Modulation of Syrian Hamster 3-Hydroxy-3-methylglutaryl-CoA Reductase Activity by Phosphorylation

ROLE OF SERINE 871

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Phosphorylation of key metabolic enzymes at specific residues has long been known to modulate their catalytic activity. Phosphorylation can result in a conformational change at the active site after phosphorylation at a remote, allosteric site (glycogen phosphorylase; Refs. 15), a conformational change of a pseudo-substrate autoinhibitory domain (pp70S6 kinase; Refs. 16 and 17), or an interaction at the active site itself (Escherichia coli isocitrate dehydrogenase; Refs. 18–20). No consensus has, however, emerged concerning whether the effects of phosphorylation are due to negative charge or to steric factors. The contributions of charge and bulk have been investigated by replacing phosphorylatable serines with bulky or negatively charged amino acids. For E. coli isocitrate dehydrogenase (18–20) and the product of the fos proto-oncogene (21), the introduction of negative charge mimics phosphorylation. However, unlike phosphorylation, the presence of acidic residues at the C terminus of the cyclic AMP-responsive element-binding protein did not abolish transcriptional activity (22), nor did substitution of glutamate for Ser871 and Ser478 of the epidermal growth factor-like protein ErbB mimic the ability of phosphorylation to suppress oncoprogenic potential (23).

Ser871 of Syrian hamster HMG-CoA reductase is located within 6 residues of His866, a residue functional in catalysis (24). By analogy to the recently solved crystal structure of Pseudomonas aerofaciens HMG-CoA reductase (25), His866 should reside in a flexible domain that approaches the active site when substrates are bound. We investigate here whether Ser871, the site of phosphorylation in the rat liver enzyme (13), is essential for catalysis. We also replaced Ser871 with charged, neutral, and bulky residues to investigate whether the effects of phosphorylation are due to negative charge or steric properties. In addition, we have investigated the residue specificity of the AMP-activated protein kinase, the kinase of prime physiologic importance for regulation of HMG-CoA reductase (26), by exploiting the presence of the protein kinase recognition sequence around Ser871 of hamster HMG-CoA reductase.

EXPERIMENTAL PROCEDURES

Chemicals—Purchased reagents included (R)-HMG-CoA, phosphoserine, and phosphothreonine (Sigma); Blue Sepharose CL-6B (Pharmacia LKB Biotechnology Inc.); T4 DNA ligase, a Sequenase® version 2 kit, and a SurePure oligonucleotide purification kit (U. S. Biochemical Corp.); restriction enzymes (New England Biolabs, Promega, or U. S. Biochemical Corp.); T4 polymerase (New England Biolabs); [γ-32P]ATP, Hyperfilm-MP™ x-ray film, and a site-directed mutagenesis kit (Amersham Corp.); [γ-32P]ATP (DuPont NEN); cellulose TLC sheets (Eastman); and Eclumce scintillation fluid (ICN Biomedicals). AMP-activated kinase (13) and its synthetic SAMS peptide substrate (HMB3AMSGLHUVRR) were generous gifts from Joe Fain and David Gibson of the Indiana University School of Medicine. The protein kinase was dissolved in Buffer K (50 mM NaF, 1.0 mM EDTA, 1 mM EGTA, 1.0 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1.0 mM benzamidine, 10% (v/v) glycerol, 0.02% Brij, 1 μM soybean trypsin inhibitor, 50 μM Tris-HCl, and 5.0 mM sodium pyrophosphate, pH 7.5). Other materials were from previously cited sources (24, 27, 28).

The reaction catalyzed by HMG-CoA reductase is a (S)-HMG-CoA + 2 NADP+ + 2 H+ → (R)-mevalonate + CoASH + 2 NADPH, constituting a major focus for control of the biosynthesis of polyisoprenoids and other mevalonate-derived metabolites. Knowledge of the mechanism of catalysis and of the regulation of the catalytic activity of HMG-CoA reductase thus is important for rational chemotherapy of certain forms of hypercholesterolemia. Regulation of higher eukaryote HMG-CoA reductases involves both changes in enzyme quantity and modulation of catalytic activity by post-translational processes that include phosphorylation (1–10). The protein kinases that phosphorylate the rat liver enzyme at serine 871 (13, 14).

The abbreviations used are: HMG, 3-hydroxy-3-methylglutaryl; PAGE, polyacrylamide gel electrophoresis.

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Phagemids, Phage, and Bacterial Strains—E. coli BL21(DE3) (29) grown on LB medium (30) plus 50 μg/ml ampicillin served as host for derivatives of phagemid pKFT7-21 (28), which encodes the catalytic domain of Syrian hamster HMG-CoA reductase. M13K07 phage (Promega) was propagated in E. coli JM101 grown on 2 × YT medium (30). Growth was at 37 °C with shaking at 300 rpm.

Site-directed Mutagenesis and DNA Sequencing—Molecular biological procedures followed Sambrook et al. (30). Isolation of single-stranded pKFT7-21 DNA used as template in site-directed mutagenesis employed helper phage M13K07 and the Proenege procedure (31). Mutagenesis employed an Amersham site-directed mutagenesis kit (32) and mutant oligonucleotides (Table I). All mutations were verified by double-stranded sequencing of phagemid DNA using the Sanger dideoxyxenase chain termination method (33) as modified by Hsiang et al. (34). Sequencing employed a Sequenase kit, [d5S1dATP, and the section of NADPH at 37 °C was monitored at 340 nm in a Hewlett-Packard model 8452 diode array spectrophotometer. Standard assays contained 150 pl: 270 mM HEPES, pH 7.5, 50 mM NaCl, 1.0 mM MgCl2, 5 mM dithiothreitol, 10% (v/v) glycerol, 50 mM Tris-HCl, pH 7.3, plus the additions and under the conditions listed in figure legends.

Assay of HMG-CoA Reductase Activity—HMG-CoA-dependent oxidation of NADPH at 37 °C was monitored at 340 nm in a Hewlett-Packard model 8452 diode array spectrophotometer. Standard assays contained, in 150 pl: 270 mM (RS)-HMG-CoA, 270 mM NADPH, 50 mM NaCl, 1.0 mM EDTA, 5.0 mM dithiothreitol, and 200 mM Na2PO4, pH 6.5. Reactions were initiated with HMG-CoA. One unit of HMG-CoA reductase catalyzes oxidation of 1 μmol of NADPH (0.5 μmol of HMG-CoA)/min. Protein determinations by the method of Bradford (35) used bovine serum albumin as standard.

Phosphorylation by AMP-activated Protein Kinase—Incubations were conducted at 37 °C in Buffer T (50 mM NaCl, 100 mM sucrose, 5 mM dithiothreitol, 10% (v/v) glycerol, 50 mM Tris-HCl, pH 7.3), plus the additions and under the conditions listed in figure legends.

Assay of Protein Kinase Activity—AMP-activated protein kinase activity was assayed by measuring incorporation of 32P from [γ-32P]ATP into the SAMS peptide (36). Incubations contained, in 30 pl, 200 μM SAMS peptide, 200 μM [γ-32P]ATP, 200 μM AMP, 5 mM MgCl2, 5 mM dithiothreitol, 6 μl (14 μg) of protein kinase in Buffer K, 11 μl of Buffer T, and either 270 μM (RS)-HMG-CoA, 270 mM NADPH, or neither. Incubations, initiated by adding MgATP, were at 37 °C for 10 min. The 32P present in the SAMS peptide was then determined as described by Davies et al. (36).

 Autoradiography and Measurement of Incorporation of 32P—Following resolution of samples by SDS-PAGE (30), gels were soaked in 10% methanol, 10% acetic acid, dried under vacuum (60 °C, 30 min), and exposed to x-ray film at −70 °C. Major radioactive bands, which corresponded in mobility to the HMG-CoA reductase subunit, were excised and counted in scintillation fluid in a Beckman model LS 1801 scintillation spectrometer.

RESULTS

Homogeneity and Physical Integrity of Wild-type and Ser71 Mutant Enzymes—Wild-type enzyme and mutant HMG-CoA reductases S871A, S871D, S871E, S871H, S871N, S871Q, and S871T, purified as previously described for the wild-type enzyme (28) were over 95% homogeneous as judged by SDS-PAGE (Fig. 1). All purified enzymes exhibited wild-type chromatographic behavior during molecular sieve high-performance liquid chromatography on a Bio-Rad Bio-Sil TSK-250 column eluted with 200 mM Na2PO4, pH 6.8, implying that each possessed wild-type quaternary structure.

Incorporation of 32P Is Accompanied by Attenuation of Catalytic Activity—MGATP plus the AMP-activated protein kinase attenuated the activity of wild-type HMG-CoA reductase in a time-dependent manner (Fig. 2). No attenuation was observed when MgATP, kinase, or both were omitted. Attenuation of activity accompanied incorporation of radioisotope from [γ-32P]ATP (Fig. 3).

HMG-CoA or NADPH Decreases the Susceptibility of HMG-CoA Reductase to Attenuation of Its Activity—HMG-CoA or NADPH decreased the rate at which the protein kinase attenuated HMG-CoA reductase activity. The time required to attenuate activity half its initial value increased from 43 min (no additions) to 120 min (+NADPH) or to 250 min (+HMG-CoA) (Fig. 4). These changes reflect effects on HMG-CoA reductase, not on the protein kinase. Kinase activity measured using the SAMS peptide, 10.2 ± 0.1 nmol/min/mg, was unaffected by either HMG-CoA or NADPH, even under conditions when the kinase was rate-limiting.

Ser71 Is Not Essential for Catalysis or Substrate Binding—Ser71 cannot be essential for catalysis since the specific activities of mutant enzymes S871A, S871N, and S871H approached wild-type values (Table II). Ser71 also does not appear essential for substrate binding since Km values for both substrates approximated wild-type values (Fig. 5, Table III).

Susceptibility of Ser71 Mutant Enzymes to Phosphorylation and Attenuation of Their Activity—The AMP-activated kinase neither affected the activity of mutant enzymes S871A, S871D, S871E, S871H, S871Q, or S871T (Fig. 6), nor catalyzed their phosphorylation (Fig. 7). The kinase thus recognized and phosphorylated only Ser71 of hamster HMG-CoA reductase.

AMP-activated Protein Kinase Can Phosphorylate Threonyl Residues—The presence in HMG-CoA reductase of the recognition sequence for the AMP-activated protein kinase permitted us to ask whether this kinase, not previously reported to catalyze phosphorylation of residues other than serine, could phosphorylate threonyl or tyrosyl residues. Mutant enzyme S871T, but not mutant enzyme S871Y or any other S871 mutant enzyme, was indeed phosphorylated (Fig. 7). Phosphorylation of threonine was confirmed by the detection of 32P-labeled phosphothreonine in an acid hydrolysate of phosphorylated mutant enzyme S871T (Fig. 8).

Phosphorylation of a Threonyl Residue Attenuates HMG-CoA
Regulation of HMG-CoA Reductase Activity

**FIG. 2.** Susceptibility of wild-type HMG-CoA reductase to attenuation of its activity by AMP-activated protein kinase. Incubations contained 200 μM ATP, 5 mM MgCl₂, 200 μM AMP, 55 μM of Buffer T, 20 μl (7.5 pg) of AMP-activated kinase in Buffer K, and 4.6 pg of wild-type HMG-CoA reductase (●) in a final volume of 100 μl. Portions removed at the indicated times were assayed for HMG-CoA reductase activity. Control incubations lacked MgATP (○), AMP-activated kinase (△), or both (□).

**FIG. 3.** Incorporation of radioactivity from [γ-32P]ATP is accompanied by attenuation of HMG-CoA reductase activity. Conditions approximated those of Fig. 2. Parallel 100-μl incubations at 37 °C employed 4.6 μg (94.4 pmol) of wild-type HMG-CoA reductase and 200 mM non-radioactive ATP or [γ-32P]ATP (specific activity 1,000–3,000 cpm/pmol). Portions (5 μl) of the non-radioactive incubation were assayed for activity, and 5-μl portions of the radioactive incubation were subjected to SDS-PAGE. Radioactivity incorporated into HMG-CoA reductase was measured as described under "Experimental Procedures." Shown is the fraction of the initial activity remaining (●) and the fraction phosphorylated (○) (pmol of 32P incorporated/pmol of HMG-CoA reductase). Inset, intensity of major radioactive bands present at the indicated times.

**FIG. 4.** NADPH and HMG-CoA decrease the susceptibility of HMG-CoA reductase to attenuation of its activity. The experiment was conducted under the conditions described for Fig. 2 except that either 270 μM HMG-CoA or 270 μM NADPH were mixed with HMG-CoA reductase prior to addition of the kinase and MgATP. Shown are: no additions (●), NADPH (△), and (RS)-HMG-CoA (○).

**TABLE II** Specific activities of wild-type and mutant enzymes

| Enzyme | Units | Specific activity |
|--------|-------|------------------|
| Wild type | 16 | 100 |
| S871A | 14 | 88 |
| S871H | 16 | 100 |
| S871N | 17 | 106 |
| S871Q | 10 | 62 |
| S871Y | 8.8 | 55 |
| S871E | 2.5 | 16 |
| S871D | 1.6 | 10 |

**DISCUSSION**

Reversible phosphorylation has long been known to attenuate the catalytic activity of the HMG-CoA reductase of higher eukaryotes (3, 6, 10). We have investigated whether Ser971, the putative site of phosphorylation of hamster HMG-CoA reductase, functions in catalysis and have assessed the relative importance of charge and bulk at position S871 in attenuating catalytic activity.

As was first shown for rat liver HMG-CoA reductase (13), the AMP-activated protein kinase phosphorylated the catalytic domain of the Syrian hamster enzyme exclusively at Ser971. Although not far from active site residue His865, Ser971 plays no role in catalysis since replacement of Ser971 by alanine, asparagine, or histidine had no significant effect on catalytic activity. We also consider it unlikely that Ser971 participates in substrate recognition since the Keq values of NADPH and HMG-CoA approximated wild-type values for all eight Ser971 mutant enzymes. While for E. coli isocitrate dehydrogenase the regulatory serine is present at the active site, unlike for isocitrate dehydrogenase, Ser971 of HMG-CoA reductase does not appear to participate in substrate binding.

While substrates protect HMG-CoA reductase against various inhibitors (37–39), the effect of substrates on the susceptibility of HMG-CoA reductase to phosphorylation has not previously been reported. The rate at which the activity of wild-type HMG-CoA reductase was attenuated by phosphorylation was decreased by factors of 3 and 6 by NADPH or HMG-CoA, respectively. These substrates do not inhibit the kinase itself since kinase activity measured using the synthetic SAMS peptide was unaffected by HMG-CoA or NADPH.
Ser\(^{871}\) of Syrian hamster HMG-CoA reductase is located within 6 residues of His\(^{869}\), a residue functional in catalysis (24). By analogy to the recently solved crystal structure of \(P, m\) evaculonii HMG-CoA reductase (25), His\(^{869}\) and Ser\(^{871}\) should reside in a flexible domain at the C-terminal region that approaches the active site when substrates bind. A decreased rate of inactivation in the presence of other substrate thus probably reflects decreased availability of Ser\(^{871}\) to the kinase consequent to a substrate-induced conformational change in the C-terminal region of the enzyme. The susceptibility of HMG-CoA reductase to phosphorylation thus might in future be exploited to study conformational changes at the C-terminal region of the enzyme.

For isocitrate dehydrogenase, replacement of Ser\(^{113}\) by glutamate or aspartate mimicked the effects of phosphorylation. Substitution of tyrosine, a residue similar in size to a phosphorylated serine, also attenuated activity, but less severely (18, 19). These investigators concluded that the effects of phosphorylation resulted primarily from electrostatic repulsion (18, 19). HMG-CoA reductase behaved in an analogous fashion. Replacement of Ser\(^{871}\) by aspartate or glutamate attenuated catalytic activity, while replacement by tyrosine attenuated activity less severely. Replacement of Ser\(^{871}\) by asparagine, glutamine, or histidine had far less effect. While neither glutamate nor aspartate attenuated activity as profoundly as phosphorylation of Ser\(^{871}\), these acidic residues introduce only a single charge, which probably is positioned somewhat differently from that of a seryl phosphate. We conclude that, while steric hindrance may contribute to some extent, the major factor responsible for the attenuation of activity that accompanies phosphorylation of Ser\(^{871}\) is the negative charge of the phosphate group.

To our best knowledge, all known phosphorylation sites for the AMP-activated protein kinase are seryl residues (40, 41). However, this kinase catalyzed phosphorylation of mutant enzyme S871T, and phosphorylation was accompanied by attenuation of activity. Since no other sites in the wild-type or mutant enzymes other than S871T are phosphorylated by this kinase, and since phosphothreonine was detected in a hydrolysate of mutant enzyme S871T, we infer that Thr\(^{871}\) was phosphorylated. The AMP-activated protein kinase, which fulfills a regulatory role in lipid metabolism (26), thus might well phosphorylate a threonyl residue of other proteins. Finally, we note that, while no known HMG-CoA reductase has a threonine at position 871 of the Syrian hamster enzyme, the activity of HMG-CoA reductase can be attenuated as well by the phosphorylation of a threonyl as of a seryl residue.

**Table III**

\(K_m\) values for wild-type and Ser\(^{871}\) mutant enzymes

| Enzyme   | (RS)-HMG-CoA (mM) | NADPH (mM) |
|----------|-------------------|------------|
| Wild type| 22 ± 4            | 42 ± 6     |
| S871A    | 22 ± 5            | 53 ± 6     |
| S871H    | 18 ± 3            | 57 ± 11    |
| S871N    | 9 ± 1             | 58 ± 18    |
| S871Q    | 10 ± 2            | 46 ± 11    |
| S871E    | 17 ± 3            | 68 ± 26    |
| S871D    | 14 ± 4            | 57 ± 6     |

Fig. 5. Double-reciprocal plots for the dependence of initial velocity on the concentration of NADPH and (RS)-HMG-CoA. Shown are data for (RS)-HMG-CoA (left) or NADPH (right) for wild-type and the indicated mutant enzymes. All assays were conducted at pH 6.5. 1/\(v\) is the reciprocal of the specific activity (\(\mu\)mol of NADPH oxidized/min/mg of protein). Where NADPH concentration was varied, the fixed concentration of (RS)-HMG-CoA was 270 \(\mu\)M (12 times the wild-type \(K_m\)). Where (RS)-HMG-CoA concentration was varied, the fixed concentration of NADPH was 270 \(\mu\)M (6.5 times the wild-type \(K_m\)).

Fig. 6. Susceptibility of Ser\(^{871}\) mutant enzymes to attenuation of their activity. Each panel displays data for wild-type enzyme (○) and for the indicated Ser\(^{871}\) mutant enzyme (□). Incubations at 37 °C contained 200 \(\mu\)M ATP, 5 \(\mu\)M MgCl\(_2\), 200 \(\mu\)M AMP, 20 \(\mu\)l (7.5 \(\mu\)g) of AMP-activated protein kinase in Buffer K, 4.6–5.3 \(\mu\)g of wild-type or mutant enzyme, and 55 \(\mu\)l of Buffer T in a final volume of 100 \(\mu\)l. Portions removed at the indicated times were assayed for HMG-CoA reductase activity. Differences in the rates of attenuation of the activity of wild-type enzyme result from kinase preparations of different activities.
Fig. 7. Susceptibility of Ser$^{871}$ mutant enzymes to phosphorylation. The reaction employed [γ-32P]ATP and the conditions of Fig. 3, except for a 10-μl incubation that contained 0.46 μg of wild-type or mutant enzyme. 2 μl (4.6 μg) of AMP-activated kinase in Buffer K, and 5.5 μl of Buffer T. Following incubation at 37 °C for 60 min, samples were subjected to SDS-PAGE and autoradiography. Lanes K and R are incubations in which either the kinase (−K) or HMG-CoA reductase (−R) was omitted. Arrows indicate the positions of molecular weight standards of the indicated size (in kDa).

Fig. 8. Phosphorylation of mutant enzyme S871T. Incorporation of 32P into 50 μg of wild-type HMG-CoA reductase or mutant enzyme S871T was conducted as in Fig. 3. A control incubation lacked HMG-CoA reductase. Incubation at 37 °C for 2.5 h was terminated by adding 25 μl of 50% trichloroacetic acid. Following centrifugation, the precipitate was washed successively with 10% trichloroacetic acid (2 times) and acetone (2 times), dried in vacuo, dissolved in 10 μl of 1% formic acid, and electrophoresed into a 4% plastic-backed cellulose TLC sheet for 4 h at 500 V. Shown is an autoradiograph of the electropherogram of hydrolysates of wild-type enzyme (WT), mutant enzyme S871T (S871T), and wild-type enzyme plus mutant enzyme S871T (WT+S871T). Arrows indicate the positions to which non-radioactive phosphothreonine (P-Thr) and phosphoserine (P-Ser) standards, detected with ninhydrin, migrated.

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REFERENCES
1. Goldstein, J. L., and Brown, M. S. (1990) Nature 345, 425-430
2. Hunter, C. F., and Rodwell, V. W. (1980) J. Lipid Res. 21, 399-405
3. Keith, M. L., Rodwell, V. W., Rogers, D. H., and Rudney, H. (1979) Biochem. Biophys. Res. Commun. 96, 969-975
4. Keith, M. L., Kennelly, P. J., and Rodwell, V. W. (1983) J. Protein Chem. 2, 209-220
5. Kennelly, P. J., Brandt, K. G., and Rodwell, V. W. (1983) Biochemistry 22, 2784-2788
6. Kennelly, P. J., and Rodwell, V. W. (1985) J. Lipid Res. 26, 903-914
7. Beg, Z. H., Stokin, J. A., and Brewer, H. B., Jr. (1989) J. Biol. Chem. 255, 8541-8545
8. Gillespie, J. G., and Hardie, D. G. (1992) FEBS Lett. 306, 59-62
9. Parker, R. A., Miller, S. J., and Gibson, D. M. (1988) J. Biol. Chem. 264, 4877-4887
10. Mackintosh, R. W., Davies, S. P., Clarke, P. R., Weekes, J., Gillespie, J. G., Gibb, B. J., and Hardie, D. G. (1992) Eur. J. Biochem. 206, 925-931
11. Beg, Z. H., Stokin, J. A., and Brewer, H. B., Jr. (1985) J. Biol. Chem. 260, 1682-1687
12. Beg, Z. H., Stokin, J. A., and Brewer, H. B., Jr. (1987) J. Biol. Chem. 262, 13228-13240
13. Clarke, P. R., and Hardie, D. G. (1990) EMBO J. 9, 2439-2446
14. Clarke, P. R., and Hardie, D. G. (1990) FEBS Lett. 269, 213-217
15. Sprang, S. R., Acharaya, K. R., Goldsmith, E. J., Staudt, D. I., Varvill, K., Fletterick, R., Madsen, N. B., and Johnson, L. N. (1988) Nature 336, 215-221
16. Barenje, P., Ahmad, M. F., Grove, J. R., Kozlosky, C., Price, D. J., and Avruch, J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8550-8554
17. Barford, D. (1991) Biochim. Biophys. Acta 1133, 55-62
18. Hurley, J. H., Dean, A. M., Sobel, J. L., Koshland, D. E., Sohl, J. L., Koshland, D. E., Hurley, J. H., Dean, A. M., Barford, D., Madsen, N. B., and Johnson, L. N. (1990) Science 249, 1012-1016
19. Thorsness, P. E., and Koshland, D. E., Jr. (1987) J. Biol. Chem. 262, 10422-10435
20. Dean, A. M., Lee, M. H. I., and Koshland, D. E., Jr. (1989) J. Biol. Chem. 264, 20482-20486
21. Ofir, R., Drwark, V. J., Rashid, D., and Verma, I. M. (1990) Nature 348, 80-82
22. Gonzalez, G. A., and Mestmin, M. R. (1989) Cell 58, 675-680
23. Theroux, S. J., Taglentian-Sian, C., Nair, N., Counteway, J. L., Robinson, H. L., and Davis, R. J. (1992) J. Biol. Chem. 267, 7967-7970
24. Darnay, B. G., and Rodwell, V. W. (1993) J. Biol. Chem. 268, 8429-8435
25. Lawrence, C. M., Rodwell, V. W., and Staufacher, C. V. (1994) Science (submitted)
26. Hardie, D. G., Carling, D., and Sim, A. T. R. (1989) Trends Biochem. Sci. 14, 20-23
27. Wang, Y., Darnay, B. G., and Rodwell, V. W. (1990) J. Biol. Chem. 265, 21634-21641
28. Frimpom, K., Darnay, B. G., and Rodwell, V. W. (1993) Protein Expression Purif. 4, 337-344
29. Studier, F. W., and Moffatt, B. A. (1986) J. Mol. Biol. 189, 113-130
30. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Promega Corp. (1991) Protocols and Applications Guide, 2nd Ed., pp. 109-113, Promega Corp., Madison, WI
32. Amersham Corp. (1983) Oligonucleotide-directed in Vitro Mutagenesis System, Version 2, pp. 1-39, Amersham Corp., Arlington Heights, IL
33. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
34. Promega Corp. (1991) Protocols and Applications Guide, 2nd Ed., pp. 109-113, Promega Corp., Madison, WI
35. Amersham Corp. (1983) Oligonucleotide-directed in Vitro Mutagenesis System, Version 2, pp. 1-39, Amersham Corp., Arlington Heights, IL
36. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
37. Pomeroy, J. A. (1981) J. Neur. Sci. 47, 193-200
38. Jordan-Stark, T. C., and Rodwell, V. W. (1989) J. Biol. Chem. 264, 17913-17918
39. Omkumar, R. V., Banerji, A., Ramakrishnakurup, C. P., and Ramasarma, T. (1981) Biochim. Biophys. Acta 670, 219-225
40. Carling, D., and Hardie, D. G. (1989) Biochim. Biophys. Acta 1012, 81-86
41. Pearson, R. B., and Kemp, B. E. (1991) Methods Enzymol. 200, 62-81