Mutagenesis of the Phosphorylation Site (Serine 19) of Smooth Muscle Myosin Regulatory Light Chain and Its Effects on the Properties of Myosin†

H. Kamisoyama, Y. Araki,‡ and M. Ikebe*

Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

Received May 27, 1993; Revised Manuscript Received October 7, 1993®

ABSTRACT: A full-length cDNA of smooth muscle regulatory light chain was obtained and the recombinant regulatory light chain was expressed in an *Escherichia coli* expression system. The recombinant regulatory light chain was introduced into myosin or HMM using a subunit exchange strategy [Morita, J., Takashi, R., & Ikebe, M. (1991) *Biochemistry 30*, 9539–9545]. The recombinant wild-type regulatory light chain exhibited the same biological properties as the natural isolate, i.e., phosphorylation at Ser-19 by myosin light-chain kinase and phosphorylation-activated actomyosin ATPase activity. To clarify whether or not the activation of the ATPase by phosphorylation is simply due to the introduction of negative charge, we produced three mutant light chains. Two of them contain Ser-19 substituted by either Asp or Ala and the third contains Asp substituted for both Thr-18 and Ser-19. Incorporation of the Asp mutant partially activated actomyosin ATPase activity but the activation level was significantly lower than that by phosphorylation. The Asp/Asp mutant further activated actomyosin ATPase activity. On the other hand, the Ala mutant did not affect the ATPase activity. Incorporation of Asp mutant slightly affected the 10S–6S conformational transition and filament formation of myosin. The Asp/Asp mutant more significantly affected the 10S–6S conformational transition and filament formation of myosin. These results suggested that the activation of smooth muscle myosin requires the introduction of negative charge in the defined spacial position. Using Ser-19 deficient mutants, the effects of Thr-18 phosphorylation on myosin function was also studied. Actin-activated ATPase activity of myosin was significantly activated by phosphorylation of Thr-18. The Thr-18 phosphorylation also stabilized the 6S conformation of myosin and induced myosin filament formation.

The activation of the contractile apparatus of vertebrate smooth muscle cells and nonmuscle cells is achieved by phosphorylation of the regulatory light chain of myosin (Hartshorne, 1987; Sellers & Adelstein, 1987). The phosphorylation site primarily responsible for the activation of actomyosin ATPase is Ser-19, and a Ca^{2+}/calmodulin-dependent specific protein kinase, myosin light-chain kinase (MLCK),[1] catalyzes this phosphorylation reaction. Although the bulk of evidence supports the phosphorylation hypothesis, it is still obscure how the phosphorylation of the regulatory light chain (20 000 Da) can affect the ATPase site to activate the cycle rate.

It has been suggested that the conformation at the head–neck junction of myosin is altered by phosphorylation and this triggers the activation of the catalytic site (Ikebe & Hartshorne, 1984, 1985a; Higashihara et al., 1989; Morita et al., 1991). Support for this notion was the finding that S-1 containing intact regulatory light chain does not exhibit actin-activated ATPase activity that is regulated by phosphorylation (Ikebe & Hartshorne, 1985a). Recently, it was also found that the deletion of the amino acid sequence at the N-terminal side of Ser-19 abolished the phosphorylation mediated regulation (Ikebe & Morita, 1991). The essential region is identified to be Arg-13–Arg-16 of the regulatory light chain. These findings suggest that some interaction between the N-terminal region of the regulatory light chain and the heavy chain at the head–neck junction may be important to achieve the communication between the regulatory site and the active site of myosin molecule.

It is known that smooth muscle myosin can form two distinct conformations in solution. These are referred to 10S and 6S myosins according to their sedimentation velocities (Suzuki et al., 1978). These conformations are visualized by electron microscopy and it is found that 10S myosin exists in a folded structure while 6S myosin is an extended conformation (Trybus et al., 1982; Onishi & Wakabayashi, 1982; Craig et al., 1983). The conformational transition is accompanied by a change in Mg^{2+}- and Ca^{2+}-ATPase of myosin and it is found that the ATPase activity is dramatically decreased upon the formation of 10S myosin (Ikebe et al., 1983); this is attributable to the trapping of the nucleotide in the active site (Cross et al., 1986). It is thought that 6S myosin readily assembles thick filaments at low ionic strength while 10S myosin does not. Therefore, the 10S–6S equilibrium can be reflected by the extent of filament formation at low ionic strength. An interesting finding is that the 10S–6S transition is affected by phosphorylation at physiological ionic conditions (Craig et al., 1983; Onishi et al., 1983; Ikebe et al., 1983; Trybus & Lowey; 1984). The mechanism by which phosphorylation of the regulatory light chain at Ser-19 favors the formation of 6S myosin, thereby stabilizing the thick filaments, is still obscure, but the addition of the negative charge may play an important role in destabilizing the folded conformation.

In addition to the phosphorylation at Ser-19, it is known that regulatory light chain in myosin molecule is also

† This work is supported from NIH Grants AR 38888, HL 37117, and AR 41653.

* To whom correspondence should be addressed.

‡ Present address: Department of Obstetrics and Gynecology, Yamagata University School of Medicine, Yamagata, Japan.

® Abstract published in *Advance ACS Abstracts,* January 1, 1994.

[1] Abbreviations: MLCK, myosin light-chain kinase; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; HMM, heavy meromyosin; S-1, heavy meromyosin subfragment 1; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside.

0006-2960/94/0433-840$04.50/0

phosphorylated at Thr-18 (Ikebe et al., 1986) by myosin light-chain kinase and at Ser-1 and -2 and Thr-9 by protein kinase C (Ikebe et al., 1987b; Begnur et al., 1987). It was shown that the phosphorylation of Thr-18 further activates actomyosin ATPase although the effects of phosphorylation at Thr-18 alone are not known. On the other hand, the phosphorylation of the regulatory light chain at Ser-1 and -2 (Ikebe et al., 1987b) and Thr-9 (Nishikawa et al., 1984; Ikebe et al., 1987b) does not activate actomyosin ATPase but rather decreases the affinity for actin of heavy meromyosin phosphorylated at Ser-19. These results suggest that the activation of ATPase activity requires phosphorylation at a defined position. In the present study, we have substituted Ser-19 and Thr-18 with aspartic acid or alanine and the effects of introduction of negative charge on myosin function are examined. We also studied the effects of Thr-18 phosphorylation on myosin function using the mutant regulatory light chain deficient in Ser-19. A preliminary report of this paper appeared in abstract form (Kamisoyama et al., 1993).

MATERIALS AND METHODS

Materials. Restriction endonucleases and modifying enzymes were purchased from Boehringer Mannheim (Indianapolis, IN) or U.S. Biochemical (Cleveland, OH). The chicken gizzard cDNA library, constructed in vector λgt11, was obtained from Clontech laboratories (Palo Alto, CA). The *Escherichia coli* expression system using T7 RNA polymerase (Tabor & Richardson, 1985; Tabor et al., 1987) was provided by Dr. S. Tabor (Harvard Medical School). Myosin light-chain kinase was prepared from frozen turkey gizzards (Ikebe et al., 1987a). Myosin was prepared from frozen turkey gizzards as described previously (Ikebe & Hartshorne, 1985b). HMM is prepared from myosin by *Staphylococcus aureus* protease digestion (Ikebe & Hartshorne, 1985a). Calmodulin was prepared from bull testes according to Walsh et al. (1983).

Cloning of the Regulatory Light Chain cDNA. A cDNA library constructed from chicken gizzard in the phage vector λgt11 was screened with degenerate [^{32}P]-end-labeled oligonucleotide probes designed according to the known amino acid sequence (Maita et al., 1981). Phage plaques were transferred to nitrocellulose filters and the DNA on the filters was hybridized at 43 °C overnight in Church's buffer (0.5M Na-P_i pH 7.5, 7% SDS, 1% bovine serum albumin, and 1 mM EDTA) containing radiolabeled oligonucleotide probes at 1 × 10^6 cpm/mL. The filters were washed four times for 5 min with 0.5× SSC (1× SSC is 150 mM NaCl/15mM sodium citrate) at room temperature, two times for 30 min with 0.5× SSC + 0.01% SDS at 43 °C, and then two times with 0.2× SSC. After drying, the filters were exposed to Kodak XAR-5 film with an intensifying screen at −80 °C overnight. Positive plaques were subjected to a second screening and several single plaques were obtained. Phage DNA was prepared by the plate lysate method and then digested by *Eco*RI to obtain the cDNA inserts of regulatory light chain. The cDNA inserts were subcloned into the *Eco*RI site of Bluescript SKII(+) (Strategene, La Jolla, CA). To determine the sequence, the deletion mutants were prepared by unidirectional digestion with exonuclease III and mung bean nuclease as described by Yanisch-Perron et al. (1985). Nucleotide sequences were determined on both strands of DNA by dideoxynucleotide termination method (Sanger et al., 1977) using Sequenase (U.S. Biochemical).

Construction of LC_{20} Expression Vector. The cDNA clone encoding the entire open reading frame of LC_{20} except the first six bases (SLC-1) (Araki & Ikebe, 1990) was subjected to the oligonucleotide-directed nucleotide insertion strategy of Taylor et al. (1985). Nucleotide sequence CCATGTCC was inserted just upstream of the 5′-end of SLC-1 (SLC-1m) (Figure 1). After the insertion of the nucleotides was confirmed by sequencing, the *Eco*RI fragment of SLC-1m containing the complete LC_{20} open reading frame plus a 3′ untranslated sequence was subcloned into the *Eco*RI site of the polylinker of the pT7-7 vector (Tabor & Richardson, 1985; Taylor et al., 1987), giving the pT7-SLC-1m expression vector (Figure 1).

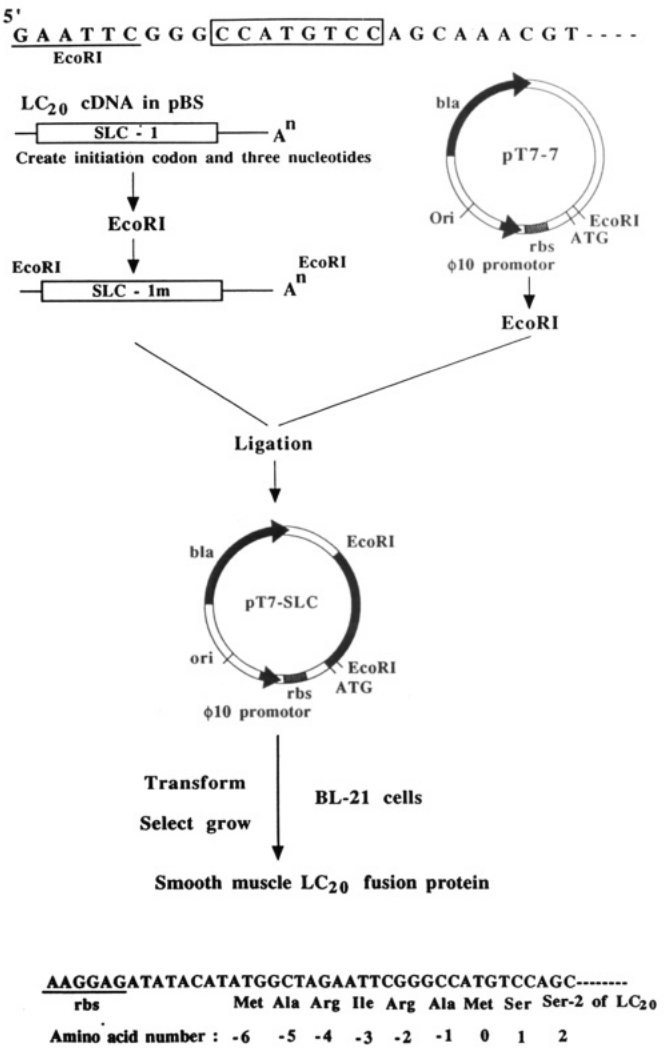

FIGURE 1: Construction of regulatory light chain expression vector. A detailed strategy to construct the expression vector is described in Materials and Methods. SLC-1 lacking an initiation codon and the first three nucleotides (TCC) was subjected to in vitro mutagenesis to insert nucleotide sequence CCA TG TCC just upstream of the 5′ end of SLC-1 (SLC1m). The *Eco*RI fragment of SLCm containing the complete open reading frame of the regulatory light chain was inserted in frame into the *Eco*RI site of vector PT7-7 (pT7-SLC). The recombinant protein contains a six-residue N-terminal tag derived from the vector sequence. pBS: pBluescript SKII(+).

Expression of Recombinant LC_{20} in E. coli. The pT7-7 expression system was used for the expression of recombinant LC_{20}. pT7-7 is a plasmid containing the T7 RNA polymerase promoter ϕ10 in front of a polylinker sequence. *E. coli* strain BL21 competent cells containing T7RNA polymerase gene under the control of the *lac* promoter were transformed with pT7-SLC-1m. The transformants were grown at 37 °C in LB medium supplemented with 25 mg/L ampicillin to OD_{600} = 1.2, and then 1 mM IPTG was added to the medium and

the cells were further cultured at 37 °C for 4 h. Cells were harvested and stored at −80 °C.

Purification of the Recombinant LC_{20}. The packed cells were suspended in buffer containing 30 mM Tris-HCl, pH 7.5, 10 mg/L leupeptin, 3% Triton X-100, and 1 mM EDTA and sonicated for 2 min on ice. After centrifugation at 10000*g* for 5 min, the pellet was washed twice with the same buffer and then washed three times with the same buffer without Triton X-100. The recombinant light chain was extracted with sonication for 2 min in buffer containing 30 mM Tris-HCl pH 7.5, 1 mM DTT, and 7 M urea. The solution was centrifuged at 10000*g* for 10 min and the supernatant (almost all recombinant light chain was recovered in the supernatant) was applied to a DE 52 column equilibrated with 30 mM Tris-HCl, pH 7.5, 1 mM DTT, and 7 M urea. The recombinant light chain was eluted with a linear NaCl gradient (0–0.5 M). The light chain fractions were pooled, dialyzed against 30 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM DTT, and then stored at −80 °C.

Oligonucleotide-Directed in Vitro Mutagenesis. Single-stranded pBS SKII(+)-SLC-1m recombinant DNA was isolated and used as template. Three different oligonucleotides, 5′-CAGCGCGCCACCGACAATGTCTTCGC-3′, 5′-AGCGCGCCACCGCCATTGTCTTC-3′, and CCCGCAGCGCGCCGACGACAATGTCTTCGCTATG, designed for mutation of Ser-19 to Asp and Ala and both Thr-18 and Ser-19 to Asp (underlined codons), respectively, were synthesized by the Case Western Reserve University Molecular Biology Core Laboratory. An oligonucleotide-directed in vitro mutagenesis system, version 2 (Amersham Corp.), was used for preparation of mutant cDNAs. Mutant clones were isolated, and cDNA sequences were reconfirmed by dideoxynucleotide sequencing. The mutant cDNAs were subcloned into the pT7-7 expression vector and expressed using the same strategy of the wild-type recombinant LC_{20}.

Phosphorylation of Recombinant Light Chains. Purified recombinant light chains were phosphorylated in the presence of 15 μg/mL MLCK and 10 μg/mL calmodulin at 25 °C for 30 min in the buffer containing 1 mM ATP, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.3 M KCl, 30 mM Tris-HCl (pH 7.5), and 100 nM Microcystine. The extent of phosphorylation was monitored by urea gel electrophoresis (Perrie & Perry, 1970). For phosphorylation of the light chain at Thr-18, 100 μg/mL MLCK, 50 μg/mL calmodulin, and 50 mM KCl were used.

Exchange of Exogenous 20 000-Da Light Chain into Myosin and HMM. The endogenous 20 000-Da light chain of myosin or HMM was exchanged with 10-fold molar excess exogenous light chain in 0.6 M KCl, 10 mM K-P_i, pH 6.8, 10 mM EDTA, 1 mM DTT, and 1 mM ATP at 42 °C for 20 min. The exchange myosin was dialyzed against 30 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, and 1 mM DTT at 4 °C for 2 h. After centrifugation, the resulting precipitate was washed three times with dialyzing buffer, dissolved in a buffer containing 0.5 M KCl, 30 mM Tris-HCl, and 1 mM DTT, and then centrifuged at 12 000 rpm for 5 min. The supernatant was myosin containing the exchanged light chain and was used for experiments. The exchanged HMM was separated from free light chains using a Sephacryl S-300 (Pharmacia) gel-filtration column equilibrated with 30 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 1 mM DTT.

Other Experimental Procedures. ATPase activity of myosin or actomyosin was measured as described previously (Ikebe & Hartshorne, 1985b). Light scattering of the light chain exchanged myosin was measured at 400 nm using Perkin-Elmer fluorescence spectrophotometer LC-3.

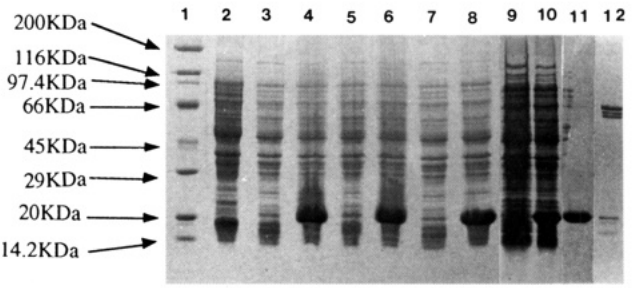

FIGURE 2: SDS–PAGE profile of the expressed recombinant regulatory light chains. The transformants were grown in LB medium with ampicillin at 37 °C. After the OD_{600} reached 1.0, 1 mM IPTG was added and cultured for 6 h at 37 °C. The cells were harvested and subjected to SDS–PAGE analysis. Lane 1, molecular mass standards; lane 2, BL-21 whole cell homogenate; lanes 3 and 4, wild-type light chain; lanes 5 and 6, Ala mutant; lanes 7 and 8, Asp mutant; lanes 9 and 10, Asp/Asp mutant. Lanes 3, 5, 7, and 9, before IPTG induction; lanes 4, 6, 8, and 10, after IPTG induction. Lane 11, purified recombinant wild-type light chain; lane 12, purified HMM exchanged with recombinant wild-type light chain.

Myosin containing the recombinant light chains was observed at various concentrations of KCl and $MgCl_2$ under an electron microscope. Samples were applied to carbon-coated copper grid and negatively stained with 1% aqueous uranyl acetate. Electron micrographs were taken by JEOL JEM 100CX at direct magnification of 33000×.

RESULTS

Expression of the Recombinant Regulatory Light Chains. The recombinant light chains were cloned into the expression vector pT7-7 (see Materials and Methods and Figure 1) and expressed in *E. coli* strain BL21 (DE3) which contains the T7 RNA polymerase gene under the control of the *lacUV5* promoter (Studier & Moffatt, 1986). The recombinant light chains contained six additional amino acid residues at their N-terminal end derived from the vector sequence. The transformants were cultured overnight at 37 °C and then recombinant light chain synthesis was induced by IPTG (Figure 2). The time course of the expression of recombinant light chain after IPTG induction was monitored and it was found that the expression of the light chain increased after 30 min of induction and reached maximum level at 6 h, and then the expression level stayed at the maximum level for at least 16 h (data not shown). The culture of the transformants was stopped after 6 h of IPTG induction and the whole cell lysates were subjected to SDS–PAGE analysis. As shown in Figure 2, a significant amount of recombinant light chain was expressed and the expression level was almost the same for wild type and mutants. The apparent molecular masses of the recombinant light chains (20 700 Da) were slightly higher than that of natural isolates (20 000 Da) due to the additional six amino acid residues derived from pT7-7. It should be noted that a small amount of the recombinant light chain was expressed even before addition of IPTG (Figure 2).

Purification of the Recombinant Regulatory Light Chains. The packed cells expressing the recombinant light chains were lysed in buffer containing 3% Triton X-100 with sonication and washed several times with buffer containing Triton X-100 and then with buffer lacking Triton X-100 (see Materials and Methods). During these steps, the majority of proteins derived from *E. coli* were removed. The recombinant light chains were extracted from the pellet in buffer containing 7 M urea and then subjected to DEAE ion-exchange chromatography. The recombinant wild-type light chain was eluted approximately at 50 mM NaCl and was free from contaminant

Table 1: Actin-Activated ATPase of HMM Exchanged with Recombinant Regulatory Light Chain

	V_{max} (nmol of P min^{-1} mg^{-1})[a]	K_{actin} (mg/mL)
DP-wild-HMM	8.9	1.5
DP-Ala-HMM	6.0	1.8
DP-Asp-HMM	20.0	2.5
P-wild-HMM	215	7.4
P-Ala-HMM	215	15.7
P-Asp-HMM	215	8.7

[a] Conditions for the ATPase assay are 30 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mg/mL HMM, 50 mM KCl, and various actin concentrations. ATPase activity in the absence of actin is subtracted.

proteins (Figure 2). The purified recombinant light chain was well phosphorylated by myosin light-chain kinase as monitored by autoradiography using ^{32}P-labeled ATP on urea gel electrophoresis, in which the phosphorylated light chain migrates faster than the dephosphorylated form (Perrie & Perry, 1970; Ikebe & Hartshorne, 1985c) (data not shown). The time course of phosphorylation of recombinant light chain was identical to that of naturally isolated light chain under identical conditions. Also, the rate of dephosphorylation of the recombinant light chain by myosin light-chain phosphatase (SMPIV) was the same as that of naturally isolated light chain (data not shown). These purified recombinant light chains were used for the following experiments.

Exchange of the Recombinant Regulatory Light Chains into Smooth Muscle Myosin. The recombinant light chains were exchanged into myosin or HMM according to the method of Morita et al. (1991). With a 10-fold molar excess of exogenous regulatory light chain, approximately 90% of the regulatory light chain was exchanged. The efficiency of the exchange was the same for the recombinant light chains and the naturally isolated regulatory light chain. HMM exchanged with the wild-type recombinant light chain was separated from free light chain by Sephacryl S-300 gel-filtration chromatography. The recombinant light chain (molecular mass of 20 700 Da) coeluted with the heavy chain and the 17 000-Da light chain of HMM, suggesting that the exogenous light chain was incorporated into HMM (Figure 2). The endogenous regulatory light chain (molecular mass of 20 000 Da) appeared at the same position as excess exogenous light chain (data not shown). These results indicate that the exogenous recombinant light chain was incorporated into HMM. The three mutant light chains were also similarly incorporated into HMM (data not shown).

ATPase Activity of HMM/Myosin Containing the Recombinant Regulatory Light Chains. The actin-activated ATPase activity of HMM exchanged with the recombinant regulatory light chains was examined. As a control, the wild-type recombinant light chain was first phosphorylated by MLCK at Ser-19 (phosphorylation was monitored by urea gel electrophoresis and the light chain was found to be 100% phosphorylated) and then exchanged into HMM. The actin-activated ATPase activity was enhanced as expected (Table 1). The actin-activated ATPase activity of HMM whose regulatory light chain was exchanged with the mutant light chain (Ser-19 → Asp) was higher than that of dephosphorylated HMM, but it was not as high as that of HMM containing phosphorylated recombinant wild-type light chain (Table 1). On the other hand, the incorporation of another mutant light chain (Ser-19 → Ala) into HMM did not activate the actin-dependent ATPase activity of HMM but the activity was rather less than that for dephosphorylated HMM (Table 1). These results suggested that the actin-activated ATPase is influenced by a negative charge at the 19th position of the regulatory light chain. The mutant light chains were also incorporated into myosin and the actin-activated ATPase activity of myosin was also studied (Table 2). Consistent with the results for acto-HMM ATPase activity, the incorporation of the mutant light chain (Asp) activated actin-activated ATPase activity to some extent but the activation was lower than that for myosin exchanged with the phosphorylated wild-type light chain. The mutant light chain (Asp/Asp) further activated actin-activated ATPase activity (Table 2). On the other hand, the incorporation of the Ala mutant did not affect the actomyosin ATPase activity. The ATPase activity measured in Tables 1 and 2 was not further increased at high ATP concentration; therefore, the values in the tables are V_{max} in terms of ATP concentration.

Table 2: Actin-Activated ATPase Activity of Myosin Exchanged with Recombinant Regulatory Light Chain[a]

	activity (nmol of P min^{-1} mg^{-1})	
myosin[b]	without F-actin	with F-actin
DP-myosin	2.1	2.2
P-myosin	12.5	62.0
PP-myosin	16.5	125.0
DP-wild-myosin	2.0	2.2
P-wild-myosin	12.2	60.5
DP-Ala-myosin	3.1	3.5
P-Ala-myosin	6.5	27.0
DP-Asp-myosin	7.0	13.5
P-Asp-myosin	8.5	51.5
DP-Asp/Asp-myosin	9.0	22.0

[a] Actin-activated ATPase activity was measured in 65 mM KCl, 30 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$ in the presence and absence of 2.0 mg/mL F-actin. [b] DP-myosin, dephosphorylated unexchanged myosin; P-myosin, unexchanged myosin phosphorylated at Ser-19; PP-myosin, unexchanged myosin phosphorylated at both Ser-19 and Thr-18; DP-wild-myosin, dephosphorylated myosin exchanged with dephosphorylated wild-type light chain; P-wild-myosin, dephosphorylated myosin exchanged with phosphorylated (Ser-19) wild-type light chain; DP-Ala-myosin, dephosphorylated myosin exchanged with dephosphorylated Ala mutant; P-Ala-myosin; dephosphorylated myosin exchanged with phosphorylated Ala mutant; DP-Asp-myosin, dephosphorylated myosin exchanged with dephosphorylated Asp mutant; P-Asp-myosin, dephosphorylated myosin exchanged with phosphorylated Asp mutant; DP-Asp/Asp-myosin, dephosphorylated myosin exchanged with Asp/Asp mutant.

It was found previously (Ikebe & Hartshorne, 1985c; Ikebe et al., 1986) that smooth muscle myosin can be phosphorylated by MLCK not only at Ser-19 but also at Thr-18 and that the phosphorylation at Thr-18 further activates actomyosin ATPase. However, the effect of phosphorylation at Thr-18 alone on actomyosin ATPase activity has not been clarified. Using the mutant light chain in which phosphorylatable Ser-19 is substituted by nonphosphorylatable residues, the effect of Thr-18 phosphorylation was studied. The mutant light chains were first phosphorylated in the presence of a high concentration of MLCK (0.1 mg/mL) and calmodulin (50 μg/mL). Phosphorylation of the light chain at the threonine site was confirmed by urea gel electrophoresis (completely phosphorylated) and by phosphoamino acid analysis (data not shown).

Actin-activated ATPase activity of HMM exchanged with the mutant light chains phosphorylated at Thr-18 was significantly higher than that of the dephosphorylated form (Table 1). It should be noted that the K_a value of HMM containing Thr phosphorylated light chains was significantly larger than that containing Ser phosphorylated light chains. Actin-activated ATPase activity of myosin exchanged with the Thr-18 phosphorylated mutant light chains is shown in

Table 2. The ATPase activity was significantly activated. The activity of myosin exchanged with phosphorylated Asp mutant was similar to myosin exchanged with Ser-19 phosphorylated wild-type light chain. The activity of myosin exchanged with phosphorylated Ala mutant was also activated although the value was lower than that for Ser-19 phosphorylated myosin. The results show that phosphorylation at Thr-18 by MLCK can activate actomyosin ATPase and is consistent with the earlier findings (Ikebe et al., 1986; Ikebe & Hartshorne, 1985c), which showed that the additional phosphorylation at Thr-18 further activates the actin-activated ATPase activity of myosin which is phosphorylated at Ser-19 of the regulatory light chain.

Thick Filament Formation and the 10S–6S Conformational Transition of Myosin Containing the Recombinant Regulatory Light Chains. It is known that smooth muscle myosin forms two distinct conformations and these are referred to as 10S and 6S myosin (see introduction). The formation of the two conformations is affected by salt concentration and the transition from 6S to 10S occurs between 0.2 and 0.3 M KCl (NaCl) concentration (Ikebe et al., 1983). The transition is paralleled by the marked depression of Mg^{2+}-ATPase activity of myosin (Ikebe et al., 1983); therefore, the conformational transition of myosin containing the recombinant light chains was monitored by measuring KCl dependence of myosin Mg^{2+}-ATPase activity (Figure 3). The Mg^{2+}-ATPase activity of dephosphorylated myosin markedly decreased below 0.3 M KCl and this confirmed the earlier results (Ikebe et al., 1983). The myosin exchanged with the wild-type dephosphorylated light chain showed the same KCl dependence of the Mg^{2+}-ATPase activity, while myosin exchanged with the phosphorylated wild-type light chain showed higher ATPase activity above 0.2 M KCl and the ATPase activity decreased below 0.2 M KCl; i.e., the 6S conformation was more stabilized. This is consistent with the earlier results (Ikebe et al., 1983) in which phosphorylation of the regulatory light chain of myosin favors the 6S conformation.

The myosin containing Ala mutant light chain showed KCl dependence of Mg^{2+}-ATPase activity similar to that of dephosphorylated myosin, suggesting that the substitution of Ser-19 for Ala does not affect the 6S–10S conformational transition of myosin. The KCl dependence of the ATPase activity of the myosin containing Asp mutant light chain shifted toward the left, suggesting that the introduction of Asp at the 19th position favors the 6S conformation of myosin. The incorporation of Asp/Asp mutant light chain further shifted the KCl dependence toward the left. On the other hand, the incorporation of the light chain mutants phosphorylated at Thr-18 significantly shifted the KCl dependence curve of the Mg^{2+}-ATPase activity to the left, which is similar to that for myosin exchanged with the phosphorylated wild-type light chain (Figure 3). These results suggest that phosphorylation at Thr-18 stabilizes the 6S conformation like the phosphorylation at Ser-19. This is also consistent with the earlier results (Ikebe et al., 1988) that phosphorylation at Thr-18 further stabilizes the 6S conformation of myosin solely phosphorylated at Ser-19.

It has been suggested that the stabilization of 6S conformation and myosin filament formation are related to each other (see introduction). Myosin filament formation was first monitored by light scattering (data not shown). The myosins containing various states of light chains were classified into three groups. The first one is the myosin containing dephosphorylated wild-type light chain or dephosphorylated Ala mutant. This group showed low light scattering intensity,

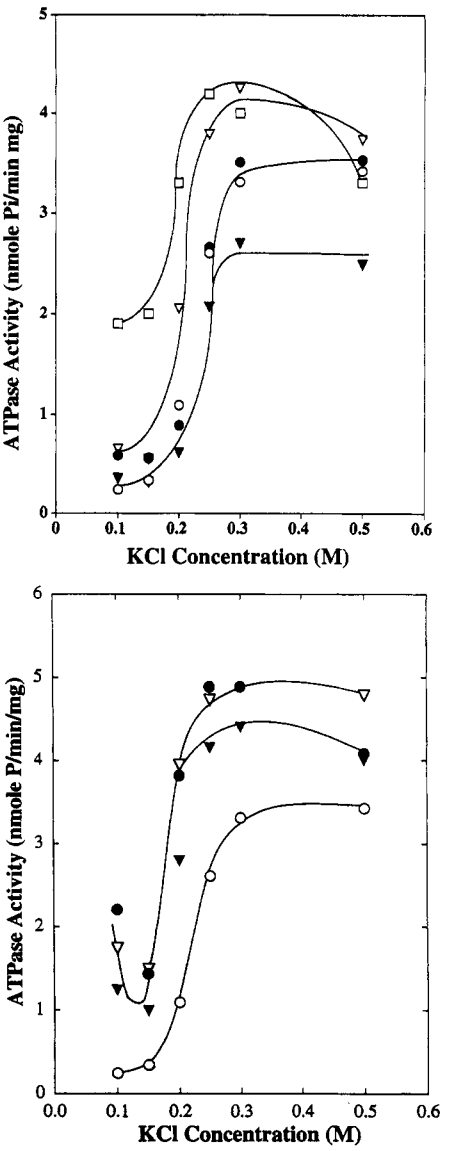

FIGURE 3: KCl dependence of Mg^{2+}-ATPase activity of myosin exchanged with recombinant regulatory light chains. Conditions for ATPase assay: 30 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mg/mL myosin, 1 mM EGTA, and various KCl concentrations. (Top panel) ○, dephosphorylated myosin; ●, dephosphorylated myosin exchanged with dephosphorylated wild-type light chain; ▽, dephosphorylated myosin exchanged with dephosphorylated Asp mutant; ▼, dephosphorylated myosin exchanged with dephosphorylated Ala mutant; □, myosin exchanged with Asp/Asp mutant. (Bottom panel) ○, dephosphorylated myosin; ●, dephosphorylated myosin exchanged with Ser 19 phosphorylated wild-type light chain; ▽, dephosphorylated myosin exchanged with Thr-18 phosphorylated Asp mutant; ▼, dephosphorylated myosin exchanged with Thr-18 phosphorylated Ala mutant.

suggesting that there are few myosin filaments. The second group is the myosin containing dephosphorylated Asp mutant and this showed slightly higher light scattering intensity than the first group, suggesting there are some filaments. The third group is the myosin containing Asp/Asp mutant, phosphorylated wild-type light chain, or phosphorylated mutant light chains (Thr phosphorylated). These myosins showed significantly higher light scattering intensities than the first group, suggesting the formation of thick filaments. The thick filaments of myosin containing the recombinant light chains are shown in Figure 4. The myosin containing dephosphorylated wild-type light chain or dephosphorylated Ala mutant light chain hardly formed filaments. The majority of myosin existed as nonfilamentous form (presumably

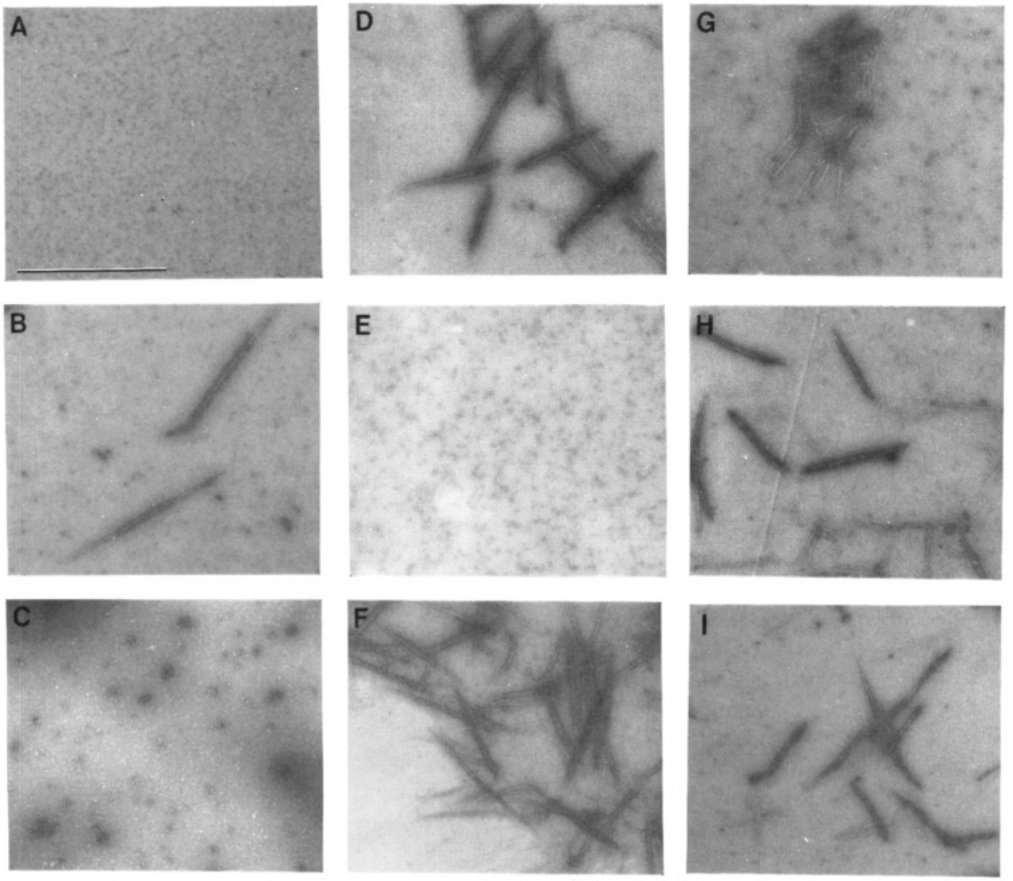

FIGURE 4: Electron micrographic images of negatively stained smooth muscle myosin exchanged with the recombinant regulatory light chains. Conditions: 0.1 mg/mL myosin, 25 mM KCl, 1 mM ATP, and 1 mM EGTA. (A) dephosphorylated myosin; (B) phosphorylated myosin; (C) dephosphorylated myosin exchanged with dephosphorylated wild-type light chain; (D) dephosphorylated myosin exchanged with Ser-19 phosphorylated wild-type light chain; (E) dephosphorylated myosin exchanged with dephosphorylated Ala mutant; (F) dephosphorylated myosin exchanged with Thr-18 phosphorylated Ala mutant; (G) dephosphorylated myosin exchanged with dephosphorylated Asp mutant; (H) dephosphorylated myosin exchanged with Thr-18 phosphorylated Asp mutant; (I) dephosphorylated myosin exchanged with Asp/Asp mutant. The bar in panel A represents approximately 1 μm.

monomers and/or dimers) and these can be seen in the negatively stained sample as background materials (Figure 4). Some filaments were found for myosin containing dephosphorylated Asp mutant light chain, while large numbers of filaments were found for myosin containing Asp/Asp mutant light chain. On the other hand, myosin containing the mutant light chains phosphorylated at Thr-18 formed significant amounts of myosin filaments which are virtually the same as those for myoxin containing Ser-19 phosphorylated light chain.

DISCUSSION

Although it is known that the phosphorylation of the regulatory light chain of myosin by MLCK activates actin-activated ATPase activity of myosin, it is obscure how the phosphorylation can activate the ATPase activity. Since MLCK-induced phosphorylation introduces a negative charge on Thr-18 and Ser-19, we examined whether or not the introduction of the negative charge on the 18th and 19th position of the regulatory light chain sequence mimics the effects of phosphorylation. It is known for calmodulin-dependent protein kinase II that the substitution of Thr-286 (autophosphorylation site of the kinase which activates the kinase activity in the absence of Ca^{2+}/calmodulin) by aspartic acid mimics the activation of the kinase by authophosphorylation at Thr-286 (Fong et al., 1989). Ser-19 of the regulatory light chain was substituted by either aspartic acid, to see the effect of negative charge, or alanine, to see the possible inhibitory effect of the side chain of the serine residue, on the ATPase activity. Substitution of the amino acid residue was achieved by site-directed mutagenesis of the cDNA of the regulatory light chain, and the recombinant proteins were expressed in *E. coli*. The recombinant proteins contained a six amino acid residue N-terminal tag, however, we think that the recombinant light chain functions as well as the natural regulatory light chain because (1) the recombinant light chain was phosphorylated by MLCK and dephosphorylated by myosin light chain phosphatase at a rate similar to the natural isolate and (2) the phosphorylated wild-type recombinant light chain incorporated in the myosin molecule activated actin-activated ATPase of myosin. The mutant light chains were introduced into myosin or HMM molecules by means of subunit exchange. The incorporation of the Asp mutant light chain activated actomyosin ATPase; however, the extent of activation was much lower than the incorporation of the phosphorylated light chain. The incorporation of the Asp/Asp mutant light chain further activated actomyosin ATPase although the activity was lower than that of the phosphorylated light chain. The results suggested that the negative charge at the 18th and 19th position of light chain is partially responsible for the activation of actomyosin ATPase. However, since the incorporation of the phosphate moiety is necessary for full activation, the results also suggest that the specific spatial position of the negative charge may be important for the activation of actomyosin ATPase activity. The Ala mutant behaves the same as dephosphorylated light chain; that is, the incorporation of Ala mutant did not activate the ATPase. This suggests that the side chain of the Ser-19 residue is not responsible for the inactivation of actin-activated

myosin ATPase activity. We also examined the effect of the substitution at the 18th and 19th position on the 10S–6S transition and thick filament formation. The substitution of Ser by Ala did not alter the 10S–6S transition of myosin conformation and myosin filament formation, while the Asp mutant slightly affected both the 10S–6S transition and thick filament formation, although the effects were much less than for phosphorylation. On the other hand, the Asp/Asp mutant more dramatically affected both the 10S–6S transition and thick filament formation. It is known that phosphorylation at Ser-19 of the regulatory light chain alters the 10S–6S conformational transition (Trybus et al., 1983; Craig et al., 1983; Ikebe et al., 1983; Onishi et al., 1983) and myosin filament formation (Suzuki et al., 1978). Confirming this earlier finding, the incorporation of the phosphorylated recombinant light chain into myosin altered the 10S–6S transition and enhanced myosin filament formation, showing that a sufficient amount of exogenous light chain was incorporated into myosin to exhibit the change in myosin conformation. Therefore, the above results suggest that the negative charge at both the 18th and 19th position is sufficient to change the conformational transition. However, negative charge at only the 19th position is not sufficient for conformational change. An alternative explanation is that the specific location of the negative charge on position 19 may be essential to abolish 10S conformation. The Asp/Asp mutant completely mimics the phosphorylation in terms of thick filament formation and the 10S–6S transition but not completely in terms of actomyosin ATPase activity, suggesting that the activation of the ATPase activity requires a more defined interaction of the negative charge on the phosphate moiety with other amino acid side chains, although phosphorylation initiates change on both the 10S–6S transition and actin activated-ATPase activity. In other words, neither the 10S–6S transition nor filament formation directly affects the actin-activated ATPase activity although they are related to each other. It is shown that the incorporation of the truncated regulatory light chain (residues 22–171) formed only a partially folded myosin (Trybus & Lowey, 1988). We also found that the incorporation of the truncated regulatory light chain (residues 17–171) which contains Ser-19 does not form 10S myosin (Ikebe & Morita, unpublished observation). These findings suggest that the N-terminal portion of the regulatory light chain contains the binding site to the tail portion of myosin heavy chain so as to form a folded conformation. It is also reported that smooth muscle myosin containing skeletal muscle regulatory light chain does not form a proper folded conformation (Trybus & Lowey, 1988). The major difference between the smooth and skeletal regulatory light chain near the phosphorylation site is that Arg-16 in smooth muscle light chain is substituted for glutamic acid in skeletal muscle light chain. Therefore, it may be hypothesized that the charge interaction between the phosphate moiety and the side chain of Arg-16 could be responsible for the change in the conformation of myosin by phosphorylation.

Using mutant light chain, we were able to prepare light chain phosphorylated only at Thr-18. Actin-activated ATPase activity of myosin was significantly activated when Thr-18 phosphorylated light chains are incorporated. It has been known that phosphorylation of the light chain at Thr-18 occurs in vivo (Colburn et al., 1988); however, phosphorylation at Thr-18 is predominantly found in diphosphorylated light chain (phosphorylated at both Ser-19 and Thr-18). Therefore, smooth muscle activation of actomyosin by threonine phosphorylation alone probably does not significantly contribute to the physiological activation of contractile machinery. It is interesting that phosphorylation at Thr-18 is effective for the activation of actin-activated ATPase activity similar to phosphorylation at Ser-19, since the three-dimensional position of the negative charge necessary for the activation presumably must be very limited. The phosphorylation at Thr-18 also influenced the 10S-6S transition as well as thick filament formation. The effects were nearly identical to those of Ser-19 phosphorylation. These results suggest that the phosphate moiety of Ser-19 and Thr-18 interact with the same moiety of certain residues. Such an amino acid residue could be on the heavy chain or 17 000-Da light chain; however, considering that the truncation of amino acids of light chain 13–16 (RPQR) abolishes the phosphorylation-dependent regulation (Ikebe & Morita, 1991), a residue interacting with the phosphate moiety is likely to be on the N-terminal side of the light chain. More detailed information to understand the interaction between the phosphate moiety and other residues requires further study using a combination of molecular biology and protein biochemistry.

REFERENCES

Araki, Y., & Ikebe, M. (1990) *Biophys. J. 57*, 159A.

Begnur, A. R., Robinson, E. A., Appella, E., & Sellers, J. R. (1987) *J. Biol. Chem. 262*, 7613–7617.

Colburn, J. C., Michnoff, C. H., Hsu, L.-C., Slaughter, C. A., Kamm, K. E., & Stull, J. C. (1988) *J. Biol. Chem. 263*, 19166–19173.

Craig, R. A., Smith, R., & Kendrick-Jones, J. (1983) *Nature 302*, 436–439.

Cross, R. A., Cross, K. E., & Sobieszek, A. (1986) *EMBO J. 5*, 2637–2641.

Fong, Y.-L., Taylor, W. Z., Means, A. R., & Soderling, T. R. (1989) *J. Biol. Chem. 264*, 16759–16763.

Hartshorne, D. J. (1987) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., Ed.) 2nd ed., Vol. 1, pp 423–482, Raven Press, New York.

Higashihara, M., Young-Frado, K.-L., Craig, R., & Ikebe, M. (1989) *J. Biol. Chem. 264*, 5218–5225.

Ikebe, M., & Hartshorne, D. J. (1984) *J. Biol. Chem. 259*, 11639–11642.

Ikebe, M., & Hartshorne, D. J. (1985a) *Biochemistry 24*, 2380–2387.

Ikebe, M., & Hartshorne, D. J. (1985b) *J. Biol. Chem. 260*, 13146–13153.

Ikebe, M., & Hartshorne, D. J. (1985c) *J. Biol. Chem. 260*, 10027–10031.

Ikebe, M., & Morita, J. (1991) *J. Biol. Chem. 266*, 21339–21342.

Ikebe, M., Hinkins, S., & Hartshorne, D. J. (1983) *Biochemistry 22*, 4580–4587.

Ikebe, M., Hartshorne, D. J., & Elzinga, M. (1986) *J. Biol. Chem. 261*, 36–39.

Ikebe, M., Hartshorne, D. J., & Elzinga, M. (1987a) *J. Biol. Chem. 262*, 9569–9573.

Ikebe, M., Stepinska, M., Kemp, B. E., Means, A. R., & Hartshorne, D. J. (1987b) *J. Biol. Chem. 262*, 13828–13834.

Ikebe, M., Koretz, J. R., & Hartshorne, D. J. (1988) *J. Biol. Chem. 263*, 6432–6437.

Kamisoyama, H., Araki, Y., & Ikebe, M. (1993) *Biophys. J. 64*, A142.

Maita, T., Chen, J. I., & Matsuda, G. (1981) *Eur. J. Biochem. 117*, 417–424.

Morita, J., Takashi, R., & Ikebe, M. (1991) *Biochemistry 30*, 9539–9545.

Nishikawa, M., Sellers, J. R., Adelstein, R. S., & Hidaka, H. (1984) *J. Biol. Chem. 259*, 8808–8814.

Onishi, H., & Wakabayashi, T. (1982) *J. Biochem. (Tokyo) 92*, 871–879.

Onishi, H., Wakabayashi, T., Kamata, T., & Watanabe, S. (1983) *J. Biochem. (Tokyo) 94*, 1147–1154.
Perrie, W. T., & Perry, S. V. (1970) *Biochem. J. 119*, 31–38.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5464.
Sellers, J. R., & Adelstein, R. A. (1987) in *The Enzymes*, ed. (Boyer, P., and Krebs, E. G., Eds.) Vol. 18, pp 381–418, Academic Press, San Diego, CA.
Studier, F. W., & Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113–130.
Suzuki, H., Onishi, H., Takahashi, K., & Watanabe, S. (1978) *J. Biochem. (Tokyo) 84*, 1529–1542.
Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074–1078.
Tabor, S., Huber, H. E., & Richardson, C. C. (1987) *J. Biol. Chem. 262*, 16212–16223.
Taylor, J. W., Ott, J., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8764–8765.
Trybus, K. M., & Lowey, S. (1984) *J. Biol. Chem. 259*, 8564–8571.
Trybus, K. M., & Lowey, S. (1988) *J. Biol. Chem. 263*, 16485–16492.
Trybus, K. M., Huiatt, T. M., & Lowey, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6151–6155.
Walsh, M. P., Hinkins, S., Dabrowska, R., & Hartshorne, D. J. (1983) *Methods Enzymol. 99*, 279–288.
Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103–109.