Biochem. J. (1991) 278, 113–117 (Printed in Great Britain)

The expression of glycogen phosphorylase in normal and dystrophic muscle

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Specific cofactor labelling was employed to determine the degradation rate of glycogen phosphorylase in normal adult C57BL/6J mice and their dystrophic counterparts (C57BL/6J$^{dy/dy}$). The rate constant for the decay of phosphorylase-bound label was 0.125 day^{-1} in normal muscle and 0.049 day^{-1} in dystrophic muscle, i.e. a lower rate of catabolism of phosphorylase in dystrophic muscle. Quantitative Northern-blot analyses of total RNA isolated from normal and dystrophic muscle indicated that the abundance of phosphorylase mRNA as a percentage of total RNA was approx. 40% lower in dystrophic muscle. The specific activity of phosphorylase in dystrophic muscle is approx. 60% lower than in normal muscle, and is elicited by a lower rate of turnover of the enzyme, i.e. both synthesis and degradation are decreased.

INTRODUCTION

Hereditary muscular dystrophy of the mouse (*dy/dy*) is an autosomal recessive disease characterized by muscle fibre necrosis. To compensate for necrosis some regeneration occurs, but this process is insufficient to replace muscle fibre loss. The steady-state level of any protein is dependent on the opposing processes of synthesis and degradation. Since the consequence of muscular dystrophy is muscle wastage, degradation must ultimately predominate over synthesis. However, some reports have claimed that overall protein synthesis is elevated in dystrophic mouse muscle (Garber *et al.*, 1980; Srivastava, 1969); an increased rate of protein turnover was determined by monitoring the loss of a radioactive tracer in normal and dystrophic muscle proteins (Simon *et al.*, 1962). Both the initial rate and the extent of amino acid incorporation were higher in cell-free systems containing polyribosomes isolated from dystrophic muscles (Srivastava, 1969; Nwagwu, 1975). Differences in the protein-synthetic capacity between normal and dystrophic muscle were abolished when the extent of incorporation was normalized to the precursor amino acid pool size (Watts & Reid, 1969; Kitchen & Watts, 1973), although the initial rate of amino acid incorporation into both total and soluble protein pools was much faster in dystrophic (129$^{dy/dy}$) mice.

An increased rate of protein degradation was demonstrated in both the myofibrillar and the sarcoplasmic fraction of several hind-limb muscles of dystrophic (*dy*^{2J}) mice by double-isotope-incorporation experiments (Goldberg *et al.*, 1977). Increased catabolism of sarcoplasmic proteins occurred in C57BL/6J$^{dy/dy}$ mice when 3-methylhistidine was used as an endogenous tracer to measure actin and myosin degradation rates (Mizobuchi *et al.*, 1985). In common with human Duchenne and avian muscular dystrophy, fast-twitch fibres are more affected by the disease process in mouse muscle than are the slow-twitch fibres (Lebherz, 1984; Petell *et al.*, 1984; Webster *et al.*, 1988). A decrease in the amount of several abundant glycolytic enzymes characteristic of 'fast'-twitch fibres occurs in skeletal muscle of C57BL/6J$^{dy/dy}$ mice (Kitahara *et al.*, 1977; Petell *et al.*, 1984). The specific activity of three enzymes involved in glycogen catabolism (phosphorylase, enolase and aldolase) were 35–50% lower in the hind-limb muscles of dystrophic mice as compared with normal mice. Short-term double-isotope-incorporation experiments conducted with muscle explants *in vitro* showed similar relative rates of synthesis of these enzymes, and of most other abundant cytosolic proteins in hind-limb muscles of both normal and dystrophic mice (Petell *et al.*, 1984). Changes in protein turnover in dystrophic muscle which lead to a decrease in catalytic activity are not clear.

The degradation of glycogen phosphorylase in mouse skeletal muscle has been measured by using the cofactor pyridoxal 5'-phosphate (PLP) as a specific label (Butler *et al.*, 1985; Cookson & Beynon, 1989; Leyland *et al.*, 1990). Labelling of the cofactor pool with a radiolabelled precursor of PLP leads to an accumulation of label in phosphorylase. The tight association between PLP and phosphorylase allows the cofactor to be released only by proteolysis of the protein, and thus the rate of loss of phosphorylase-bound label is an index of the rate of catabolism of the enzyme. In this investigation we have measured the turnover parameters of glycogen phosphorylase in adult normal and dystrophic mouse skeletal muscle. The model used in this study is the C57BL/6J$^{dy/dy}$ mouse, for which the primary defect is unknown. Unlike the *mdx* dystrophic mouse, which lacks dystrophin and which shows early muscle damage, followed by recovery, the *dy/dy* mutant undergoes progressive and extensive muscle atrophy in the hind-limb and back muscles. As such, it is a more severe model system to monitor muscle wasting.

MATERIALS AND METHODS

Experimental animals

Adult C57BL/6J inbred mice of either sex were obtained from the Departmental animal unit. Animals were maintained on a 12 h light/12 h dark cycle and allowed free access to food and water. Owing to the limited number of dystrophic animals, adult mice of different ages were used. The normal (C57BL/6J$^{+/+}$) mice were littermates aged 7 months; the dystrophic (C57BL/6J$^{dy/dy}$) mice were aged between 6 and 10 months.

Antibodies

A monoclonal antibody to PLP, E6(4)1, prepared by Viceps-Madore *et al.* (1983), was generously provided by Dr. J. A. Cidlowski, Department of Physiology, Biochemistry and Nutrition, University of North Carolina, Chapel Hill, NC,

Abbreviation used: PLP, pyridoxal 5'-phosphate.
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U.S.A. Ascites fluid was used as a source of IgG without further purification. Rabbit anti-mouse horseradish peroxidase-conjugated immunoglobulins were purchased from Dako Ltd., High Wycombe, Bucks., U.K.

Materials

[α-^{32}P]dCTP (110 TBq/mmol), uridine 5'-[α-^{35}S]thiotriphosphate (15.2 TBq/mmol) and [^{3}H]pyridoxine hydrochloride (49.2 GBq/mmol) were from Amersham International, Amersham, Bucks, U.K. Liquid-scintillation counting of radioisotopes used OptiPhase 'Safe' obtained from Pharmacia–LKB, Milton Keynes, U.K. Autoradiography was on X-ray film (NIF-RX) purchased from Fuji, London N.W.3, U.K. *Pst*1 restriction endonuclease was purchased from BCL, Uxbridge, Middx., U.K. Hybond-N nylon membranes were from Amersham. Nitrocellulose (pore size 0.2 μm) was from Schleicher and Schuell, Dassel, Germany. Sephadex G-25 (fine grade) and G-50 (medium grade) and 5'-AMP–Sepharose 4B were from Pharmacia. All other chemicals were of analytical grade.

Assays

Phosphorylase was assayed in the direction of glycogen synthesis by monitoring the release of P_i from glucose 1-phosphate (Carney *et al.*, 1978). Protein was assayed with a dye-binding assay (Bio-Rad Laboratories, Watford, Herts., U.K.).

Determination of the degradation rate of glycogen phosphorylase

The animals received a subcutaneous injection of 1.85 MBq (50 μCi) of [^{3}H]pyridoxine in 0.9% (w/v) NaCl (0.1 ml). The precursor pools of radioactivity were allowed to decline for a period of 12 days after radioisotope administration. At various times after this the animals were killed, and hind-leg and back muscle were dissected out and homogenized in 9 vol. of ice-cold 5 mM-Hepes/1 mM-EDTA/14 mM-2-mercaptoethanol, pH 7.5. The homogenate was centrifuged at 14800 *g* for 15 min and filtered through a glass-fibre filter to remove particles of the floating fatty pellicle.

AMP–Sepharose affinity chromatography of muscle supernatants was at room temperature on a column (V_t = 2.5 ml) previously equilibrated in a buffer consisting of 5 mM-Hepes, 1 mM-EDTA and 14 mM-2-mercaptoethanol, pH 7.5. Supernatant (1 ml) was applied to the column, and a flow rate of 12 ml/h was maintained. After 7 fractions each of volume 1 ml had been collected, the buffer was modified to include 10 mM-AMP, and elution was continued. Radioactivity in each fraction was determined by counting 0.8 ml of each fraction in 10 ml of scintillant. Sephadex G-25 chromatography was performed at room temperature on a column (V_t = 28.5 ml) previously equilibrated in 5 mM-Hepes/1 mM-EDTA/14 mM-2-mercaptoethanol, pH 7.5. Supernatant (1 ml) was applied to the column, and a flow rate of 20 ml/h was maintained; 1 ml fractions were collected, and the radioactivity was measured by counting 0.8 ml of each fraction in 10 ml of scintillant. Recoveries of protein/radioactivity from both columns were greater than 95%.

The data describing the decay of protein-bound label were analysed as a single-exponential decay curve of the form $A_t = A_0 \cdot \exp(-kt)$, where A_t represents the total radioactivity at time t, A_0 is the value at $t = 0$ and k represents the first-order rate constant for the decay of the pool. The equation was fitted to the data by using a non-linear curve-fitting method to a parameter accuracy of better than 0.1% by using the program P.Fit (Biosoft, Cambridge, U.K.). The data were corrected for body weight of the animal to give a constant dose of radioactivity for the muscle mass. This software yields both errors on fitted parameters and 95% confidence intervals on the fitted curve.

Western blots of SDS/polyacrylamide gels

The technique of Western blotting of SDS gels on to nitrocellulose membranes has been described previously (Leyland *et al.*, 1990).

Isolation of RNA

Mouse hind-leg and back muscle was dissected out and homogenized in 10 vol. of a buffer comprising 0.1 M-Tris, pH 8, 10 mM-EDTA, 1% (w/v) LiCl, 0.5% (w/v) SDS, 6% (v/v) phenol [phenol was redistilled and equilibrated with 0.1 M-Tris(pH 8)/10 mM-EDTA; 0.1% (w/v) 8-hydroxyquinoline was added as an anti-oxidant]. An equal volume of phenol/chloroform (1:1, v/v) was immediately added and mixed vigorously until an emulsion formed. The aqueous and organic phases were separated by centrifugation at 16000 *g* for 10 min (Sorvall RC-2B, HB-4 rotor). The aqueous phase was pipetted off and the organic phase was re-extracted with an equal volume of buffer. The pooled aqueous phases were again extracted with an equal volume of phenol/chloroform (1:1, v/v). This was followed by extraction with chloroform alone until a clear interface was obtained. Total RNA was precipitated from the aqueous phase by addition of 0.1 vol. of 3 M-sodium acetate, pH 5.3, and 2.5 vol. of ethanol at −20 °C. Precipitated RNA was recovered by centrifugation at 20000 *g* for 15 min (HB-4 rotor) and was dissolved in water (pre-treated with 0.2% diethyl pyrocarbonate). RNA recoveries were monitored by addition to the homogenate of RNA labelled with uridine 5'-[α-^{35}S]thiotriphosphate by transcription *in vitro* of a 382 bp *Xenopus laevis* histone sequence cloned into a pSP64 vector, prepared by a modification of the method of Melton *et al.* (1984).

Electrophoresis of RNA through formaldehyde/agarose gels, Northern-blot hybridization and quantification of membrane-bound probe were as described previously (Leyland *et al.*, 1990). A rat muscle phosphorylase cDNA clone was used to prime the synthesis of a ^{32}P-labelled probe by random-primed DNA labelling (Feinberg & Vogelstein, 1983).

RESULTS AND DISCUSSION

Phosphorylase levels in normal and dystrophic muscle supernatants

Glycogen phosphorylase activity was assayed in 14800 *g* muscle supernatants prepared from normal and dystrophic muscle homogenates. The mean specific activity (±S.E.M.) of phosphorylase in dystrophic muscle was 25±2.5 nkat/mg, compared with a mean value of 60±5.6 nkat/mg measured in normal muscle supernatants. The difference in specific activities between the two groups was statistically significant by Student's unpaired t test ($P < 0.001$, $n = 13$).

Determination of the degradation rate of glycogen phosphorylase

After administration of [^{3}H]pyridoxine, the precursor pools of radiolabel were allowed to decay for a period of 12 days. After this time free pools of label attain low steady-state levels, and the rate of decay of protein-bound label is largely due to the decay of phosphorylase-bound label, with a minor contribution from other PLP-binding proteins (Cookson & Beynon, 1989). Fig. 1 represents the decay of protein-bound label in normal and dystrophic mouse skeletal muscle. The rate constant for the decay of phosphorylase-bound label (±S.E.M.) was 0.125±0.022 day^{-1} ($t_{\frac{1}{2}}$ = 5.5 days) for normal muscle and

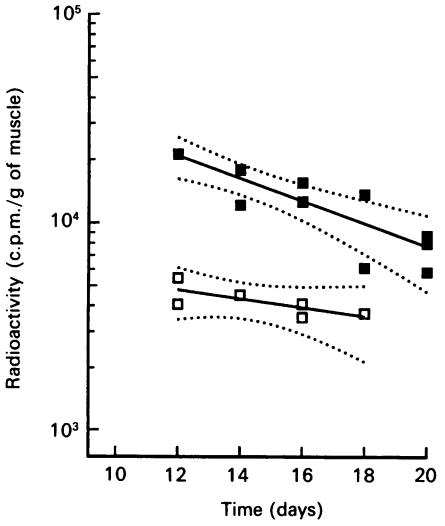

Fig. 1. Degradation of glycogen phosphorylase in normal and dystrophic muscle

Groups of normal and dystrophic C57BL/6J mice were each injected subcutaneously with 50 μCi of [^{3}H]pyridoxine. Between 12 and 20 days after injection the animals were killed and hind-leg and back muscle was dissected out and homogenized in 9 vol. of 5 mM-Hepes/1 mM-EDTA (pH 7.5)/14 mM-2-mercaptoethanol at 4 °C. The homogenates were centrifuged at 14800 *g* for 15 min, and 1 ml of supernatant was applied to a 5'-AMP–Sepharose column as described in the Materials and methods section. Radioactivity was determined in the phosphorylase pool and expressed as c.p.m./g wet wt. of tissue. The line represents the decay of label obtained by non-linear curve fitting of the untransformed data, and the dotted lines bordering each data set represent the 95% confidence limits for the fitted curve. Key: normal (■; $n = 10$), dystrophic (□; $n = 7$). Each data point represents a single animal.

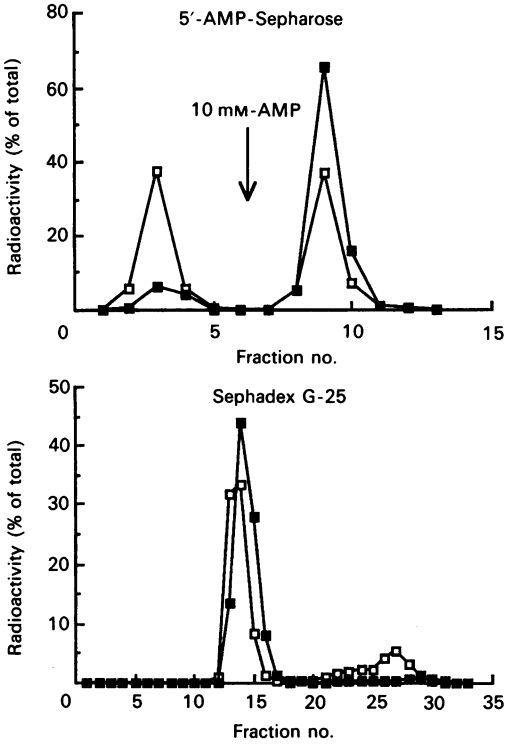

Fig. 2. Distribution of radioactivity in normal and dystrophic mouse skeletal muscle

Two normal and two dystrophic mice were each injected with 200 μCi of [^{3}H]pyridoxine. At 12 days after injection, muscle supernatants were prepared and chromatographed on 5'-AMP–Sepharose and Sephadex G-25 as described in the Materials and methods section. The elution profiles show the mean value for each fraction calculated from chromatographic runs performed with two normal and dystrophic supernatants. The peak values for the duplicate runs were within 90% of each other. Key: normal (■); dystrophic (□).

0.049 ± 0.024 day^{-1} ($t_{\frac{1}{2}} = 14.1$ days) for dystrophic muscle. Fig. 1 shows the 95% confidence limits for the fitted curves. Thus phosphorylase is degraded more slowly in dystrophic muscle, a result that is at variance with a preliminary study that indicated an increased rate of catabolism of phosphorylase in C57BL/6J$^{dy/dy}$ mice (Butler *et al.*, 1985). In those earlier experiments, however, protein-bound radioactivity was separated from low-molecular-mass/free radioactivity by gel filtration on Sephadex G-25. Protein-bound radioactivity was taken as being equivalent to phosphorylase-bound radioactivity, a valid assumption for normal muscle, as over 90% of PLP in normal skeletal muscle is bound to phosphorylase. However, Western blots of dystrophic muscle supernatants probed with the anti-PLP monoclonal antibody indicated that the proportion of PLP bound to phosphorylase was less than in normal muscle (Fig. 3). Thus protein-bound radioactivity cannot be interpreted as being equivalent to phosphorylase-bound radioactivity in this abnormal state.

The distribution of radiolabel in normal and dystrophic muscle was examined further. A pair of normal mice and a pair of dystrophic mice were each injected subcutaneously with 7.4 MBq (200 μCi) of [^{3}H]pyridoxine. At 12 days after injection, the animals were killed and muscle supernatants were prepared and chromatographed on Sephadex G-25 and AMP–Sepharose (Fig. 2). Table 1 shows the distribution of radioactivity between the phosphorylase pool (AMP–Sepharose-bound), the non-phosphorylase protein pool (the difference in radioactivity between G-25-excluded and AMP–Sepharose-bound) and the low-molecular-mass pool (G-25-included) calculated for both normal and dystrophic muscle supernatants. In normal muscle approx. 90% of the protein-bound label was bound to phosphorylase; however, in dystrophic muscle approx. 50% of the label was associated with phosphorylase and 25% with non-phosphorylase protein, putatively the aminotranferases. Thus total protein-bound label derived from gel filtration of muscle supernatants on Sephadex G-25 would not be equivalent to phosphorylase-bound label in dystrophic muscle. The present data, describing the decay in radioactivity in affinity-purified phosphorylase, should therefore be taken as more accurate.

Sarcoplasmic proteins isolated from normal and dystrophic muscle were subjected to SDS/PAGE. Prior treatment with sodium cyanoborohydride was necessary to reduce the Schiff-base linkage of the cofactor to the protein (Leyland *et al.*, 1990); this reduction step causes a shift in mobility of phosphorylase to a position of higher molecular mass. The gel was electroblotted on to nitrocellulose and probed with a monoclonal antibody to the cofactor PLP (Fig. 3). The blot shows decreased levels of PLP-binding protein in the position of phosphorylase in dystrophic muscle (Fig. 3). The anti-PLP antibody also identified two other proteins at 40–45 kDa, putatively aminotransferases, that also bind PLP, and the levels of these proteins are slightly increased in dystrophic compared with normal muscle. Both normal and dystrophic skeletal muscle were homogenized in 10 vol. of buffer, and equal volumes of the muscle supernatants were applied to the gel. Therefore dystrophic muscle contains less phosphorylase per wet weight of tissue than normal muscle, consistent with the lower specific activity. The levels of

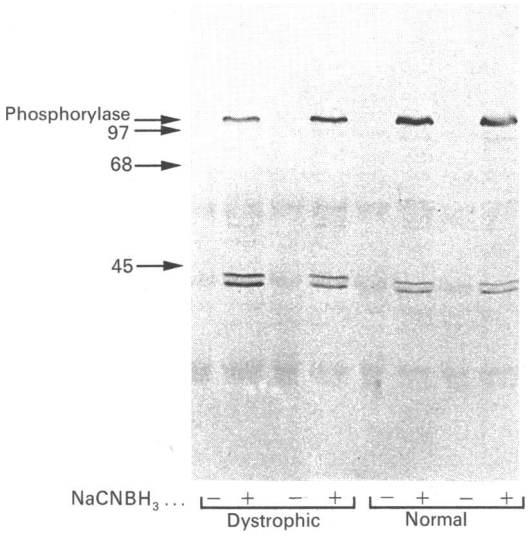

Fig. 3. Association of PLP with skeletal-muscle proteins in normal and dystrophic muscle

Hind-leg and back muscle were dissected from a pair of normal C57BL/6J$^{+/+}$ and dystrophic C57BL/6J$^{dy/dy}$ mice. The muscle from each animal was homogenized in 9 vol. of 5 mM-Hepes/1 mM-EDTA (pH 7.5)/14 mM-2-mercaptoethanol at 4 °C. The homogenates were centrifuged at 14800 *g* for 15 min and the supernatants were filtered through a glass-fibre filter. The samples indicated by (+) were treated with $NaCNBH_3$ before SDS/PAGE to stabilize the aldimine linkage between cofactor and protein. The gel was electroblotted on to nitrocellulose and probed with a monoclonal antibody to PLP. Positions of molecular-mass markers are indicated in kDa (97, phosphorylase; 68, albumin; 45, ovalbumin).

Table 1. Partitioning of radioactivity into the muscle vitamin B-6 pools of normal and dystrophic mice following subcutaneous injection of [^{3}H]pyridoxine

Two normal and two dystrophic mice were each injected with 200 μCi of [^{3}H]pyridoxine. At 12 days after injection, muscle supernatants were prepared and chromatographed on 5′-AMP–Sepharose and Sephadex G-25 as described in the Materials and methods section. Radiolabel associated with the phosphorylase (AMP–Sepharose-bound), non-phosphorylase protein pool (G-25 excluded – AMP–Sepharose-bound) and the low-molecular-mass pool (G-25 included) was calculated by using combined data from chromatography on both columns.

Animal	Distribution of radioactivity (c.p.m./g of muscle; % total)		
	Total protein-bound	Phosphorylase-bound	Non-phosphorylase-protein-bound
C57BL/6J$^{+/+}$ normal			
Mouse 1	165499 (96%)	158360 (92%)	7139 (4%)
Mouse 2	117720 (95%)	112455 (91%)	5265 (4%)
C57BL/6J$^{dy/dy}$ dystrophic			
Mouse 1	20041 (77%)	14079 (54%)	5962 (23%)
Mouse 2	17401 (75%)	11922 (51%)	5479 (24%)

the two other PLP-binding proteins emphasize a decrease in phosphorylase levels in dystrophic muscle and an altered distribution of PLP.

In the degradation study, the phosphorylase-associated radioactivity can be back-extrapolated to zero time, which gives a very approximate indication of the relative rates of synthesis of the

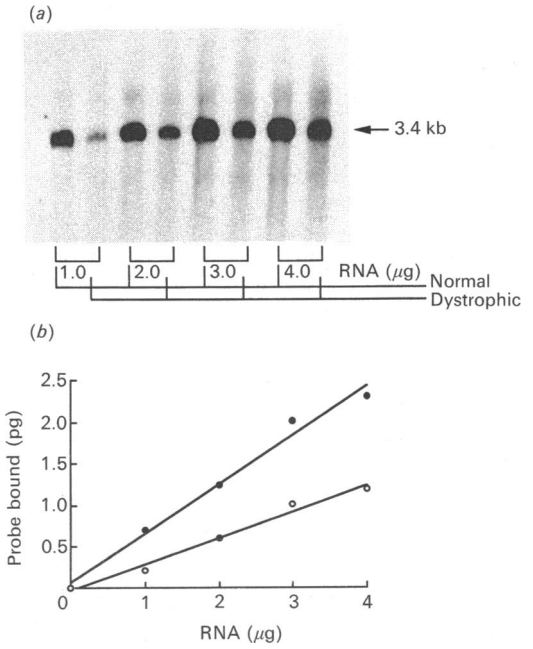

Fig. 4. Quantitative Northern-blot analysis of phosphorylase mRNA in normal and dystrophic mouse skeletal muscle

(*a*) Total RNA was isolated from the hind-leg and back muscle of normal and dystrophic C57BL/6J mice. The RNA was size-fractionated on a formaldehyde/agarose gel, transferred to a nylon membrane and probed with a rat muscle phosphorylase cDNA probe. The specific radioactivity of the radiolabelled probe was 3×10^8 c.p.m./μg of DNA. (*b*) Membrane-bound probe was quantified by scintillation counting of the portion of the membrane corresponding to the region of hybridization. Data are corrected for background radioactivity on the filter. Key: normal (●); dystrophic (○).

enzyme in the normal and the mutant strain. Although these data should not be over-interpreted, it is apparent that, even when the data are corrected for the phosphorylase pool size, the enzyme is labelled to a lower degree in the dystrophic mouse, an observation that is consistent with a lower rate of synthesis and the lower amount of the enzyme.

Phosphorylase mRNA in normal and dystrophic muscle

Total RNA was isolated from normal and dystrophic mouse skeletal muscle and size-fractioned on formaldehyde/agarose gels. The gels were capillary-blotted on to nylon membranes and probed with radiolabelled probes generated by random priming of a rat muscle phosphorylase cDNA clone (Hwang *et al.*, 1985). Phosphorylase mRNA was approx. 3.4 kb in both normal and dystrophic muscle; no bands of smaller size were detected that would correspond to degradation products or other cross-hybridizing sequences (Fig. 4*a*). To obtain a quantitative estimate of the abundance of phosphorylase mRNA in normal and dystrophic muscle, membrane-bound probe was quantified by scintillation counting of the portion of membrane corresponding to the region of hybridization, which was identified by autoradiography and cut out for scintillation counting. These data were corrected for background radioactivity that was present on the membrane. In Fig. 4(*b*) the radioactivity associated with each hybridization band is expressed as weight of probe bound (pg), since the specific activity of the radiolabelled probe was known. The relationship between the amount of probe bound and the amount of total RNA (1–4 μg) loaded on to the original gel was linear ($r = 0.98$ and 0.97 for data for normal and

dystrophic muscle respectively). Phosphorylase mRNA levels in dystrophic muscle were approx. 60% of those measured in normal muscle. By comparison, the specific activity of phosphorylase was 40% of that in normal muscle. The decreased levels of phosphorylase mRNA in dystrophic muscle may be due to a decrease in transcription of the phosphorylase gene or enhanced degradation of the mRNA. Muscle phosphorylase transcripts of different sizes were found in rat soleus and extensor digitorum longus muscles (Gorin *et al.*, 1989). Although the Northern blots in the present study would not be able to reveal small differences in size of the mature phosphorylase mRNA, there is no evidence for dramatic size changes. Alkaline RNAase II activity was increased in the skeletal muscle of 129B6F1/J dystrophic mice (Little & Meyer, 1970), introducing the possibility of enhanced degradation of phosphorylase mRNA.

A close correspondence between muscle phosphorylase mRNA and the amount of the corresponding protein was demonstrated in adult rat tissues, from which it was inferred that a major level of control of muscle phosphorylase expression was at the level of maintenance of muscle phosphorylase mRNA (David & Crerar, 1986). However, in pathological muscle such controls may be lost. In C57BL/6J$^{dy/dy}$ dystrophic muscle there is an approx. 40% decrease in phosphorylase mRNA, a 60% decrease in phosphorylase protein and a 60% decrease in the rate of degradation of the enzyme. The decrease in translatable phosphorylase mRNA would contribute in part to a lowered rate of synthesis, which is supported by the lower value for back-extrapolated radioactivity (Fig. 1). Turnover of a protein can be represented in very simple terms by the equation $k_s = k_d \cdot [E]$, where k_s represents the rate of protein synthesis (a zero-order process), k_d represents the fractional rate of protein degradation (a first-order process), and [E] represents enzyme concentration. If the rate of synthesis k_s is considered to be a composite term; the product of mRNA level and the overall translational activity, it is possible to obtain a value for the ability of dystrophic muscle to translate the phosphorylase mRNA. Our data demonstrate that the overall translational capacity of dystrophic muscle is approx. 25% that of normal muscle, and that the muscle of adult C57BL/6J$^{dy/dy}$ mice is markedly down-regulated in terms of phosphorylase expression. Whether this down-regulation extends to other protein/mRNA couples remains to be established.

Received 11 January 1991/8 April 1991; accepted 17 April 1991

D.M.L. is grateful to the Muscular Dystrophy Group of Great Britain and Northern Ireland for the provision of a research studentship. We are indebted to Dr. M. Crerar, York University, Ontario, Canada, for the provision of a rat muscle phosphorylase cDNA clone. This work was also supported in part by a grant from the Medical Research Council (Grant No. G88/10631SB).

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