tions of the Moreton subspecies. The polymorphic nature of these populations has been interpreted as evidence of past hybridization events during the westerly movement of the zone as predicted by the asymmetrical introgression of Torresian chromosomes (*15*). Again, it seems plausible that they may have arisen as a consequence of hybridization.

Another pertinent example has been found in a population sample from North Queensland where the Torresian and Daintree species are sympatric. One individual was heterozygous for a fusion involving chromosomes 5 and 1 (Fig. 1x), similar to the fusions seen in the backcrosses and in the hybrid zone. However, the most interesting feature of this individual was that in addition to the fusion, it was simultaneously heterozygous for two pericentric rearrangements involving chromosomes 1 and 6 and also contained a small acrocentric chromosome, the origin of which could not be ascertained. The karyotype of this individual suggests that it is a hybrid between the Torresian and Daintree species which has undergone a complex series of chromosomal rearrangements, similar to those observed in some of the backcross individuals.

From these data one can conclude that hybridity *per se* may indeed represent a dynamic evolutionary factor capable of generating extremely high levels of genetic variability. It may also provide a plausible explanation for the persistence of hybrid zones in nature. Thus it is conceivable that in these zones of hybridization a higher mutation rate may permit hybrid populations to attain new adaptive peaks. Studies on hybridization and hybrid zones provide substantial corroborative evidence to support this notion (*10, 20–26*). Quite clearly, the generality of the phenomenon of hybrid-induced chromosomal mutation would imply that previous hypotheses concerning the establishment of chromosomal rearrangements in the homozygous condition may no longer be appropriate (*12*). If chromosomal mutation is not a random event but one which occurs repeatedly at specific sites in the genome, as we have shown, then one can easily envisage a situation in which the establishment and fixation of a rearrangement could depend upon (i) the frequency of hybridization between two divergent taxa, (ii) the probability distribution of breakage-reunion events throughout the genome, and (iii) the relative fitness of the hybrid progeny. Thus, any reduction in population density due to hybrid inviability could produce a situation in which individuals carrying novel rearrangements may undergo positive assortative mating and hence facilitate the establishment of chromosomal homozygosity in small isolated communities. Furthermore, should the frequency of chromosome mutation events prove to be positively correlated with the intensity of hybrid breakdown then the relevance of the above scheme becomes even more plausible.

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Myosin Light Chain Phosphorylation Does Not Modulate Cross-Bridge Cycling Rate in Mouse Skeletal Muscle

Abstract. *An attempt was made to determine whether phosphorylation of the myosin light chain represents a thick filament–associated mechanism for modulating the rate of cross-bridge cycling in mouse skeletal muscle. When the degree of light chain phosphorylation was varied independently of tetanus duration, there was no correlation of phosphorylation with cross-bridge turnover rate, as measured by the shortening velocity of the muscle. It is concluded that in intact skeletal muscle phosphorylation of the myosin light chain does not in itself modulate cross-bridge cycling rate and that previously reported changes in cycling rate were due to other factors that may vary with tetanus duration.*

The regulatory light chain of skeletal muscle myosin in mammals can be phosphorylated by a calcium and calmodulin-dependent protein kinase (*1*), and cycles of phosphorylation and dephosphorylation have been observed in response to contraction and relaxation in whole muscle (*2*). Phosphorylation of the light chain is not essential for actin-activated adenosinetriphosphatase activity of myosin (*3*), but there are conflicting reports as to whether this phosphorylation may play a role in increasing or decreasing the rate of actomyosin adenosinetriphosphatase activity in isolated protein and skinned fiber systems (*4*).

Recently, Crow and Kushmerick (*5*) measured the maximum velocity of shortening and the degree of myosin light chain phosphorylation in intact mouse skeletal muscle after various periods of isometric tetanus. They found that, as the degree of phosphorylation increased with the duration of stimulation, the maximum velocity of shortening decreased. During prolonged stimulation, when light chain phosphorylation had increased to 50 percent there was a 50 percent decrease in the maximum velocity of shortening. Bárány (*6*) showed that the maximum velocity of shortening was proportional to the intrinsic rate of actin-activated adenosinetriphosphatase activity of myosin. Crow and Kushmerick (*5*) thus interpreted the relation between light chain phosphorylation and maxi-

mum velocity of shortening to mean that phosphorylation of the light chains in skeletal muscle leads to a reduction in the rate of cross-bridge turnover in vivo.

To test the validity of this hypothesis, we measured the velocity of shortening in mouse skeletal muscle under conditions in which the degree of light chain phosphorylation was varied independently of tetanus duration. We found that light chain phosphorylation did not decrease the rate of cross-bridge turnover and that the previously reported changes in turnover rate were due to other factors that vary with tetanus duration.

Phosphorylation of the 18,000-dalton light chain was increased above its low value in the resting intact muscle by an initial stimulation. Manning and Stull (*2*) showed that, during the period of rest following a short isometric tetanus, the phosphate content of the light chain continued to increase for approximately 8 seconds. We previously found that in the extensor digitorum longus (EDL) muscle of mice, a 2-second isometric stimulation followed by 8 seconds of rest resulted in 50 percent phosphorylation of the regulatory light chain (*7*). This degree of phosphorylation was equivalent to that measured after 7 seconds of continuous stimulation.

We measured the velocity of shortening of mouse EDL muscle under isotonic conditions in which the force was less than 10 percent of maximum isometric force (*8*). Shortening was initiated 0.5 second after the beginning of a tetanic stimulation. In one case the muscle was stimulated for 2 seconds ending 8 seconds before the test stimulus to increase the state of phosphorylation of the light chain to approximately 50 percent. In the other case there was no prior stimulation, and shortening velocity was measured under conditions in which the light chain was about 10 percent phosphorylated. This design enabled a direct comparison of relative cross-bridge turnover rates, as estimated from shortening velocities measured at the same time after initiation of a tetanic stimulation but under conditions of high or low phosphorylation.

Figure 1 shows a typical graph of muscle length versus time. The muscle shortens with the initiation of isotonic conditions, and shortening velocity is determined from the slope of the initial linear portion of the curve. When the muscle was stimulated before the test stimulus to increase the degree of light chain phosphorylation, the velocity of shortening was slightly higher than when the muscle was not subjected to prior stimulation. In nine muscles, the ratio of shortening velocity under normal conditions to velocity with prior stimulation in the same muscle was 0.91 ± 0.03 (*9*). The active force outputs of each muscle just before the releases under the two conditions were nearly identical (ratio, 1.02 ± 0.02; $N = 9$). Measurements of the degree of light chain phosphorylation (*10*) under conditions identical to those used in this study have verified that the light chain is 9 ± 2 percent ($N = 6$) phosphorylated at 0.5 second of contraction when the muscle is not previously stimulated, while it is 50 ± 4 percent ($N = 8$) phosphorylated when the muscle is subjected to a 2-second stimulation ending 8 seconds before the test stimulus.

These results suggest that under conditions in which the 18,000-dalton light chain is more highly phosphorylated there is a small increase in the shortening velocity of the muscle. It is unlikely, however, that light chain phosphorylation gives rise to this change in shortening velocity, since, as shown in Fig. 1, there is a decrease in velocity of shortening when the release is initiated 9 seconds after the start of the isometric tetanus. At this time the degree of light chain phosphorylation is high (*2, 5, 7*). These results, which show a decrease in velocity of shortening with increasing tetanus duration, are very similar to those of Crow and Kushmerick (*5*).

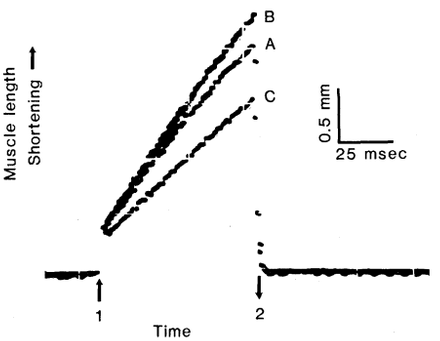

Fig. 1. Length of mouse EDL muscle as a function of time after initiation of an isotonic shortening at 22°C. Digitized traces from three different experimental designs are shown for the same muscle. The muscle was initially stimulated isometrically at the length, L_0, at which active force was maximum. At arrow 1 the servo control system (*8*) was switched to the isotonic mode and the muscle was allowed to shorten under a load of 1 g for 70 msec. At arrow 2 muscle length was returned to L_0 and maintained. Trace A is the response initiated 0.5 second after the beginning of an isometric tetanus. Trace B is the response initiated 0.5 second after the beginning of an isometric contraction when the muscle had been given a 2-second isometric stimulation ending 8 seconds before. Trace C is the response to a shortening initiated after 9 seconds of isometric stimulation. Velocity of shortening was determined from the slope of the initial linear portion of the curves and was 29, 33, and 23 mm/sec for traces A, B, and C, respectively. Active force just before release of the muscle was 19.0, 19.5, and 16 g, respectively.

Measurements of the maximum shortening velocity of mouse EDL by means of the slack test (*11*) were also made after 0.5 second of tetanic stimulation in muscles stimulated for 2 seconds ending 8 seconds before the test stimulus and in the same muscle not given prior stimulation. The ratio of the maximum velocity of shortening when light chain phosphorylation was low to that when it was high is 0.97 ± 0.02 ($N = 4$). This is further evidence that light chain phosphorylation does not decrease the rate of cross-bridge cycling.

We conclude that there is no consistent relation between the cross-bridge cycling rate, as measured by velocity of shortening, and the degree of myosin light chain phosphorylation in mammalian skeletal muscle. Similar measurements of the energetics of mouse and rat EDL muscles support this conclusion (*7*). This study does support the concept that cross-bridge turnover rate can change under different mechanical conditions, even though such changes are not caused by light chain phosphorylation. The factors responsible for alterations in cross-bridge cycling rate are not known, but may depend on the metabolic state of the muscle or on the concentration of calcium in the sarcoplasm at different times during stimulation.

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8. Male CD-1 mice (Charles River) 28 days to 3 months of age were used. All experiments were performed at 22°C in a Krebs solution containing 118 m*M* NaCl, 4.7 m*M* KCl, 1.18 m*M* $MgSO_4$, 1.2 m*M* KH_2PO_4, 1.9 m*M* $CaCl_2$, 25 m*M* $NaHCO_3$, and 11 m*M* glucose. The solution was gassed with a mixture of 95 percent O_2 and 5 percent CO_2 to obtain a *p*H of 7.4. The muscles

were stimulated directly through platinum electrodes with a Grass S44 stimulator. Stimulation frequency was either 66 or 100 Hz. No differences in results were found between these two frequencies. Muscle length was controlled with a Cambridge Technology dual-mode servo system (model 300 H). The switch from length control to force control was made with an appropriate timing circuit. Force was monitored by the Cambridge Technology system or a Kistler-Morse force transducer (model DSK-3), and both force and length signals were recorded on a Gould Brush recorder (model 440) and a Gould digital oscilloscope (model OS4000). Length and time data were transferred to a Tektronix storage oscilloscope for comparison.

9. All values given are means ± standard errors.
10. Myosin light chain phosphorylation was measured in muscles frozen with modified Wallenberger tongs cooled with liquid nitrogen. This method (*7*) involves extraction of the muscle with $HClO_4$ and isoelectric focusing of the proteins in a urea-containing polyacrylamide gel. The phosphorylated form of the 18,000-dalton light chain has a lower isoelectric point than does the nonphosphorylated form. The gels are stained with Coomassie blue and scanned at 570 nm. This method has been found to give results nearly identical to those obtained with the more common two-dimensional technique. In all experiments a rest period of at least 30 minutes was allowed between procedures on the same muscle. This period was sufficient for light chain phosphorylation to return to a value of less than 10 percent.
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Selective Reduction of Forskolin-Stimulated Cyclic AMP Accumulation by Inhibitors of Protein Synthesis

Abstract. *Inhibiting protein synthesis by incubating C6-2B rat astrocytoma cells with cycloheximide or emetine for periods up to 24 hours caused a progressive decrease in the accumulation of adenosine 3′,5′-monophosphate (cyclic AMP) when the cells were challenged for 30 minutes with 100* μM *forskolin. In contrast, cholera toxin–stimulated (6 n*M*, 3 hours) cyclic AMP accumulation was not diminished in cycloheximide-treated cells, and cyclic AMP was only minimally diminished in response to a 30-minute challenge with 10* μM *(−)-isoproterenol. These experiments suggest the presence of a previously unrecognized cyclase component, which is essential for forskolin-stimulated cyclic AMP accumulation and has a shorter half-life than the* β*-adrenergic receptor, the guanine nucleotide regulatory proteins, or the cyclase catalytic component.*

The mechanism of hormone-stimulated adenosine 3′,5′-monophosphate (cyclic AMP) accumulation in eukaryotic cells is currently thought of as involving three components of the membrane-bound adenylate cyclase system: hormone receptors, guanine nucleotide regulatory proteins (abbreviated G/F or N), and a catalytic component (*1*). Adenylate cyclase activity, although most commonly increased by hormonal interaction with receptors, can occur without hormone receptors—for example, by the direct action of cholera toxin on G/F (*2*) or by the action of the diterpene forskolin on the catalytic moiety (*3*). Cholera toxin is believed to act by catalyzing the adenosine diphosphoribosylation of G/F component peptides (*4*). Forskolin is believed to act by direct stimulation of the catalytic component, because it stimulates cyclase activity in G/F-deficient mutants of S49 murine lymphoma cells (*4*) and in other cyclase preparations apparently devoid of hormone receptors or G/F (*5*).

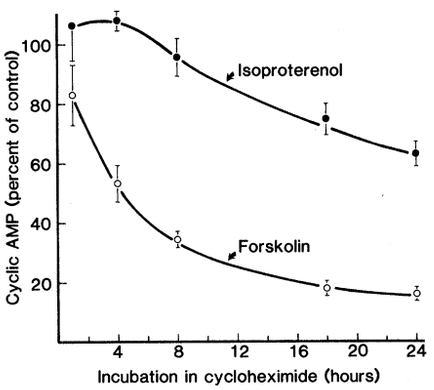

Fig. 1. Time course of the effect of cycloheximide (5 μg/ml) on a 30-minute challenge with 100 μ*M* forskolin or 10 μ*M* (−)-isoproterenol in C6-2B cells. Cells were treated with cycloheximide for the indicated times and intracellular cyclic AMP was measured after a 30-minute challenge with either 100 μ*M* forskolin or 10 μ*M* (−)-isoproterenol. Results are the means ± standard error (S.E.M.) of five to ten separate experiments, with three culture wells used at each time point. The results are expressed as the percentage of the cyclic AMP response in cells not incubated with cycloheximide.

We report that prolonged inhibition of protein synthesis in C6-2B rat astrocytoma cells reduces cyclic AMP accumulation stimulated by forskolin but does not reduce that stimulated by cholera toxin, and either does not reduce or minimally reduces that stimulated by catecholamines. These data suggest that forskolin acts on a rapidly turning over site of the adenylate cyclase system that is not necessary for cyclic AMP accumulation stimulated by cholera toxin or hormone but is essential for that stimulated by forskolin.

Confluent monolayers of C6-2B rat astrocytoma cells (passages 12 through 28) were grown (16-mm wells) in a humidified atmosphere at 37°C in Ham's F-10 medium (Gibco) supplemented with 10 percent donor calf serum (*6*). The cells were placed in serum-free medium 24 hours before the initiation of the experiments. Inhibitors of protein and RNA synthesis were added for various times up to 24 hours. At the end of these time periods the cells were stimulated with maximal concentrations of the following agents to test the functionality of cyclase components after inhibition of protein synthesis. The cells were challenged with either 100 μ*M* forskolin or 10 μ*M* (−)-isoproterenol for 30 minutes or with 6 n*M* cholera toxin for 3 hours. The responses of the cells were compared with those of cells not previously treated with the protein synthesis inhibitors. Cellular cyclic AMP content was determined as described (*7*, *8*). The efficacy of the protein synthesis inhibitors and of RNA synthesis inhibitors was tested by incubating cells with [^{3}H]leucine and [^{3}H]uridine, respectively, and then measuring radioactivity incorporated into cell homogenates precipitated with trichloroacetic acid.

Incubation of C6-2B cells with cycloheximide (5 μg/ml) caused a progressive and pronounced loss of forskolin-stimulated cyclic AMP accumulation (Fig. 1). After 8 hours of treatment with cycloheximide, the cells had lost more than 65 percent of their responsiveness to forskolin, and by 24 hours only 15 percent of the forskolin response remained. In contrast, the responsiveness to (−)-iso-

Table 1. Effect of 3-hour challenge with 6 n*M* cholera toxin on cyclic AMP accumulation (measured as picomoles per milligram of protein) in cells previously incubated with cycloheximide (5 μg/ml) for 24 hours. Cycloheximide (5 μg/ml) was included in the 3-hour challenge so that comparison could be made with cells incubated with cycloheximide for 24 hours. Earlier studies (*9*) have demonstrated that cycloheximide increases the response to cholera toxin after 3 hours. Cholera toxin increased cyclic AMP to 210 ± 10 after 3 hours in the absence of cycloheximide. As shown in Table 2, cycloheximide had no effect on basal levels of cyclic AMP.

Treatment	Cyclic AMP (pmole/mg)	
	Control	Cholera toxin
None	25 ± 3	1121 ± 189
Cycloheximide	31 ± 4	1551 ± 138