Adenovirus-mediated delivery into myocytes of muscle glycogen phosphorylase, the enzyme deficient in patients with glycogen-storage disease type V

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The feasibility of using adenovirus as a vector for the introduction of glycogen phosphorylase activity into myocytes has been examined. We used the C2C12 myoblast cell line to assay the impact of phosphorylase gene transfer on myocyte glycogen metabolism and to reproduce *in vitro* the two strategies proposed for the treatment of muscle genetic diseases, myoblast transplantation and direct DNA delivery. In this study, a recombinant adenovirus containing the muscle glycogen phosphorylase cDNA transcribed from the cytomegalovirus promoter (AdCMV-MGP) was used to transduce both differentiating myoblasts and non-dividing mature myotube cells. Muscle glycogen phosphorylase mRNA levels and total phosphorylase activity were increased in both cell types after viral treatment although more efficiently in the differentiated myotubes. The increase in phosphorylase activity was transient (15 days) in myoblasts whereas in myotubes

higher levels of phosphorylase gene expression and activity were reached, which remained above control levels for the duration of the study (20 days). The introduction of muscle phosphorylase into myotubes enhanced their glycogenolytic capacity. AdCMV-MGP-transduced myotubes had lower glycogen levels under basal conditions. In addition, these engineered cells showed more extensive glycogenolysis in response to both adrenaline, which stimulates glycogen phosphorylase phosphorylation, and carbonyl cyanide *m*-chlorophenylhydrazine, a metabolic uncoupler. In conclusion, transfer of the muscle glycogen phosphorylase cDNA into myotubes confers an enhanced and regulatable glycogenolytic capacity. Thus this system might be useful for delivery of muscle glycogen phosphorylase and restoration of glycogenolysis in muscle cells from patients with muscle phosphorylase deficiency (McArdle's disease).

INTRODUCTION

The mobilization of muscle glycogen stores provides the metabolic energy for muscular contraction. This glycogenolytic process is controlled by glycogen phosphorylase. Three different isoenzymes of glycogen phosphorylase have been identified in mammals: muscle [1], liver [2] and brain [3]. In fetal muscle, the brain isoenzyme is expressed, whereas the muscle form is induced in the late phases of muscle maturation [4,5]. The muscle form is the sole isoenzyme expressed in adult skeletal-muscle tissue. Muscle glycogen phosphorylase is regulated through two different mechanisms: phosphorylation of the enzyme by phosphorylase kinase to produce the active *a* form, and allosteric regulation. The phosphorylation mechanism is mainly controlled by extracellular factors, whereas allosteric regulators are signals of the intracellular energetic state (see ref. [6] for review). One of the key allosteric effectors of the muscle form is AMP, which potentially activates muscle phosphorylase *b* [7,8].

McArdle's disease (or type V glycogenosis) is a genetically determined metabolic myopathy characterized by a deficiency in muscle glycogen phosphorylase. As a result of the lack of this enzyme activity, muscle cells are unable to mobilize muscle glucose stores in response to exercise, and symptoms of myalgia, cramps, early fatigue and myoglobinuria develop after physical effort in affected patients [9–11]. Restoration of the glycogenolytic capacity of muscle cells from patients with McArdle's disease could conceivably be achieved by the restoration of muscle glycogen phosphorylase activity by means of gene augmentation.

Gene-therapy strategies for the correction of genetically inherited diseases in muscle include (i) direct delivery of the gene of

interest into intact muscle by injection [12–15] and (ii) the isolation of myoblasts from muscle tissue for genetic modification *ex vivo* and reimplantation [16,17]. Adenovirus has emerged as a very promising tool for mediating gene transfer into mammalian cells. Replication-deficient recombinant adenovirus has several advantages over other gene-transfer vehicles, such as its ability to mediate DNA transfer into non-proliferating cells and its capacity to deliver genes to a wide variety of animal cells [13,14,18–20]. Previous work has demonstrated that recombinant adenovirus is able to direct *in vivo* expression of *lacZ* reporter [13,15] or minidystrophin [14] genes in muscle tissue after intramuscular injection.

A description of adenoviral-mediated gene delivery into both differentiating myoblasts and non-proliferating multinucleated myotubes is presented. Our results demonstrate that adenovirus can mediate the efficient introduction of muscle phosphorylase into both cell systems, although higher and more persistent levels of expression and activity are reached when non-proliferating myotubes are infected instead of differentiating myoblasts. We also show that the introduction of muscle glycogen phosphorylase enhances myotube glycogenolytic capacity in a regulated way. This study represents a first approach to the therapy of McArdle's myopathy.

EXPERIMENTAL

Cell culture

C2C12 myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Whittaker). Differentiation into myotubes was

Abbreviations used: FBS, fetal bovine serum; HS, horse serum; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; DMEM, Dulbecco's modified Eagle's medium; 1 × SSC, 0.15 M NaCl/0.015 M sodium citrate; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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induced by incubating the cells in DMEM containing 10% horse serum (HS) (Gibco). Fused myotubes were observed 6 days after the medium change.

Construction of recombinant adenovirus AdCMV-MGP and cell infection

The construction of AdCMV-MGP has been described [18]. Briefly, a transcriptional unit formed by the cytomegalovirus promoter, a 2.56 kb fragment of the rabbit muscle glycogen phosphorylase cDNA including the entire coding region [1] and a fragment of the simian virus 40 genome including the polyadenylation signal, was inserted into the genome of a replication-defective deletion mutant of adenovirus type 5. The recombinant viruses were obtained by homologous recombination in 293 cells co-transfected with plasmid pACCMV-pLpA and plasmid PJM17. Viral stocks of 5×10^7 – 50×10^7 plaque-forming units/ml were prepared in 10% FBS/DMEM. Gene delivery into C2C12 myocytes was performed at different stages of differentiation by exposing the cells to stocks of recombinant adenovirus-expressing simian virus 40 T-antigen (Ad1613) or AdCMV-MGP, for 1 h at a multiplicity of infection of 5.

Preparation of cell extracts for enzyme activity assays

Extracts were prepared by scraping cell monolayers into a buffer consisting of 10 mM Tris/HCl (pH 7.0), 150 mM KF, 15 mM EDTA, 15 mM 2-mercaptoethanol, 10 µg/ml leupeptin, 1 mM benzamidine and 1 mM phenylmethanesulphonyl fluoride, and subsequent sonication. Homogenates were centrifuged at 10000 g for 15 min and the resulting supernatants used for the determination of enzyme activity. Protein concentration was measured as described by Bradford [21], using the Bio-Rad protein assay reagent and BSA as standard.

Creatine kinase activity

Total creatine kinase activity was determined by a spectrophotometric assay with a commercially available kit (Boehringer Mannheim) in a Cobas-Bio autoanalyser. One unit of enzyme activity is the amount of enzyme catalysing the formation of 1 µmol of NADPH/min at 25 °C.

Glycogen phosphorylase activity

Total glycogen phosphorylase activity in cell extracts was determined by the incorporation of [^{14}C]glucose 1-phosphate into glycogen in the presence of the allosteric activator AMP (5 mM), as previously described [22]. Glycogen phosphorylase isoenzymes were separated by electrophoresis in 7.5% precast polyacrylamide gels in a Pharmacia PhastSystem apparatus as described previously [23]. Glycogen phosphorylase activity was revealed by incubation of the gels with a solution containing 25 mM β -glycerophosphate (pH 6.8), 5 mM EDTA, 0.1% glycogen, 50 mM glucose 1-phosphate, 5 mM dithiothreitol and 5 mM AMP, for at least 15 h. Newly formed glycogen was detected by staining with iodine solution.

Glycogen content

Cell monolayers were scraped into 30% KOH and homogenates boiled for 15 min and centrifuged (5000 g, 15 min). A portion of the supernatant was used for the measurement of protein concentration. Supernatants were spotted on to Whatman 31-ET papers and glycogen was precipitated by immersing the papers in ice-cold 66% ethanol. After two additional washes, papers were

dried and incubated with α -glucosidase and the glucose released was measured enzymically.

Northern-blot analysis of RNA

Total RNA was isolated from C2C12 myocytes by a single-step guanidinium thiocyanate/phenol/chloroform method [24], fractionated by electrophoresis in 1.5% agarose gels and transferred to nitrocellulose membranes. Hybridization with an anti-sense ^{32}P -labelled riboprobe derived from a 1.3 kb *AccI*–*HindIII* fragment of rabbit muscle glycogen phosphorylase cDNA [4] was performed at 65 °C in the presence of 50% formamide. Filters were washed at 65 °C to a final stringency of $0.1 \times \text{SSC}$ (where $1 \times \text{SSC}$ is 0.15 M NaCl/0.015 M sodium citrate) and 0.1% sodium lauryl sulphate and subjected to autoradiography.

β -Galactosidase assay

For β -galactosidase staining, cells were fixed at room temperature in 2% paraformaldehyde freshly prepared in PBS for 15 min and extensively washed in PBS. β -Galactosidase activity was detected by incubating cells with 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal)-staining solution [5 mM $\text{K}_4\text{Fe}(\text{CN})_6$ /5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ /2 mM MgCl_2 /1 mg/ml X-Gal] for 24 h at 37 °C. Results are expressed as the percentage of blue-stained myocyte nuclei.

RESULTS

Muscle glycogen phosphorylase delivery into differentiating myoblasts

C2C12 myoblasts (myogenic proliferating cells) were induced to differentiate into myotubes (multinucleated and non-proliferating cells) by replacing the incubation medium, 10% FBS/DMEM, with 10% HS/DMEM. Morphological evidence of cell fusion was obtained after 4 days of culture in differentiating medium (Figure 1c) and multinucleated myotubes were observed at day 7 (Figure 1d). The differentiation process was also

Figure 1 Differentiation of C2C12 cells

Phase-contrast micrographs of C2C12 cells cultured in growth medium (DMEM with 10% FBS) (a) or differentiating medium (DMEM with 10% HS) for 2 (b), 4 (c) and 7 (d) days.

Figure 2 Analysis of RNA from C2C12 myoblasts and myotubes

Northern blots of total RNA isolated from C2C12 control (C, □) or AdCMV-MGP-infected (M, ■) cells were hybridized with a ^{32}P -labelled rabbit muscle glycogen phosphorylase riboprobe. (a) C2C12 myoblasts grown in 10% FBS/DMEM were infected with AdCMV-MGP virus and the medium was replaced by differentiating medium (10% HS/DMEM). Results on different days after infection and induction of differentiation are shown. A representative Northern blot and the mean $\pm$ S.E.M. of the data from four independent experiments are presented in arbitrary units compared with control cells at day 0 (1.0 unit). (b) C2C12 myotubes cultured for 7 days in 10% HS/DMEM were infected with AdCMV-MGP virus. Results on different days after infection are shown. A representative Northern blot and the mean $\pm$ S.E.M. of the data from four independent experiments are presented in arbitrary units compared with control cells at day 1 (1.0 unit).

monitored by studying total creatine kinase activity, which rose from 59 (proliferating myoblasts) to 1150 units/mg of protein after 7 days of cell culture in the presence of HS.

Muscle glycogen phosphorylase cDNA was delivered into C2C12 proliferating myoblasts which were simultaneously induced to differentiate (see Figure 1). Adenovirus containing the

Figure 3 Glycogen phosphorylase activity in C2C12 differentiating myoblasts

C2C12 myoblasts grown in 10% FBS/DMEM were induced to differentiate by incubation with 10% HS and simultaneously were exposed to Ad1613 (△) or AdCMV-MGP (○) virus, or left untreated (□). At the indicated days after differentiation induction and infection, cells were harvested and glycogen phosphorylase activity was measured in the presence of 5 mM AMP by a radiometric assay. Data represent the mean $\pm$ S.E.M. of five independent experiments.

β -galactosidase reporter gene (AdCMV- β Gal) [25] was used to evaluate the efficiency of adenoviral infection of differentiating myoblasts. In our hands, an efficiency of infection of $59 \pm 3\%$ was found 7 days after infection with this recombinant virus. C2C12 myoblasts infected with an equivalent number of Ad1613 or AdCMV-MGP recombinant virions were harvested at different intervals after cell infection and fusion induction, and total RNA was isolated for analysis of muscle phosphorylase expression. Consistent with a previous study [26], expression of the endogenous muscle glycogen phosphorylase gene was regulated during the differentiation process in uninfected control cells. No expression of endogenous muscle phosphorylase was detected in proliferating myoblasts whereas the muscle phosphorylase transcript was clearly observed 2 days after the medium switch (Figure 2a). Constant levels of expression were reached after 6 days of incubation in fusion medium. In cells that had been exposed to AdCMV-MGP, a 2.5-fold increase in the expression of muscle phosphorylase mRNA was measured at day 2 after infection. Increasing amounts of the muscle phosphorylase transcript were detected by 6 days after infection, and levels of muscle glycogen phosphorylase mRNA were still high (about 2-fold) relative to control cells 15 days after infection.

Total phosphorylase activity was also examined. In control cells (uninfected or wild-type-infected), a time-dependent increase in enzyme activity was observed after replacement of the proliferating medium by differentiating medium. At day 7, a maximum activity was reached which then remained constant for the duration of the study (20 days) (Figure 3). Infection of myoblasts with AdCMV-MGP recombinant virus resulted in a detectable increase in muscle phosphorylase activity relative to control cells 3 days after infection, with a maximal increase of 1.3-fold achieved at 10–15 days after infection. The increase in glycogen phosphorylase activity was less marked than that of the phosphorylase transcript, which might be explained by the involvement of post-transcriptional mechanisms of regulation. By 20 days, however, enzyme activity had returned to control levels.

Transfer of muscle glycogen phosphorylase into C2C12 myotubes

The efficiency of adenovirus-mediated gene transfer into non-proliferating myotubes was also studied. For this purpose, C2C12 myotubes cultured for 7 days in 10% HS/DMEM were infected

Figure 4 Glycogen phosphorylase activity in C2C12 myotubes

C2C12 myotubes cultured for 7 days in 10% HS/DMEM were studied uninfected (□) or after infection with Ad1613 (△) or AdCMV-MGP (○) virus. At the indicated days after infection, cells were harvested and glycogen phosphorylase activity was measured in the presence of 5 mM AMP by a radiometric assay. Data represent the mean $\pm$ S.E.M. of five independent experiments.

Figure 5 Non-denaturing gel assays of phosphorylase activity

C2C12 myotubes cultured for 7 days in 10% HS/DMEM were uninfected (control) or infected with virus carrying muscle glycogen phosphorylase cDNA (AdCMV-MGP). Cells were harvested at 7 or 10 days after infection. Extracts prepared from C2C12 cells and from rat brain, rat skeletal muscle and rat heart tissues were submitted to electrophoresis. Phosphorylase activity was revealed by staining newly formed glycogen with iodine solution.

with AdCMV- β Gal, Ad1613 or AdCMV-MGP virus (day 0). The histochemical detection of β -galactosidase after 7 days of infection showed that $78 \pm 4\%$ of C2C12 myotube nuclei expressed this enzyme activity. Total RNA was prepared at intervals after infection with Ad1613 and AdCMV-MGP. As seen in Figure 2(b), uninfected differentiated myotubes expressed muscle phosphorylase. In 20-day-old myotubes, a decrease in the message was apparent, which might be related to general phenotype changes occurring in myotubes as a function of time of culture. Myotube experiments could not be extended for more than 20 days after induction of differentiation because cells began to detach from the plates. Delivery of muscle phosphorylase cDNA into myotubes resulted in clear increases in muscle glycogen phosphorylase mRNA at day 4 after infection (about 2.5-fold increase), which increased with time for the duration of the study (up to a 4-fold increase 10 days after infection).

Table 1 Glycogen content in C2C12 myotubes

C2C12 myotubes cultured for 7 days in 10% HS/DMEM were studied uninfected (control) or after infection with AdCMV-MGP. At 7 days after infection, cells were preincubated for 1 h in DMEM with no serum and then treated for the indicated times with no effectors (basal), 1 μ M adrenaline or 1 μ M CCCP. After the incubation period, cellular glycogen levels were measured. Data represent the means $\pm$ S.E.M. of three independent experiments performed in duplicate. All data from infected cells significantly differ ($P \leq 0.005$ or lower) from their corresponding values in uninfected cells.

		Glycogen (μ g of glucose/mg of protein)			
Time (min) ...		20	40	60	90
Uninfected	Basal	25.2 $\pm$ 1.3	23.7 $\pm$ 1.5	22.0 $\pm$ 1.7	21.3 $\pm$ 1.3
	Adrenaline	23.4 $\pm$ 2.1	19.3 $\pm$ 1.3	16.0 $\pm$ 0.9	14.7 $\pm$ 1.3
	CCCP	16.3 $\pm$ 2.2	11.4 $\pm$ 1.7	7.7 $\pm$ 0.03	5.4 $\pm$ 0.1
	Basal	19.3 $\pm$ 0.7	17.4 $\pm$ 1.5	18.9 $\pm$ 0.1	17.4 $\pm$ 0.3
AdCMV-MGP	Adrenaline	11.2 $\pm$ 0.6	7.4 $\pm$ 0.5	4.4 $\pm$ 0.03	1.3 $\pm$ 0.07
	CCCP	7.8 $\pm$ 0.3	2.6 $\pm$ 0.3	1.9 $\pm$ 0.01	0.2 $\pm$ 0.06
	Basal	19.3 $\pm$ 0.7	17.4 $\pm$ 1.5	18.9 $\pm$ 0.1	17.4 $\pm$ 0.3

Total glycogen phosphorylase activity in uninfected or wild-type adenovirus-infected myotubes was unchanged over the time period studied (from day 1 to 20) (Figure 4). In contrast, in cells infected with AdCMV-MGP, phosphorylase activity increased with time after transfection, reaching a maximum increment (more than 2-fold) at day 20. It is worth noting that the relative and absolute increments in glycogen phosphorylase levels in myotubes were much higher than those obtained in differentiating C2C12 myoblasts. Thus muscle glycogen phosphorylase delivered into C2C12 cells appears to reach higher levels of expression and to persist longer when non-proliferating myotubes are infected rather than differentiating myoblasts.

The pattern of phosphorylase isoenzyme expression in myotubes was also studied by non-denaturing gel electrophoresis (Figure 5). In control cells (uninfected 14-day-old myotubes), one major band of activity, which co-migrated with the activity band expressed in brain tissue, and a minor band, which corresponded to the muscle form, were seen. Therefore C2C12 myotubes showed a pattern of phosphorylase isoenzyme expression that is similar to that found in many fetal tissues [4,5]. In cells transfected with muscle phosphorylase (7 or 10 days after infection), a clear increase was detected only in the band that co-migrated with the muscle isoenzyme, demonstrating that this is the enzyme form that had been transferred.

Myotube glycogen levels after delivery of muscle glycogen phosphorylase

We next examined whether the increase in myotube glycogen phosphorylase activity could be correlated with a modified glycogenolytic capacity. The time course of glycogen degradation in control and infected cells in response to effectors is shown in Table 1. After incubation of the cells with a glycogenolytic agent such as adrenaline, a larger response to the hormone was observed in the genetically engineered cells. AdCMV-MGP-infected cells showed a 75% reduction in their glycogen content after 1 h of hormone treatment compared with a 27% decrease obtained in uninfected cells ($P < 0.002$). Similar results were obtained when myotube glycogenolysis was stimulated with the metabolic uncoupler CCCP. After 1 h of CCCP treatment the polysaccharide content was reduced by 90% in AdCMV-MGP-infected cells compared with a 65% decrease in uninfected cells ($P < 0.001$). It is worth noting that the infection process did not affect the

glycogenolytic response of the C2C12 myotubes as assessed by using Ad1613 (results not shown).

DISCUSSION

This work represents a first approach to the development of a gene-therapy strategy for overcoming McArdle's disease using adenovirus as a gene-delivery system. One of the issues addressed is whether the introduction of muscle glycogen phosphorylase into myotubes results in physiologically active protein able to regulate the glycogenolytic process.

Our results indicate that basal glycogen levels in myotubes transduced with AdCMV-MGP virus are decreased, but even more importantly, the introduced phosphorylase can be activated through the two different mechanisms of regulation, phosphorylation and allosteric stimulation. In cells challenged with adrenaline, which activates glycogen phosphorylase by a cyclic AMP-mediated increase in its phosphorylation state, a larger glycogenolytic response was observed in engineered cells than in control cells. These results suggest that the delivery of muscle phosphorylase to myotubes *in vivo* would allow a normal glycogenolytic response to stimuli that signal via phosphorylation of the enzyme, such as muscular contraction or adrenaline challenge. The results obtained when cells were treated with CCCP, a metabolic uncoupler known to raise intracellular AMP levels [27], suggest that the phosphorylase delivered may also be sensitive to allosteric stimulation. The fact that muscle phosphorylase can be precisely regulated when introduced into muscle cells contrasts with our previous observations in hepatocytes [18]. Engineered hepatocytes containing muscle glycogen phosphorylase presented an enhanced glycogenolytic response to the metabolic inhibitor CCCP but not to glucagon, which activates phosphorylase kinase by raising hepatocyte cyclic AMP levels. These results reinforce the hypothesis that the presence of certain allosteric inhibitors or the absence of a muscle-specific factor in the hepatocytes impedes the full expression of the muscle phosphorylase activity in these cells.

We have also examined *in vitro* the adequacy of the two different strategies previously described for the correction of genetic diseases of muscle cells, namely, the reimplantation of *ex vivo* genetically modified myoblasts and the direct *in vivo* delivery of the gene of interest into the tissue. Accordingly we chose to perform our studies on the myoblast cell line C2C12, which retains the ability to differentiate into non-proliferating myotubes [28].

The possibility of transplanting adenovirus-transduced myoblasts has been investigated *in vitro* by infecting proliferating C2C12 myoblasts with adenovirus-containing muscle glycogen phosphorylase, and subsequently inducing myoblast differentiation into fused myotubes by switching fetal serum for horse serum. This protocol mimics the reimplantation of myoblasts genetically modified *ex vivo* into the muscle by injection, where they are induced to differentiate and fuse, contributing to endogenous fibres [28]. The efficiency of adenovirus-mediated gene transfer into myoblasts, tested with AdCMV- β Gal, was lower than into myotubes. These results can be explained by the fact that, even though myoblasts are induced to differentiate, a few rounds of cell division may occur before fusion into myotubes. In a dividing population of C2C12 myoblasts, since adenovirus remains as an episomal DNA [15], the introduced gene will gradually be diluted. However, the possibility that myoblasts possess fewer adenovirus receptors than myotubes cannot be excluded. Adenovirus-mediated delivery of muscle glycogen phosphorylase into differentiating myoblasts resulted in a moderate and transient increase in glycogen phosphorylase

activity, which returned to basal levels by 20 days after infection. Again, this transient expression of muscle glycogen phosphorylase is probably explained by the fact that adenovirus does not efficiently integrate into genomic DNA.

Our second protocol, the transfer of muscle glycogen phosphorylase by means of adenovirus into non-proliferating multinucleated myotubes, was designed as a model for the direct delivery of the enzyme into muscle tissue. In this case, phosphorylase activity reached higher levels and the increase persisted. After 20 days of infection with AdCMV-MGP, glycogen phosphorylase activity remained more than twice that in control cells. Therefore our results indicate that direct delivery of the muscle gene into mature muscle cells may be the method of choice for restoration of glycogenolytic capacity in patients with McArdle's disease. Further encouragement for this approach comes from recent studies in which adenovirus has been shown to mediate the *in vivo* expression of *lacZ* or the minidystrophin gene after intramuscular injection [13–15].

In summary, our results indicate that myotubes appear to be a better system for adenoviral-mediated gene delivery than differentiating myogenic cells and suggest that direct delivery of glycogen phosphorylase into skeletal-muscle cells would be more suitable than implantation of adenovirus-engineered myoblasts. Our data also show that the delivery of muscle phosphorylase activity into myotubes augments their glycogenolytic capacity both in basal conditions and after challenge with different stimuli. Taken together these experiments suggest that the restoration of phosphorylase activity in the deficient muscle cells of patients with McArdle's disease will allow glycogenolysis to take place in a regulated way.

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REFERENCES

- Nakano, K., Hwang, P. K. and Fletterick, R. J. (1986) FEBS Lett. **204**, 283–287
- Newgard, C. B., Nakano, K., Hwang, P. K. and Fletterick, R. J. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8132–8135
- Newgard, C. B., Litman, D. R., Van Gederen, C., Smith, M. and Fletterick, R. J. (1988) J. Biol. Chem. **263**, 3850–3856
- Martinuzzi, A., Askanas, V., Kobayashi, T., Engel, W. K. and DiMauro, S. (1986) J. Cell. Biol. **103**, 1423–1429
- Newgard, C. B., Norkiewicz, B., Hughes, S. D., Frenkel, R. A., Coats, W. S., Martiniuk, F. and Johnston, J. M. (1991) Biochim. Biophys. Acta **1090**, 333–342
- Newgard, C. B., Hwang, P. K. and Fletterick, R. J. (1989) Crit. Rev. Biochem. Mol. Biol. **24**, 69–99
- Browner, M. F. and Fletterick, R. J. (1992) Trends Biochem. Sci. **17**, 66–71
- Coats, W. S., Browner, M. F., Fletterick, R. J. and Newgard, C. B. (1991) J. Biol. Chem. **266**, 1613–1619
- DiMauro, S. and Bertolin, N. (1986) Phosphorylase Deficiency, in Myology (Banker, I. E., ed.), pp. 1585–1601, McGraw-Hill, New York
- Gautron, S., Daegelen, D., Menneier, F., Dubocq, D., Kahn, A. and Dreyfus, J.-C. (1987) J. Clin. Invest. **79**, 275–281
- McConchie, S. M., Coakley, J., Edwards, R. H. T. and Beynon, R. J. (1991) Biochim. Biophys. Acta **1096**, 26–32
- Wolff, J. A., Malone, R. W., Williams, W. C., Acsadi, G., Jani, A. and Felgner, P. L. (1990) Science **247**, 1465–1468
- Quantin, B., Perricaudet, L. D., Tajbakhsh, S. and Mandel, J.-L. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 2581–2584
- Ragot, T., Vincent, N., Chafey, P., Vigne, E., Gilgenkrantz, H., Couton, D., Cartaud, J., Briand, P., Kaplan, J.-C., Perricaudet, M. and Kahn, A. (1993) Nature (London) **361**, 647–650
- Stratford-Perricaudet, L. D., Makeh, I., Perricaudet, M. and Briand, P. (1992) J. Clin. Invest. **90**, 626–630
- Gussoni, E., Pavlath, G. K., Lancot, A. M., Sharma, K. R., Miller, R. G., Steinman, L. and Blau, H. M. (1992) Nature (London) **356**, 435–438

- 17 Partridge, T. A., Morgan, J. E., Coulton, G. R., Hoffman, E. P. and Kunkel, L. M. (1989) *Nature (London)* **337**, 176–179
- 18 Gomez-Foix, A. M., Coats, W. S., Baqué, S., Alam, T., Gerard, R. D. and Newgard, C. B. (1992) *J. Biol. Chem.* **267**, 25129–25134
- 19 LaSalle, G. L. G., Robert, J. J., Berrard, S., Ridoux, V., Stratford-Perricaudet, L. D., Perricaudet, M. and Mallet, J. (1993) *Science* **259**, 988–990
- 20 Lemarchand, P., Jaffe, H. A., Danel, C., Cid, M. C., Kleinman, K., Stratford-Perricaudet, L. D., Perricaudet, M., Pavirani, A., Lecocq, J.-P. and Crystal, R. G. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6482–6486
- 21 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 22 Gilboe, D. P., Larson, K. L. and Nuttal, F. Q. (1972) *Anal. Biochem.* **47**, 20–27
- 23 Mayer, D. and Letsch, I. (1991) *Electrophoresis* **12**, 297–302
- 24 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 25 Herz, J. and Gerard, R. D. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2812–2816
- 26 Lockyer, J. M. and McCracken, J. B. (1991) *J. Biol. Chem.* **266**, 20262–20269
- 27 Carabaza, A., Guinovart, J. J. and Ciudad, C. J. (1986) *Arch. Biochem. Biophys.* **250**, 469–475
- 28 Hughes, S. and Blau, H. (1992) *Cell* **68**, 659–671

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