Muscle Biochemistry and a Genetic Study of Myotonic Distrophy

The study of the molecular genetics of myotonic muscular dystrophy (DM) has proceeded rapidly, with the discovery of a variable trinucleotide repeat interrupting the 3′ untranslated region of myotonin–protein kinase (Mt-PK). Despite the elegance and sophistication of molecular genetic methods, certain control experiments should be performed when comparing diseased and normal (control) muscle samples. Fu *et al.* (*1*) provide conclusions and interpretations of gene expression data, but do not take into account relevant methods of muscle biochemical analyses.

The reverse transcription polymerase chain reaction (RTPCR) data with which Fu *et al.* perform polymerase chain reaction (PCR) across the variable repeat [figure 3 in (*1*)] raise some problems. Fu *et al.* quantitatively relate the amount of PCR product to the amount of the expressed normal and repeat-containing allele mRNA, rather than relating variations in PCR efficiency with increasing repeat sizes. A distinction between the two alleles that used coding sequence polymorphisms would have been more convincing. The experiments (*1*) were also performed in lymphoblasts with extrapolation to muscle, although using dissected muscle fiber would have been feasible.

The comparison between Mt-PK mRNA and immunoreactive protein in DM and normal muscle [figures 3 and 5 in (*1*)] should have included experiments that controlled for variations that can result from (i) other pathological changes in the biopsied muscle, including fibrosis and fatty infiltration; (ii) methods of biopsy and time to freezing; (iii) length of time stored and method of tissue storage; (iv) differences between sampled muscles (age, fiber type, conditioning, and so on), and (v) selection and handling of normal control muscle samples. The use of "μg of total protein" [figure 5 in (*1*)] as a denominator in calculations in such studies has been shown to produce spurious results (*2*, *3*).

Comparative molecular experiments are not fundamentally different from biochemical comparisons. There are excellent studies of skeletal muscle comparisons, especially of measurements in dystrophic muscle (*2*–*4*). Despite the sophistication of the molecular measurements reported by Fu *et al.* (*1*), there was a lack of characterization in their study of the experimental variables with regard to samples from their patient or from normal people. Indeed, half of the measures of mRNA taken from healthy muscle tissue were equivalent to those obtained from DM patients [figure 3 in (*1*)]. Without attention to such variables, it is difficult to interpret statements by Fu *et al.* about quantitative decreases in DM. Conclusions regarding genetic mechanisms operating in DM based on these data should be weighed cautiously.

Allen D. Roses
Division of Neurology,
Duke University Medical Center,
Durham, NC 27710, USA

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Response: The RTPCR data described in our report (*1*) had two components. First, primers that did not span the repeat region were used in order to obtain a quantitative measure of the total Mt-PK mRNA in muscle tissue from normal adults and from adults with DM. Moreover, an internal control was used to correct for differences in the efficiency of PCR conducted with different samples. Therefore, the determination of the steady-state amount of Mt-PK mRNA present in these issues was not subject to error resulting from variations in PCR efficiency with different repeat sizes. Second, after we demonstrated that muscle tissue from adult patients with DM had a decreased total amount of Mt-PK mRNA, we performed PCR across the repeated region in order to distinguish the level of expression of the normal as opposed to the affected allele. The amount of mRNA from the normal allele was found to be normal, whereas that from the affected allele was deficient. This result, and the fact that the amount of mRNA from the two alleles was equal to the total detected in the original assay, is internally consistent and supports the concept that the defective allele was under-expressed. These conclusions have been reached by three research groups using different methods (*2*).

The results reported were not obtained with lymphoblasts exclusively, but with the use of muscle tissue from adults with and without DM. The muscle tissue was obtained by biopsy, which was carried out by highly qualified physicians with extensive experience in neuromuscular disease. Tissues from affected and unaffected individuals were processed identical to eliminate variations.

We reported the baseline used to compare various protein samples analyzed for their content of Mt-PK protein as "μg of protein." However, we also stained for total proteins in polyacrylamide gels that were run with samples of the various tissue protein extracts; these showed that the pattern and amount of protein was identical and provided an internal control. Some of the resulting figures were not included in the final short report, but were used during review.

Most investigators who conduct molecular genetic experiments to investigate muscle biology, as well as many other subjects, are aware that the techniques have limitations and that one must be cognizant of them when interpreting results. Extensive discussions of such limitations are seldom found in regular papers, but are often part of review articles or technical papers that focus on the techniques themselves.

In our study, muscle biopsies were taken from three different groups of patients from different countries. Each group contained adult controls of the same race. The samples were frozen in liquid nitrogen and were stored frozen until use in our measurements. Samples were assayed upon thawing and were refrozen and thawed only to reconfirm results in subsequent experiments. Reassay of samples stored as homogenates provided the same results as did assay of sample aliquots that had undergone only a single freeze-thaw cycle. The variation in the control samples was less than 10% of the mean, which suggests that the samples were stable and the data reproducible. Taken together, these observations suggested to us that these samples were suitable for our initial measurements. We agree with Roses that cellular characterization of these parameters is the next logical step in the study of molecular pathogenesis of DM.

Y.-H. Fu
J. A. Pearlman
A. Pizzuti
R. G. Fenwick Jr.
Institute for Molecular Genetics,
Baylor College of Medicine,
Houston, TX 77030, USA
D. L. Friedman
M. B. Perryman
Section of Cardiology,
Baylor College of Medicine

S. Richards
R. A. Gibbs
Institute for Molecular Genetics and Human Genome Center, Baylor College of Medicine
T. Ashizawa
Department of Neurology, Baylor College of Medicine, and Veterans Administration Medical Center, Houston, TX 77030, USA
G. Scarlato
Istituto di Clinica Neurologica, Universita di Milano, Centro Dino Ferrari Via F. Sforza 35, 20122 Milan, Italy

C. T. Caskey
Institute for Molecular Genetics and Howard Hughes Medical Institute, Human Genome Center, Baylor College of Medicine

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A Truncated Erythropoietin Receptor and Cell Death: A Reanalysis

We reported that human bone marrow cells express both a truncated and a full-length form of erythropoietin receptor (EPOR-T and EPOR-F, respectively), with the truncated form predominating in immature progenitors and the full-length form predominating in late-state progenitors (*1*). Transfection experiments revealed no difference in binding affinity to erythropoietin (EPO) between the two, and showed that both EPOR-F and EPOR-T could transduce mitogenic signals. However, when compared with cells expressing EPOR-F, those expressing EPOR-T were more likely to undergo apoptosis at lower concentrations of EPO. On the basis of these data, we speculated that the signaling pathway for prevention of apoptosis is different from the one for mitogenesis (*1*). R. Schwall proposed a different interpretation of the data, that is, EPOR-T does not selectively fail to prevent programmed cell death, but it transduces EPO actions inefficiently (*2*).

Our experiments, like many others, were performed with a mouse pro-B cell line, Ba/F3, as recipient cells. Recently, it was reported that a small amount of endogenous EPOR is expressed in Ba/F3 cells (*3*). Other investigators reported that JAK2 kinase has a critical role in EPOR-mediated signal transduction by binding to the cytoplasmic portion of the receptor (*4*). These observations prompted us to rule out the possibility that the endogenous mouse EPOR is responsible for EPO-responsive cell proliferation in EPOR-T expressing transfectants, Ba/F3-(EPOR-T). Unexpectedly, RT-PCR of mRNA obtained from EPOR-T–transfected Ba/F3 cells showed presence of both EPOR-T and EPOR-F messages (Fig. 1A, lane 2). When these transfectants were cultured under EPO for a week, the amount of EPOR-F messages increased (Fig. 1A, lane 3). Subcloning and sequencing of the PCR products identified this fragment as human EPOR-F rather than mouse endogenous EPOR. The complementary DNA (cDNA) clone that we used for construction of an expression vector for EPOR-T (pEPOR-TF) contained an intron 7 and the entire exon 8 (Fig. 1B). Since transfection of the same construct into L cells resulted in production of only a trace amount of EPOR-F (Fig. 1A, lane 5), it seems that the intron 7 of EPOR-T type mRNA transcribed from pEPOR-TF is spliced out by some cell-specific mechanism to produce EPOR-F in Ba/F3 cells. Thus, the response of Ba/F3-(EPOR-T) to EPO shown in our previous report (*1*) could have been mediated by coexisting EPOR-F. To rule out this possibility, we constructed an expression vector with EPOR-T7 (pEPOR-T7) (Fig. 1B), which does not contain exon 8 and thus never produces EPOR-F. When this construct was transfected, Ba/F3 cells did not respond to EPO (Fig. 2). Expression of endogenous mouse EPOR (Fig. 1A, lane 1) seems to be responsible for low-grade response at higher concentrations of EPO. From these data, we conclude that EPOR-T cannot transduce mitogenic signals.

How, then, does the expression of EPOR-T affect apoptosis of cells under lower concentrations of EPO? It is believed that EPOR acts as a homodimer to bind with EPO and to transduce signals (*5*). Several experiments using mouse EPOR deletion mutants showed that cytoplasmic portion of EPOR is not essential for cell surface expression and EPO binding (*6*). Therefore, it is possible that EPOR-T forms not only a homodimer, but a heterodimer with EPOR-F. To investigate the influence of EPOR-T co-expression on EPOR-F–expressing cells, we transfected Ba/F3-(EPOR-F) cells with pEPOR-T7 (Ba/F3-EPOR-(F+T7)). The response of Ba/F3-EPOR-(F+T7) was lower than that of Ba/F3-(EPOR-F) (Fig. 2). When Ba/F3-EPOR-

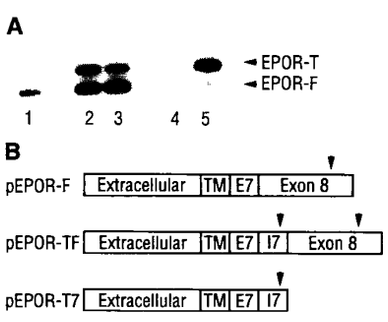

Fig. 1. (**A**) Southern (DNA) hybridization of the reverse transcriptase polymerase chain reaction (RT-PCR) products. mRNA obtained from Ba/F3 parent cells (lane 1), Ba/F3 cells transfected with pEPOR-TF and cultured under IL-3 and G418 (lane 2), Ba/F3 cells transfected with pEPOR-TF and cultured under EPO for a week (lane 3), L cells (lane 4), and L cells transfected with pEPOR-TF (lane 5) were subjected to RT-PCR and Southern blot analysis after gel electrophoresis (*8*). Both human and mouse EPOR cDNA were used as probes. The shorter band (457 bp) represents the full-length EPOR (EPOR-F) and the longer band (552 bp) represents the truncated form (EPOR-T). (**B**) Structure of cDNAs used for construction of an expression vector. pEPOR-F has all exons and encodes full-length EPOR. pEPOR-TF is an alternatively spliced form of EPOR that contains intron 7 and exon 8. pEPOR-T7 contains intron 7 but exon 8 is deleted at the exon-intron junction by PvuII restriction enzyme treatment. Arrows indicate stop codon. Extracellular, extracellular domain; TM, transmembrane region; E7, exon 7; I7, intron 7.

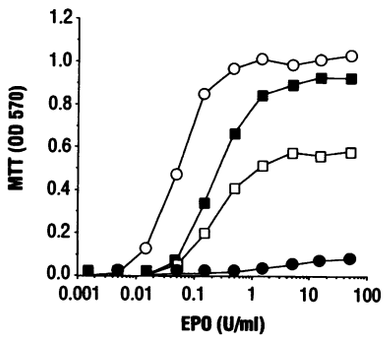

Fig. 2. Cell growth studies on Ba/F3 transfectants expressing different human EPORs. Over-transfection and selection of Ba/F3-(EPOR-F) by pEPOR-T7 was analyzed. The transfectants were cultured in the presence of different concentrations of EPO (*9*): Ba/F3-(EPOR-F) (○), Ba/F3-[EPOR-F(Hygro)+EPOR-T7(G418)] just after the F418 selection (□), Ba/F3-[EPOR-F(Hygro)+EPOR-T7(G418)] after 1 week culture in the presence of EPO (■) and Ba/F3-(EPOR-T7) (●). U, unit.