIB and IIC fibers precluded appropriate determination of their areas. The greater percentage and area of type I fibers in T than C mice resulted in a greater ($P < 0.003$) relative area for this fiber type in the former group (Table I).

TABLE I. HISTOCHEMICAL CHARACTERISTICS OF SOLEUS MUSCLE

Group	I (%)	I (area)	I (area %)	IIA (%)	IIA (area)
T	77.2 ± 5.1^b	2925 ± 269^c	82.4 ± 5.2^c	21.4 ± 4.7^a	2137 ± 137^d
C	58.4 ± 2.5	2192 ± 50	64.3 ± 2.4	39.6 ± 2.3	1788 ± 65

Note. Values are means ± SE. T, transgenic ($n = 4$). C, control ($n = 6$). I or IIA (%), percentage of type I or IIA fibers. I or IIA (area), area of type I or IIA fibers in μm^2. I (area %), relative area of type I fibers.
[a-d] Significant group effect ($P < 0.001$, <0.002, <0.003, and <0.02, respectively).

Discussion. Implantation of steroidal pellets (18), daily injection (19), injection of cell tumors (20), and arterial infusion (21) techniques, for example, have been used to study the *in vivo* effects of substances such as toxins, drugs, and hormones on various tissues and physiologic processes. These techniques are valuable, but are invasive, probably inconvenient, and in long-term studies are fraught with the potential of developing immune reactions in recipient animals. We report in this study the effect of hGH, produced endogenously in transgenic animals, on histochemical characteristics of mouse skeletal muscle. The transgenic mice synthesize hGH-messenger RNA and hGH, as evidenced by the presence of the hormone in their plasma. Nontransgenic littermates served as controls and hGH was not detectable in their plasma.

Body and soleus weights were greater (approximately 55 and 25%, respectively) in transgenic (T) than control (C) mice and the former group showed more rapid weight gain. The magnitude of the effect of hGH on total body mass of transgenic mice is comparable to that reported by Palmiter *et al.* (6). The larger increase in body than soleus weight has been reported in rats inoculated with pituitary tumor cells (GH3) secreting growth hormone (20). This response may reflect greater enlargement of nonmuscle (e.g., liver, kidney, and spleen) than muscle tissue (20, 22).

Type I and type IIA fibers of the soleus muscle were larger in T than C mice (approximately 33 and 19%, respectively). Rats exposed to growth hormone-secreting pituitary tumor cells (20) and individuals afflicted with acromegaly also show hypertrophy of skeletal muscle fibers (23). It is reasonable that these responses are a direct effect of a growth hormone-induced increase of protein synthesis (e.g., (24–26)).

The most significant result of the present study was the greater percentage of type I fibers in the soleus of T than C mice (Table I). It is generally accepted that histochemical methods are appropriate for determining the type I (slow-twitch) and type II (fast-twitch) fiber composition of skeletal muscle and the reversal of staining intensity of type I and II fibers with alkaline and acid preincubation further substantiates the validity of this method. Neural factors, particularly the nature of impulse activity, are generally believed to determine the fiber composition of skeletal muscle (see (1, 2)). Hormonal factors in concert with and/or independent of neural factors, however, may also influence skeletal muscle fiber composition. The data of Ianuzzo *et al.* (12), for example, indicate that the thyroid state alters the character of skeletal muscle myosin.

A potentially confounding response to growth hormone administration is an increase in body/muscle weight (present study, 20). This may have imposed differences in the functional demands of muscle between the T and C mice, and, thereby, influenced skeletal muscle mass (27), fiber composition (8, 11), or both (9, 10). It is doubtful, however, that differences in this factor between groups, if evident, could solely account for the results of the present study. Muscle enlargement induced by growth hormone (GH3 tumor cells) appears to occur via a mechanism independent of alterations in functional demand (28) and is evident to a similar extent in weight-bearing and non-weight-bearing muscles (20). Nevertheless, differences in functional demand between the T and C mice may have contributed, in part, to the findings.

Our results, in summary, suggest growth hormone has the potential for altering the phenotype of skeletal muscle myosin and muscle mass. Although the growth-promoting effect of this hormone probably occurs after differentiation of skeletal muscle (29), it is not clear when the effect of growth hormone on

fiber type composition is exerted with respect to differentiation.

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