Control of glycogen synthesis is shared between glucose transport and glycogen synthase in skeletal muscle fibers

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1Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110; 2Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202; and 3Departments of Pharmacology and Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Azpiazu, Iñaki, Jill Manchester, Alexander V. Skurat, Peter J. Roach, and John C. Lawrence, Jr. Control of glycogen synthesis is shared between glucose transport and glycogen synthase in skeletal muscle fibers. Am. J. Physiol. Endocrinol. Metab. 278: E234–E243, 2000.—The effects of transgenic overexpression of glycogen synthase in different types of fast-twitch muscle fibers were investigated in individual fibers from the anterior tibialis muscle. Glycogen synthase was severalfold higher in all transgenic fibers, although the extent of overexpression was twofold greater in type IIB fibers. Effects of the transgene on increasing glycogen and phosphorylase and on decreasing UDP-glucose were also more pronounced in type IIB fibers. However, in any grouping of fibers having equivalent malate dehydrogenase activity (an index of oxidative potential), glycogen was higher in the transgenic fibers. Thus increasing synthase is sufficient to enhance glycogen accumulation in all types of fast-twitch fibers. Effects on glucose transport and glycogen synthesis were investigated in experiments in which diaphragm, extensor digitorum longus (EDL), and soleus muscles were incubated in vitro. Transport was not increased by the transgene in any of the muscles. The transgene increased basal [14C]glucose into glycogen by 2.5-fold in the EDL, which is composed primarily of IIB fibers. The transgene also enhanced insulin-stimulated glycogen synthesis in the diaphragm and soleus muscles, which are composed of oxidative fiber types. We conclude that increasing glycogen synthase activity increases the rate of glycogen synthesis in both oxidative and glycolytic fibers, implying that the control of glycogen accumulation by insulin in skeletal muscle is distributed between the glucose transport and glycogen synthase steps.

The relative importance of glucose transport and glycogen synthase in controlling the rate of glycogen synthesis has been debated over the years. Recently, much attention has focused on the importance of glucose transporters in determining the rate of glycogen biosynthesis, and a widely held view is that most of the control is at the level of the transporter. For example, studies with transgenic mice overexpressing the glucose transporters GLUT-1 and GLUT-4 have provided compelling evidence that increasing glucose transport is sufficient to increase glycogen synthesis in skeletal muscle (10, 32). The glycogen content of muscles from these transgenic animals was markedly increased, but the activation state of glycogen synthase in the transgenic muscles did not appear different from that in the control muscles. On the basis of these results, it was concluded that glucose transport is strictly rate limiting for glycogen synthesis (32). Mice heterozygous for a disrupted GLUT-4 allele, expressing some 50% of the wild-type level of GLUT-4, also exhibited impaired glycogen synthesis during euglycemic hyperinsulinemic clamps (34). However, the results from animals with genetically modified expression of GLUT-1 or GLUT-4 do not exclude the possibility that activation of glycogen synthase has an important role in the stimulation of glycogen synthesis by insulin. Indeed, our recent
finding that glycogen was markedly elevated in muscles of transgenic mice overexpressing glycogen synthase supports the view that increasing glycogen synthase activity is sufficient to promote glycogen accumulation (27).

The heterogeneity of the fibers that comprise skeletal muscle is a complicating factor in all studies of muscle metabolism. Even adjacent fibers in a muscle may have markedly different contractile and metabolic properties (30, 42). A method involving histochemical staining of myosin ATPase activity at different H+ concentrations has been widely used to distinguish three types of fibers, designated types I, IIA, and IIB (1). Fiber type assignments have also been made on the basis of the activities of representative enzymes of glycolytic and oxidative energy metabolism found in different fibers (14, 26, 30, 42). Type I fibers have relatively low levels of glycolytic enzymes and high levels of oxidative enzymes. These fibers also have a slow twitch speed and are much less fatigable than type II fibers, because most of their energy is derived from oxidative metabolism. Type IIB fibers have a fast twitch speed and depend largely on glycogen metabolism to provide the energy to fuel rapid and forceful contractions. These fibers have relatively high levels of glycolytic enzymes and low levels of enzymes of oxidative energy metabolism. Type IIA fibers have a rapid contractile speed and high levels of both glycolytic and oxidative enzymes. One of four myosin heavy-chain isoforms is usually expressed either exclusively or predominantly in a single fiber (35). The ability to detect the different heavy-chain isoforms led to the identification of a third type of fast-twitch fiber, designated type IID (or 2X). The oxidative enzyme content of these fibers is higher than that of IIB fibers but generally lower than that of IIA fibers (35).

Single fiber analyses and immunohistochemical studies have demonstrated that the amount of GLUT-4 correlates directly with the levels of enzymes of oxidative energy metabolism, such as malate dehydrogenase (MDH) (19, 28). This finding is consistent with observations that the effect of insulin on glucose transport is enhanced in muscles composed predominantly of oxidative fiber types (20). Whether glycogen synthase levels correlate with those of GLUT-4 in different fibers is not known, but it is possible that the relative contributions of glucose transport and glycogen synthase to glycogen synthesis could differ among fibers.

The present study extends our previous work (27) on transgenic mice that overexpress glycogen synthase under the control of promoter/enhancer elements of the mouse muscle creatine kinase gene. The transgenic animals overexpress a mutant form of rabbit muscle glycogen synthase having Ser to Ala mutations in two key inactivating phosphorylation sites, sites 2 and 3a (39). We had previously correlated expression of the transgene with elevated glycogen accumulation in various muscles. We now present results from single fiber analyses that allow direct comparison between transgene-stimulated glycogen accumulation and the oxidative capacity of a fiber, as assessed by measurements of MDH activity. In addition, we describe the effects of the transgene on glucose transport and the rates of glycogen synthesis in muscles having different fiber-type compositions.

**METHODS AND MATERIALS**

**Animals**

Transgenic mice expressing glycogen synthase (GS) (with mutations in sites 2 and 3a) have been described previously (27). Expression of the transgene was driven by promoter/enhancer elements from the skeletal muscle creatine kinase gene, which favors expression in fast-twitch fibers. Two transgenic lines, GSL3 and GSL30, were used in the experiments presented. Heterozygous male mice were mated to wild-type (C57BL6 × CBA)F1 females. Transgenic pups were identified by the PCR to amplify chloramphenicol acetyltransferase sequences in tail DNA. All mice were used ~2 mo after birth. At this time the weights of the transgenic mice and the nontransgenic littermates, which were used as controls, were approximately equal (Table 1).

**Experimental Protocols**

Single fiber analyses. Transgenic and age-matched wild-type animals were fed ad libitum and then anesthetized by subcutaneous injection (1 ml/kg) of a mixture of ketamine (40 mg/ml), xylazine (10 mg/ml), and acepromazine (1.5 mg/ml). Hindlimb muscles were exposed and frozen in situ between two stainless steel blocks (1 cm × 2.5 cm × 2 cm) that had been chilled in liquid nitrogen. The muscles were freeze-dried at −35°C and then stored under vacuum at −70°C before use. Single fibers were manually dissected from the muscle samples (26) and weighed using a fish pole balance (29). Glucose and G-6-P (13), G-1-P (29), and the activities of creatine kinase (12) and MDH (14) were measured in pieces of individual fibers. Phosphorylase activity and levels of glycogen and UDPG were measured as described by Henry and Lowry (11).

To determine the amount of GS, a piece of fiber was weighed and dissolved in buffer (80 µl/µg dry weight of fiber) containing sodium dodecylsulfate (SDS) (21). Samples (20 µl) of the dissolved fibers and samples containing known amounts of purified rabbit skeletal muscle GS were subjected to PAGE in the presence of SDS (21). The proteins were then electrophoretically transferred to Immobilon (Millipore) membranes, and GS was detected by immunoblotting as described previously (27). Optical densities of the bands corresponding to GS were determined using a scanning laser densitometer (Molecular Dynamics). Integrated values from the samples of purified GS were used to generate a standard curve, from which the amount of GS protein present in the fiber samples was determined.

Incubation of muscles in vitro. All media used for muscle incubations were directly gassed by bubbling with a mixture of 95% O2-5% CO2. Incubations were conducted essentially as described previously.

| Table 1. Average age and weights of wild-type and transgenic mice providing muscles for incubations in vitro |
|-------------------------------------------------|----------|----------|
| No. of Animals | Age, days | Weight, g |
| Wild type | 36 | 58 ± 4 | 22.3 ± 0.7 |
| GSL3 | 28 | 58 ± 3 | 21.9 ± 0.7 |
| GSL30 | 21 | 62 ± 6 | 24.4 ± 1.0 |

Values are means ± SE.
Materials. Porcine insulin (27 U/mg) was obtained from Eli Lilly. 2-Deoxy-[1,2-3H]glucose, [U-14C]mannitol, and [U-14C]glucose were obtained from New England Nuclear. Most commonly used chemicals and reagents were from Sigma Chemical.

RESULTS

Single Fiber Analyses

In previous work, three lines of transgenic mice overexpressing GS were studied, of which line GSL30 had the highest level of overexpression (27). The transgenic enzyme is present in high levels in anterior tibialis muscles, which are composed of the three types of fast-twitch fiber types (9, 15). To determine whether the transgene affected glycogen accumulation to different extents in these three types of fibers, enzymes and metabolites were measured in single fibers that were manually dissected from freeze-clamped samples of muscles from control and GSL30 animals.

Muscle fiber classification. Creatine kinase and MDH activities were measured to assess differences between fibers. Type IIB fibers have the lowest levels of MDH activity of the different types of fibers (14), and in a plot of creatine kinase vs. MDH activity, the data points from IIB fibers were tightly clustered and well separated from those derived from the oxidative fibers (Fig. 1). Type IIA fibers generally have higher levels of enzymes of oxidative metabolism than IID fibers (9). Consequently, we believe that the fibers with the highest MDH activities in Fig. 1 are IIA fibers. However, some IID fibers have high levels of oxidative enzymes; therefore, we have assigned fibers having an MDH activity higher than 0.25 nmol·min⁻¹·mg⁻¹ to a type IIA\IID category. By this criterion, 53% of the control fibers analyzed were IIB, and 47% were IIA\IID fibers. These proportions agree well with the fiber type composition of mouse anterior tibialis muscles estimated by other methods. For example, in a recent study in which fiber type was assigned on the basis of reactivity of fibers with isoform-specific myosin heavy-chain antibodies, the deep region (from which the present fibers were obtained) of the muscle was found to contain 47% IIB fibers, 24% IIA fibers, and 27% IID fibers (15). The proportions of IIB and IIA\IID fibers (50% of each) in transgenic muscles were essentially unchanged from IIB fibers were tightly clustered and well separated from the control fibers (Fig. 1).

Creatine kinase and malate dehydrogenase (MDH) activities were measured in individual anterior tibialis fibers from control and GSL30 mice. Anterior tibialis muscles in right hindlimb of 3 GSL30 mice (GSL30 M1-M3) and 3 wild-type littermates (Control M1-M3) were freeze-clamped in situ before samples of tissue were freeze-dried at −35°C. Nos. of fibers analyzed from the different muscles were as follows: Control, M1 (n = 16), M2 (n = 16), M3 (n = 15); GSL30, M1 (n = 16), M2 (n = 14), and M3 (n = 27). Creatine kinase and MDH activities were measured in pieces of each fiber, and remaining pieces of fibers were retained for measurements of metabolites and other enzymes (see Figs. 2–7). Fibers were assigned to type IIB and IIA\IID groups as described in text.

![Fig. 1. Creatine kinase and malate dehydrogenase (MDH) activities in individual anterior tibialis fibers from control and GSL30 mice. Anterior tibialis muscles in right hindlimb of 3 GSL30 mice (GSL30 M1-M3) and 3 wild-type littermates (Control M1-M3) were freeze-clamped in situ before samples of tissue were freeze-dried at −35°C. Nos. of fibers analyzed from the different muscles were as follows: Control, M1 (n = 16), M2 (n = 16), M3 (n = 15); GSL30, M1 (n = 16), M2 (n = 14), and M3 (n = 27). Creatine kinase and MDH activities were measured in pieces of each fiber, and remaining pieces of fibers were retained for measurements of metabolites and other enzymes (see Figs. 2–7). Fibers were assigned to type IIB and IIA\IID groups as described in text.](http://ajpendo.physiology.org/DownloadedFrom10.1152/ajpendo.00723.2016/fig1)
the same as in control muscles. Thus the transgene did not elicit any major change in the overall fiber composition of the muscle.

Effect of transgenic expression of GS on the glycogen content of single fibers. Consistent with other studies of single muscle fibers (14, 26), the average levels of creatine kinase were approximately twice as high in IIB fibers as in the oxidative fast-twitch fiber types (Fig. 1). Because transgenic expression of GS was driven by promoter/enhancer elements derived from the muscle creatine kinase gene, levels of the transgene would also be expected to be higher in type IIB fibers. GS levels were measured by immunoblotting in control and transgenic fibers (Fig. 2A). As observed in samples of whole muscle (27), GS from single wild-type fibers appeared as electrophoretically distinguishable species (Fig. 2A), most likely because of differences in phosphorylation, which retards the electrophoretic mobility of the GS subunit. In fibers from wild-type muscle, the amount of GS estimated from optical density scanning of the immunoblot was approximately twice as great in IIA\IID fibers as in IIB fibers (Fig. 2B). GS in the transgenic IIB and IIA\IID fibers was much higher than in the respective fibers from wild-type animals, and the level in transgenic IIB fibers was higher than that in transgenic IIA\IID groups. We believe that immunoblotting tends to overestimate the expression of the transgenic rabbit protein relative to the endogenous mouse GS, because the antibodies used were raised against rabbit GS. We previously observed that the increase due to transgenic expression of GS as estimated by immunoblotting extracts of whole anterior tibialis muscle was greater than that predicted from the increase in total synthase activity in the same extracts (27). Nevertheless, because of the extent of overexpression, the results indicate that the amount of GS in transgenic IIB fibers was approximately twice that in the IIA\IID fibers.

The glycogen content of almost all transgenic IIB fibers was higher than that of the control IIB fibers (Fig. 3). Indeed, only one control IIB fiber was found in the transgenic domain. The mean glycogen content of the transgenic IIA\IID fibers was lower than that of the transgenic IIB fibers, consistent with the higher expression of the transgene in IIB fibers. In the IIA\IID groups, there was some overlap in glycogen levels between the control and transgenic fibers. However, in fibers having approximately equal MDH activity, the glycogen content of the large majority of the transgenic IIA\IID fibers was higher than that of the control fibers.

Increased phosphorylase activities in transgenic fibers. Glycogen levels in fibers are ultimately determined by the rates of both synthesis and degradation. Therefore, we investigated the possibility that the higher levels of glycogen achieved in the IIB fibers were due to a reduction in phosphorylase, the enzyme that mediates glycogen degradation. Instead of having less phosphorylase, the transgenic IIB fibers had approximately fourfold higher levels of the enzyme than the control IIB fibers (Fig. 4). Interestingly, there was a very strong inverse correlation ($r = -0.95$ by linear regression) between MDH and phosphorylase activities in the transgenic fibers. Thus, in the most oxidative fibers, there was very little difference between the levels of phosphorylase in control and transgenic fibers (Fig. 4). There was also a correlation, albeit positive ($r = 0.80$), between phosphorylase and glycogen among the different fibers. Thus, the fibers with the most glycogen tended to have the highest levels of phosphorylase (Fig. 5).

Effect of the transgene on metabolites in the GS pathway. To investigate the mechanisms involved in the increase in glycogen mediated by the transgene,
each of the metabolites in the pathway leading to GS was measured. We had previously found that UDPG, the immediate precursor to glycogen, was lower in transgenic anterior tibialis muscles (27). UDPG was also lower in both the transgenic IIB and IIA\IID fibers than in the corresponding types of control fibers (Fig. 6). Indeed, at equivalent MDH activities, the UDPG level in only one transgenic IIB fiber was found in the control domain, and the UDPG content of all of the transgenic IIA\IID fibers was lower than in control fibers of these types. Other precursors in the pathway leading to glycogen were not decreased in the transgenic fibers (Fig. 7).

Glucose levels associated with the single fibers were modestly higher in the transgenic fibers than in the control fibers. It should be noted that this measurement includes not only intracellular glucose but also a fraction of the interstitial glucose that is recovered with the fibers. G-6-P levels were ~50% higher in both groups of transgenic fibers than in the control groups. A similar difference in G-6-P levels in samples of anterior tibialis muscles from control and transgenic mice was observed previously (27). No difference in G-1-P was observed between control and transgenic fibers in either the IIB or the IIA/IID groups.

Experiments with Muscles Incubated in Vitro

To analyze GS and glucose transport, it was necessary to utilize intact muscle, and experiments were conducted in vitro with diaphragm, EDL, and soleus muscles. These muscles may be maintained in a viable state during short-term incubations, and they have different fiber type compositions, allowing some correlation with data obtained from dissected fibers. On the basis of staining with antibodies against different myosin isoforms, mouse soleus muscles were found to contain approximately equal numbers of type I and IIA

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Fig. 4. Relationship between phosphorylase and oxidative capacity in control and GSL30 fibers. Phosphorylase was measured in fibers analyzed in Fig. 1 and plotted against MDH. Linear regression analyses of phosphorylase vs. MDH were performed on values obtained from control (r = -0.72, P < 0.0001) and transgenic (r = -0.95, P < 0.0001) fibers.

Fig. 5. Relationship between phosphorylase and glycogen in control and GSL30 fibers. Phosphorylase is plotted against glycogen measured in the same fibers. The correlation coefficient (r = 0.80, P < 0.0001) was determined by linear regression analysis.

Fig. 6. Reduced uridine diphosphoglucose (UDPG) levels in IIB and IIA/IID fibers from transgenic muscles. UDPG was measured in the fibers analyzed in Fig. 1. Each value is plotted vs. MDH activity measured in the same fiber.

Fig. 7. Crossover analyses of the glycogen synthesis pathway in IIB and IIA/IID fibers. Metabolites in pathway leading to synthesis of glycogen were measured in the individual fibers analyzed in Fig. 1. G6P, glucose 6-phosphate (G-6-P); G1P, glucose 1-phosphate (G-1-P). Results from transgenic IIB and IIA/IID fibers are means ± SE and are expressed as percentages of values measured in corresponding types of wild-type fibers. Levels of metabolites (nmol/mg dry weight) in control IIB fibers and control IIA/IID fibers, respectively, were as follows: glucose, 5.3 ± 0.5, 5.7 ± 0.7; G-6-P, 1.4 ± 0.2, 0.87 ± 0.2; G-1-P, 0.28 ± 0.06, 0.16 ± 0.4; UDPG, 0.071 ± 0.003, 0.099 ± 0.007; and glycogen, 104 ± 7, 111 ± 15.
fibers (15). The following proportions of fibers were found in EDL muscles: 1% type I, 12% type IIA, 68% type IIB, and 19% type IID fibers (15). In mouse diaphragm the proportions were as follows: 1% type I, 56% type IIA, and 35% of a group containing IIB and IID fibers (31). Although the IIB and IID fibers were not specifically identified by the myosin heavy-chain antibodies used in this study (31), it is likely that very few IIB fibers were present, because almost all fibers in the mouse diaphragm appear to be oxidative types (8).

GS activities in muscles incubated in vitro. Transgenic overexpression of GS driven by the muscle creatine kinase regulatory gene cassette is highest in the EDL, followed by the diaphragm and soleus muscles (27). This expression pattern may be seen by comparing the total GS activities measured after in vitro incubations of muscles from wild-type animals and two lines of transgenic mice (Fig. 8).

GS is activated by dephosphorylation, which increases the $-G-6-P/+/G-6-P$ activity ratio. Monitoring changes in the activity ratio provides a convenient means for monitoring the activation of GS by insulin. In the three muscles investigated in the present study, the diaphragm was most responsive to insulin. In wild-type diaphragm muscles, insulin increased the activity ratio by fourfold. When incubated without insulin, the activity ratios were higher in the transgenic diaphragm (Fig. 8A) and EDL muscles (Fig. 8B) than in the respective wild-type muscles. As discussed previously (27), this is most likely due to the fact that sites 2 and 3a in the transgenic GS had Ser to Ala mutations, which limit phosphorylation and inactivation of the enzyme. The activity ratios in the transgenic soleus muscles were not significantly different from those in the wild-type soleus muscles (Fig. 8C), likely reflecting the lower level of expression of the transgene. GS is a multimeric enzyme, and so hybrids can form between mutant and wild-type subunits of the enzyme. Because it is thought that phosphorylated subunits negatively affect unphosphorylated neighbors (44), the relative level of expression of the mutant enzyme is important in determining the activity ratio.

There is evidence that dephosphorylation of sites 2 and 3a is involved in the activation of GS by insulin in rat diaphragm (22). Results obtained with mouse diaphragms are consistent with this view, because the effect of insulin on increasing the activity ratio was less in muscles from GSL30 animals than in muscles from wild-type animals (Fig. 8A). Findings in the EDL were less clear-cut, possibly because the effect of insulin was less pronounced in the wild-type EDL than in the diaphragm (Fig. 8B). However, no indication of diminished activation of GS was apparent in the transgenic soleus muscles (Fig. 8C). An implication of these results is that the activation of GS in response to insulin may involve dephosphorylation of additional sites.

Effect of insulin on 2-deoxyglucose uptake in control and transgenic muscles incubated in vitro. Glucose transport was assessed by measuring the initial rate of 2-deoxyglucose uptake. Basal rates of uptake were approximately equal in diaphragm, EDL, and soleus muscles from wild-type animals (Fig. 9). The effect of insulin on increasing glucose transport in these muscles was most pronounced in the diaphragm, where the hormone produced an eightfold increase in the initial rate of 2-deoxyglucose uptake (Fig. 9A). Insulin increased transport activity by $\sim 2$-fold and $\sim 2.5$-fold in the EDL (Fig. 9B) and soleus muscles (Fig. 9C), respectively. Insulin-stimulated rates of 2-deoxyglucose uptake in muscles from GSL3 and GSL30 mice were not significantly different from the rates in muscles from wild-type animals, and basal rates of transport were not higher in any of the transgenic muscles than in

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**Fig. 8.** Glycogen synthase total activities and activity ratios after in vitro incubation of diaphragm, extensor digitorum longus (EDL), and soleus muscles from wild-type and transgenic mice with insulin. Muscles from wild-type, GSL3, and GSL30 mice were incubated for 45 min in DMEM to remove endogenous hormones, then transferred to Krebs-Henseleit buffer containing 5 mM glucose, and finally incubated at 37°C without insulin or with 20 mU/ml insulin. After 30 min muscles were frozen in liquid nitrogen. GS activities were measured in muscle extracts in both the absence and the presence of 10 mM G-6-P. Activity ratios ($-G-6-P/+G-6-P$) of synthase from control and insulin-treated muscles and total GS activities are presented. Values for total glycogen synthase activities (in nmol·min$^{-1}$·mg wet wt$^{-1}$) obscured by insulin-stimulated activity ratio bars were as follows: insulin-treated wild-type EDL, 1.08 ± 0.18; insulin-treated wild-type soleus, 1.16 ± 0.13; and insulin-treated GSL3 soleus, 1.54 ± 0.31. Results for activity ratios and total activities are mean values ± SE from 5 experiments.
wild-type muscles. In EDL muscles from GSL 30 mice, basal 2-deoxyglucose uptake was significantly less than the basal uptake in wild-type EDL muscles (Fig. 9B).

Increased glucose incorporation into glycogen in transgenic muscles incubated in vitro. To investigate the effect of the transgene on glycogen synthesis, [U-14C]glucose incorporation into glycogen was measured in diaphragm, EDL, and soleus muscles from GSL 3 animals incubated with increasing concentrations of insulin (Fig. 10). In all three muscles and at all insulin concentrations, the mean values for [14C]glycogen synthesis were higher in the GSL 3 muscles than in muscles from wild-type animals, although statistical significance was not achieved in all cases. The greatest effect of insulin was observed in diaphragm muscles (Fig. 10A), where [14C]glycogen synthesis was increased 24-fold by 2 mU/ml insulin. The difference between the wild-type and transgenic muscles reached statistical significance at the 20 µU/ml concentration, where the rate in the transgenic diaphragms was 2.5-fold higher than in the wild-type muscles. In EDL muscles from wild-type mice, [14C]glycogen synthesis was increased fivefold by 2 mU/ml insulin (Fig. 10B). The rates of [U-14C]glucose incorporation into glycogen observed either without insulin or with 20 µU/ml insulin were approximately twofold higher in the GSL 3 muscles than in muscles from wild-type mice. [14C]glycogen synthesis in the GSL 3 soleus was approximately twice that observed in the wild-type soleus when muscles were incubated with 200 µU/ml insulin (Fig. 10C).
DISCUSSION

An important conclusion from the present study is that increasing glycogen synthase enhances glycogen synthesis in both the glycolytic and oxidative types of fast-twitch fibers. This conclusion is supported by results obtained from microanalytical measurements of glycogen and metabolites in single fibers derived from control and transgenic muscles that had been freeze-clamped in situ and from measurements of the rates of 2-deoxyglucose uptake and glucose incorporation into glycogen in muscles incubated in vitro.

Transgenic overexpression of glycogen synthase was associated with increased glycogen accumulation in both the IIB and the IIA/IID groups of fibers from anterior tibialis muscles (Fig. 3). Although we did not attempt to distinguish between IIA and IID fibers, in any grouping of fibers of approximately equal oxidative capacity, as reflected by MDH activity, glycogen was higher in the transgenic fibers. Because approximately equal numbers of IIA and IID fibers should have been present in the population of fibers that we analyzed (15), it seems likely that the transgene increased glycogen in both of these types of fibers, although to a lesser degree than in type IIB fibers. In addition, the UDPG concentrations were decreased in all groupings of fibers of nearly equal MDH activities, consistent with increased conversion of UDPG to glycogen as a result of increased glycogen synthase (Fig. 6). Expression of the transgene did not increase basal glucose transport or enhance insulin-stimulated glucose transport in diaphragm, EDL, or soleus muscles incubated in vitro (Fig. 9). Therefore, we infer that the increased glycogen synthase activity, rather than an increase in glucose transport, accounted for the increase in glycogen accumulation.

In the present study we also sought to identify fiber-specific differences in glycogen metabolism in response to transgene expression by performing cross-over analysis of the precursors in the synthetic pathway leading to glycogen. Crossover plots for the IIB and IIA/IID groups were remarkably similar, and in both, crossover occurred between G-1-P and UDPG (Fig. 7). Thus, with overexpression of glycogen synthase, UDPG pyrophosphorylase became more rate determining for glycogen synthesis in both the IIB and IIA/IID groupings of fibers. The major differences between the two groups were the greater effects of the transgene on increasing glycogen and on decreasing UDPG in the IIB fibers. It seems reasonable to conclude that the higher level of transgenic glycogen synthase observed in the IIB fibers contributed to these differences (Fig. 2).

Increased glucose transport would increase glycogen precursors, including UDPG, and oxidative fibers contain more GLUT-4 than IIB fibers (19). Thus the higher overall capacity for glucose uptake might partly explain why UDPG concentrations were not decreased as much by the transgene in the IIA/IID fibers as in IIB fibers. However, by the same token, the greater glucose transport capacity of IIA/IID fibers would not explain why these fibers accumulated less glycogen than IIB fibers in response to overexpression of glycogen synthase, again indicating that glycogen levels and glucose transport are not always strictly correlated.

As with all transgenic animal models, there is the issue of adaptive changes that accompany expression of the transgene. In our previous analyses of whole muscles from the overexpressing mice (27), we had noted that increased levels of glycogen synthase were accompanied by an increase in the amount of phosphorylase. Another secondary effect resulting from expression of the transgene is the hyperaccumulation of glycogen, which itself has been viewed as a possible regulator of metabolic processes. For example, Danforth (3) described many years ago an inverse correlation between glycogen synthase activity ratio and the amount of glycogen in muscle. It is interesting to speculate that increased glycogen may serve as a signal for increasing phosphorylase. The significant correlation observed between levels of glycogen and phosphorylase activity across fiber types would be consistent with this type of regulation (Fig. 5). One hypothesis is that glycogen upregulates expression of the phosphorylase gene, but in principle glycogen could act either by increasing the synthesis of phosphorylase or by decreasing the rate of phosphorylase degradation. Decreasing the spontaneous contractile activity of myotubes with TTX, which would be expected to increase glycogen stores by reducing energy expenditure of the cells, markedly decreased the rate of phosphorylase degradation (23). Regardless of the mechanism by which phosphorylase is increased in the transgenic fibers, it is unlikely that it is the increase in phosphorylase, which degrades glycogen, that leads to increased glycogen accumulation.

There have also been suggestions that glycogen levels could feed back to inhibit glucose transport (7, 12, 16). In the present study, basal 2-deoxyglucose uptake was significantly lower in EDL muscles from GSK-30 mice than in control muscles (Fig. 9). This result was not unexpected, because we had previously found that GLUT-4 levels in the transgenic EDL muscles were ~50% lower than in control muscles (27). Thus our results support the view that accumulated glycogen may decrease glucose transport, at least under certain conditions. However, the existence of a secondary effect of glycogen to inhibit glucose transport would not appear to affect our interpretation that glycogen accumulation in the transgenic animals is due to the increase in glycogen synthase activity, because a decrease in transport would be expected to limit glycogen accumulation by reducing the availability of substrate for glycogen synthesis.

When muscles were incubated in vitro with $[^{14}C]$glucose, the rate of $[^{14}C]$glycogen synthesis was higher in the transgenic EDL than in the wild-type EDL (Fig. 10B). This finding provides additional support for the conclusion that elevated glycogen synthase increases glycogen synthesis in IIB fibers, as these fibers account for the large majority of fibers in the EDL. Likewise, the finding of increased $[^{14}C]$glycogen synthesis in diaphragm muscles (Fig. 10A) strengthens the conclusion that increasing glycogen synthase increases glu-
cose transport in IIA and IID fibers, because the diaphragm is composed primarily of these fiber types. In the presence of insulin, [14C]glycogen synthesis was also increased in the transgenic soleus muscles. However, this increase could have occurred in either the type I or IIA fibers that comprise this muscle. Because single fiber analyses of type I fibers were not performed in the present study, the effect of the transgene in type I fibers remains to be established.

Although we believe that the present results strongly support the conclusion that activation of glycogen synthase by insulin has an important role in the stimulation of glycogen synthesis, we certainly do not mean to imply that glucose transport is unimportant. There is strong evidence that glucose transport is the principal rate-determining step for glucose metabolism under basal conditions, as there appears to be very little free glucose in resting skeletal muscle fibers. Under these conditions, increasing glycogen synthase could not increase net glucose uptake by increasing the driving force for the facilitative diffusion of glucose into the muscle fiber. Thus any increase in glycogen synthase would come at the expense of other pathways involved in glucose utilization. This could explain why transgenic overexpression of glycogen synthase did not increase the basal rate of glucose incorporation into glycogen in diaphragms and soleus muscles (Fig. 10, A and C). On the other hand, glucose transport did not appear to be strictly rate limiting for glycogen synthase in EDL muscles even under basal conditions, as the rate of glycogen synthase was increased in response to the increase in glycogen synthase in muscles incubated without insulin (Fig. 10B). In the presence of insulin, more glucose becomes available for glycogen synthase as a result of increased glucose transport, and transgenic overexpression of glycogen synthase increased the rate of [U-14C]glucose incorporation into glycogen in all three muscles (Fig. 10).

Results obtained using transgenic animal models in which either GLUT-1 or GLUT-4 levels were manipulated suggested that the rate of glucose entry into cells exerted an important influence on glycogen accumulation (10, 32). Shulman et al. (38) came to similar conclusions through the application of metabolic control analysis. There is no contradiction between our results and those just cited, because more than one step in a multistep metabolic pathway can contribute to determining the flux (6). Indeed, from a theoretical analysis of glycogen metabolism by use of metabolic control analysis, Schulz (36) reached the conclusion that the control of glycogen synthase is distributed among different steps that assume more or less importance depending on the physiological conditions. More recently, Jucker and Shulman (17) analyzed rats during hyperinsulinemic clamp and used metabolic control analysis to apportion the control of glucose disposal among glucose transport, glycogen synthase, and glycolysis. The result was that glucose transport/phosphorylation had a control coefficient of 0.55 and glycogen synthase that of 0.30, meaning that both steps substantially affect glucose disposal. It is interesting to compare these coefficients with the effects of insulin on increasing glucose transport and glycogen synthase activity. In the present experiments, the largest effects of insulin were observed in the diaphragm muscles. Incubating hemidiaphragms with a maximally effective concentration of insulin increased the rate of [14C]glycogen incorporation into glycogen by 24-fold (Fig. 10A), an effect much greater than either the increase in 2-deoxyglucose uptake (Fig. 9A) or the increase in 4-fold in the G-6-P/G-6-P activity ratio of glycogen synthase (Fig. 8A). These results are consistent with the view that the stimulation of glycogen synthesis involved increases in both glucose transport and glycogen synthase.

In conclusion, control of glycogen accumulation by insulin is distributed between transport and synthesis, and increasing either in skeletal muscle leads to increased glycogen accumulation in vivo. The activation of both glucose transport and glycogen synthase by insulin ensures the efficient storage of glucose as glycogen, and thus defects in either may compromise the process of glycogen synthesis.

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