have multiple modes of regulation, including temporal control of expression (like PGC-1) and regulation of inherent activity through transactivation factor docking and signal transduction systems.

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7. Expression of SRC-1, p300, CBP, and p/CAF did not activate transcription when it was coexpressed with GAL4 DBD. In addition, the expression of these coactivators did not affect protein levels of GAL4–PGC-1.
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13. R. T. Nolte *et al.*, *Nature* **395**, 137 (1998); L. Gelman *et al.*, *J. Biol. Chem.* **274**, 7681 (1999). Using a GST-PPARγ (amino acids 128 to 229) fusion protein, we also confirmed that this region of PPARγ (amino acids 128 to 229) did not interact with in vitro translated [^{35}S]SRC-1 and [^{35}S]p300. PPARγ and NRF-1 alleles had no effect on the transcriptional activity of GAL4–PGC-1 construct containing only the activation domain, amino acids 1 to 170. Expression levels of GAL4–PGC-1 were similar in different transient transfections.
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16. Whole-cell extracts were prepared by lysing the cells in a buffer containing 100 mM tris (pH 8.5), 250 mM NaCl, 1% NP-40, 1 mM EDTA, protease inhibitors (Boehringer Mannheim), and 0.1 mM phenylmethylsulfonyl fluoride. Whole-cell lysates were incubated with monoclonal antibodies against SRC-1 or CBP/p300 for 2 hours at 4°C, followed by an overnight incubation with protein A/G Sepharose beads. Immunoprecipitates were extensively washed with the lysis buffer, resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and protein immunoblot with specific antibodies against GAL4 DBD monoclonal antibodies.
17. Whole-cell lysates were incubated with polyclonal antibodies against GAL4 DBD for 2 hours at 4°C, followed by an overnight incubation with protein A/G Sepharose beads. Immunoprecipitates were washed three times with the lysis buffer and used for HAT assays in solution with histones as substrates [J. E. Brownell and C. D. Allis, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6364 (1995)].
18. GST–SRC-1 constructs were made as described [T. E. Spencer *et al.*, *Nature* **389**, 194 (1997)]. GST-p300 constructs were made as described in (*8*). GST pull-down assays were performed as described in (*5*).
19. GAL4–PGC-1 deletions were performed by ligating PGC-1 polymerase chain reaction (PCR) fragments into Sal I–Eco RV cloning sites of pCMX expression vector. Expression levels for these different deletions in transfected cells were similar. GAL4-p/CAF was performed by ligating p/CAF PCR full-length fragment into Eco RI and Eco RV.
20. GST–PGC-1 fragments were generated by cloning the PCR fragments of PGC-1 into PGEX vectors. We made GST proteins in *Escherichia coli*, using manufacturer's instructions (Pharmacia). His-tagged PPARγ (amino acids 128 to 229) and His-tagged GFP were made by cloning PCR products into a PET30 vector. We expressed His-tagged proteins in *E. coli* and purified them under nondenaturing conditions, using manufacturer's protocol (Qiagen). Proteins were renatured by dialyzing against the binding buffer [20 mM Hepes (pH 7.7), 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% NP-40, 2 mM dithiothreitol, and 10% glycerol] and kept at −80°C until further use. In vitro binding assays were performed as described in (*5*), with the same amount of GST fusion proteins (analyzed by Coomassie staining).
21. Flag-tagged PGC-1 [in vitro translated with the TnT kit (Promega)], 0.1 μg of His-tagged purified proteins, and different amounts of trypsin (from 1 to 5 μg) were mixed with a buffer (50 mM NH_4HCO_3) in a total volume of 30 μl and incubated at 25°C for 10 min. The reaction was stopped by adding SDS-PAGE sample buffer. Digested fragments were analyzed by immunoblotting with a monoclonal antibody to flag (Sigma).

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Evidence for Autoregulation of Cystathionine γ-Synthase mRNA Stability in *Arabidopsis*

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Control of messenger RNA (mRNA) stability serves as an important mechanism for regulating gene expression. Analysis of *Arabidopsis* mutants that overaccumulate soluble methionine (Met) revealed that the gene for cystathionine γ-synthase (CGS), the key enzyme in Met biosynthesis, is regulated at the level of mRNA stability. Transfection experiments with wild-type and mutant forms of the CGS gene suggest that an amino acid sequence encoded by the first exon of CGS acts in cis to destabilize its own mRNA in a process that is activated by Met or one of its metabolites.

Genetic studies of metabolic pathways in bacteria and yeast have revealed important regulatory mechanisms. For example, studies of amino acid biosynthesis operons in bacteria led to an understanding of mRNA attenuation (*1*), and the histidine biosynthesis pathway of yeast led to an understanding of the complex interplay between general and pathway-specific controls (*2*). With the exception of tryptophan biosynthesis in *Arabidopsis* (*3*), genetic methods have not been extensively used to analyze amino acid biosynthesis in plants. To study the molecular mechanisms for regulation of methionine biosynthesis in plants, we used *Arabidopsis* mutants, termed *mto1*, that overaccumulate soluble Met (*4*).

Met, a sulfur-containing amino acid, functions not only as a protein component but also as a precursor of *S*-adenosylmethionine, the primary methyl donor in many transmethylation reactions and, in plants, a precursor of the phytohormone ethylene (*5*). Met is an essential dietary amino acid for mammals. Studies with the aquatic plant *Lemna* have shown that the cellular concentration of soluble Met remains unchanged over a 3000-fold range in sulfur availability (*6*), indicating that Met biosynthesis is tightly regulated in plants. Cystathionine γ-synthase (CGS) catalyzes the first committed step in Met biosynthesis, and it has been suggested to be a key regulatory site of the pathway (*5*). Indeed, CGS activity in *Lemna* and barley is regulated positively and negatively in response to the availability of Met (*7*).

Analyses of CGS expression in *mto1-1* mutant plants revealed that the steady-state levels of CGS mRNA, protein, and enzyme activity are three- to fivefold higher than in wild-type plants (Fig. 1, A to C). Application of Met to wild-type plants reduced the amount of mRNA for CGS, whereas no such effect was observed in the *mto1-1* mutant (Fig. 1D). This suggests that wild-type plants down-regulate the level of CGS mRNA in response to exogenous Met, and this regulation is impaired in the *mto1* mutant.

A liquid callus culture system (*8*) was used for further studies. As with whole plants, the steady-state level of CGS mRNA was reduced by feeding calli from wild-type plants with Met

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(Fig. 1E), although the response was less significant than in whole plants (*9*). In addition to an overall reduction in CGS mRNA, a minor band about 500 bases shorter than the full-length transcript was evident in Met-treated wild-type calli (*9*). Two lines of evidence indicate that this minor mRNA species is a form truncated at the 5′ end of the transcript. The minor band is observed after polyadenylate selection (*10*) and a probe covering the 3′ untranslated region hybridizes to it, whereas one covering the 5′ region does not (Fig. 1E). This truncated transcript may be an intermediate in the degradation of CGS mRNA (see below).

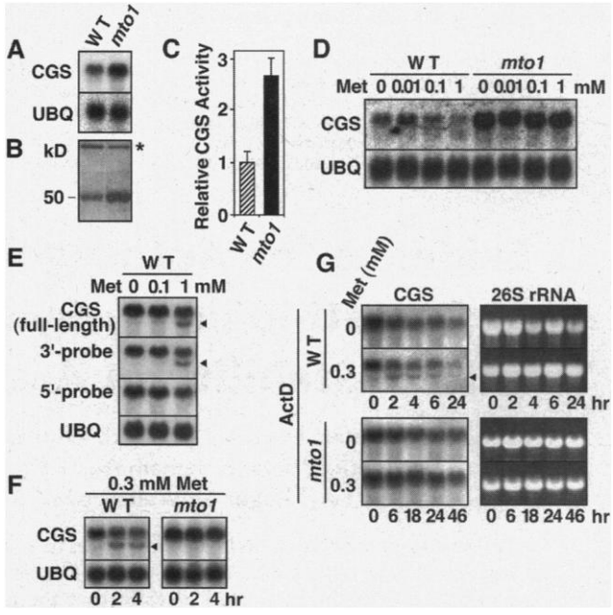

Fig. 1. CGS expression in wild-type (WT) and *mto1-1* mutant (*mto1*). (**A** to **C**) Levels of CGS mRNA (A), protein (B), and enzyme activity (C) in leaves of 3-week-old plants (*27*). Total RNA (10 μg) was analyzed by RNA blot hybridization (*21*) with CGS cDNA used as a probe (*28*). The membrane was rehybridized (*26*) with a control ubiquitin (UBQ) cDNA (*28*) (A). Protein extracts (2 μg) were subjected to immunoblot analysis (*21*) by using rabbit antiserum to CGS. CGS protein migrated at about 50 kD. Asterisk indicates a band that was also detected with the control serum (B). CGS activities (*29*) relative to wild-type activity (5.28 milliunits/mg) are shown. Error bars indicate standard deviations of three independent samples (C). (**D** and **E**) Effect of Met on CGS mRNA accumulation. Various concentrations of Met were applied to plants (*27*) (D) or callus culture (*8*) (E) for 3 days and total RNA (10 μg) was analyzed as in (A). The 3′ and 5′ regions of CGS cDNA (*28*) were also used as probes in (E). (**F** and **G**) Time course of Met effect in callus culture (*8*). Samples were withdrawn at time points as indicated after Met (0.3 mM) alone (F) or Met (0 or 0.3 mM) and ActD (100 μg/ml) (*11*) treatments (G), and total RNA (10 μg) was analyzed as in (A). Control in (G) is ethidium bromide staining of 26*S* ribosomal RNA. Arrowheads indicate positions of truncated mRNA (E to G). Representative results of at least triplicate experiments are shown (A to G).

The *mto1* mutation and the CGS gene mapped close to each other on chromosome 3 (*13*). Sequence analyses of five *mto1* mutants (*14*) revealed single base changes in the CGS coding region, giving rise to alterations in the amino acid sequence (Table 1). The mutations were clustered in a small region of eight amino acids located about 80 residues from the NH_2-terminus, with two of the independent mutations being identical. Hereafter, the wild-type amino acid sequence defined by the *mto1* mutations is referred to as the MTO1 region and the corresponding nucleotide sequence is *MTO1*.

The role of the MTO1 region was studied by transient expression experiments. The coding region from exon 1 (amino acids 1 to 183) of CGS (*15*), with or without *mto1* mutations, was fused in-frame to the 5′ end of the *Escherichia coli* β-glucuronidase (GUS) reporter gene. The constructs were placed under the control of a cauliflower mosaic virus (CaMV) 35*S* RNA promoter and used in transfection experiments. Reporter activity was lower for the construct carrying wild-type exon 1 than for those carrying *mto1* mutations and reporter activity was repressed by incubation with Met (Fig. 2A). In contrast, reporter activity was insensitive or less sensitive to Met treatment for the constructs carrying *mto1* mutations (*16*) or for a construct carrying only the first four amino acids of exon 1 (Δ5–183) (*17*). Thus, wild-type exon 1 contains a sequence that is both necessary and sufficient for down-regulation of reporter gene activity in response to applied Met.

To test whether the nucleotide or amino acid sequence is important for this regulation, we mutated the sequence of exon 1 by introducing

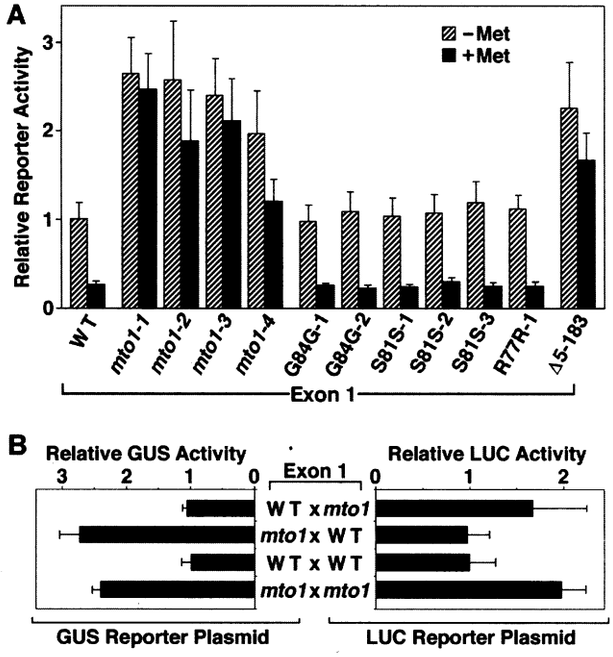

Fig. 2. Role of CGS exon 1. We used cells from liquid callus cultures (*8*) for these experiments. (**A**) Effect of wild-type and mutant exon 1 on reporter activity in a transient expression system. We transfected wild-type protoplasts (*30*) with 10 μg of plasmid carrying the GUS reporter. The plasmids carried CGS exon 1 from either wild type (WT) or mutants as indicated (*31*). Transfected protoplasts were incubated for 48 hours with (filled box) or without (hatched box) 0.1 mM Met (*9*). A control plasmid (10 μg) carrying the LUC reporter (*31*) was cotransfected as an internal standard, and GUS activities were normalized with LUC activities. The GUS/LUC values relative to the wild-type exon 1 construct in the absence of Met are shown. Error bars indicate standard deviations of at least five experiments. (**B**) Cotransfection experiments. Wild-type protoplasts were transfected with 10 μg each of two plasmids carrying wild-type (WT) or *mto1-1* mutant (*mto1*) CGS exon 1 fused to GUS or LUC reporters (*31*). Cotransfection was carried out in all four combinations. After 48 hours of incubation, GUS and LUC activities were determined and normalized with protein content. Reporter activities relative to those of wild-type exon 1 combination are shown. Error bars indicate standard deviations of three to five experiments.

base changes into the *MTO1* region that do not alter the amino acid sequence (silent mutations) (Table 1). Transfection experiments showed that the silent mutants behaved as did the wild type (Fig. 2A), which suggests it is the amino acid sequence that plays a role in regulation.

We examined whether the MTO1 region functions in cis or in trans by cotransfecting plasmid constructs carrying different reporter genes and exon 1 of CGS from wild type or the *mto1-1* mutant. Neither the wild-type nor mutant exon 1 affected the reporter activity of the other (Fig. 2B), which indicates that the mutation acts in cis.

Comparison of the CGS amino acid sequence from four plant species showed that the region encompassing exon 1 of *Arabidopsis* CGS has a low overall homology and variable length (Fig. 3A). A notable exception was a stretch of 38 amino acids that includes the MTO1 region (Fig. 3B). Conservation of the MTO1 region among widely different plant species suggests that it plays a functional role (*18*).

These results suggest that the exon 1 polypeptide of CGS acts in cis to down-regulate its own mRNA stability in response to excess Met. Although it is unusual for a polypeptide to act in cis, a plausible explanation is that the regulation occurs during translation when the nascent polypeptide and its mRNA are in close proximity. A model for such a regulation mechanism predicts a role for the exon 1 polypeptide of CGS in destabilizing its own mRNA during translation and an activation of this process by Met or one of its metabolites (*19*). Alterations in the amino acid sequence of the MTO1 region abolish either of the reactions. The mechanism may generate an intermediate mRNA species with a truncated 5′ end (Fig. 1E).

The β-tubulin gene in animals (*20*) has a similar mechanism in that an amino acid sequence acts in cis to regulate its own mRNA stability, although the responsible amino acid sequences are placed differently between the two systems. The stability of β-tubulin mRNA is down-regulated by the unassembled β-tubulin subunits, with the NH_2-terminal tetrapeptide of nascent β-tubulin being responsible for the regulation (*20*). The presence of another system reported here, together with the genetics of *Arabidopsis* and identification of a possible degradation intermediate, will help in defining the molecular mechanisms of this mode of mRNA regulation.

Much is known about the feedback regulation at the level of enzyme activity in metabolic pathways in plants (*5*), but very little is known about feedback regulation at the level of gene expression. The system reported here adds another mechanism to the repertoire of metabolic controls (*1, 2, 5*).

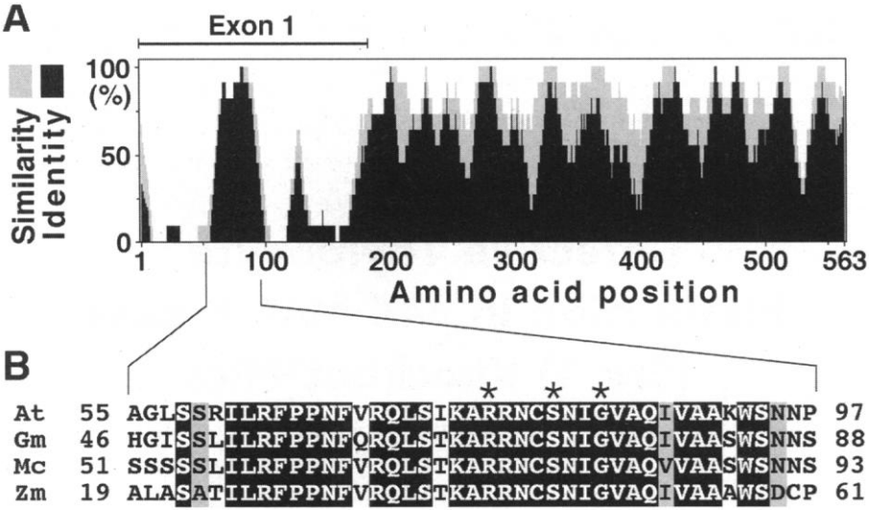

Fig. 3. Alignment of CGS amino acid sequence. **(A)** We aligned the deduced amino acid sequences of the complete coding region for CGS from *Arabidopsis* (GenBank database accession no. AB010888), soybean (*Glycine max*; accession no. AF141602), common ice plant (*Mesembryanthemum crystallinum*; accession no. AF069317), and maize (*Zea mays*; accession no. AF007786) by using the CLUSTALW program (*32*). The alignment was scanned with a window of 11 amino acids, and the identical (filled box) or similar (shaded box) amino acids among the four plant species were counted. **(B)** Alignment of amino acid sequence for the highly conserved region covering the MTO1 region. Those amino acids that are identical (reversed) or similar (shaded) among the four plant CGS are marked. At, *Arabidopsis*; Gm, soybean; Mc, common ice plant; Zm, maize. Positions of *mto1* mutations are marked with asterisks.

Table 1. Nucleotide and amino acid changes in *mto1* and silent mutations. The *mto1-3* and *mto1-5* mutants are independent of each other because they were isolated from different batches of mutagenized population.

Mutation	Amino acid position	Nucleotide change*	Amino acid change
mto1 mutations			
mto1-1	84	GGT → AGT	Gly → Ser
mto1-2	81	AGC → AAC	Ser → Asn
mto1-3, 5	84	GGT → GAT	Gly → Asp
mto1-4	77	CGT → CAT	Arg → His
Silent mutations			
G84G-1	84	GGT → GGA	Gly (no change)
G84G-2	84	GGT → GGC	Gly (no change)
S81S-1	81	AGC → TCA	Ser (no change)
S81S-2	81	AGC → TCT	Ser (no change)
S81S-3	81	AGC → TCG	Ser (no change)
R77R-1	77	CGT → CGG	Arg (no change)

*Nucleotide changes are underlined.

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8. Liquid callus cultures were prepared as described in P. Guzman and J. R. Ecker [*Nucleic Acids Res.* **16**, 11091 (1988)] as modified in (*21*), except that the culture condition was 22°C under continuous white fluorescent light at about 100 $\mu E\ m^{-2}\ s^{-1}$.
9. Because of the difference in experimental systems, some inconsistencies in dose response were apparent. The response to Met in calli seems to be less significant than in whole plants or electroporated protoplasts. This may result from a difference in the ability to take up Met. Also, the truncated RNA was hardly visible in whole plant samples, possibly because such RNA is more labile in whole plants than in calli.
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12. At 24 hours after ActD treatment, the quantities of CGS mRNA in the absence of Met were reduced to about 30% and 60%, in wild-type and *mto1-1* mutant, respectively. These values in the presence of Met were about 15% and 60% for wild-type and *mto1-1* mutant, respectively.
13. Mapping with simple sequence length polymorphism (SSLP) markers [C. J. Bell and J. R. Ecker, *Genomics* **19**, 137 (1994)] located the *mto1* mutation about 3 centimorgans (cM) north of *nga172*, which is at 6.83 cM on chromosome 3 (http://nasc.nott.ac.uk/new_

ri_map.html). In this regard, the gene order in the previous report (*4*) is corrected to *mto1-hy2-abi3*. The CGS gene was mapped about 4 cM north of *nga172* (*22*).
14. We amplified a 6651–base pair region covering the CGS gene from the *mto1-1* mutant by polymerase chain reaction (PCR) and compared the nucleotide sequence of the amplified DNA with the wild-type sequence (GenBank database accession no. AB010888). The primers were designed after the wild-type sequence. Sequencing was carried out with an ABI PRISM dye terminator cycle sequencing kit and model 377 DNA sequencer (Perkin-Elmer). Additional alleles of *mto1* mutants were isolated as described in (*4*), and *mto1-2* through *mto1-5*, which are independent of each other, were used for further study after they were back-crossed three times to wild type. Because the *mto1* mutation is semidominant over wild type (*4*), complementation tests were not applicable. Mapping with a SSLP-type marker in the 5′-upstream region of CGS (*22*) indicated that they all mapped within a few centimorgans from *mto1-1*. These mutants were sequenced for the exon 1 region.
15. The CGS gene has 11 exons encoding 563 amino acids (*23*).
16. The level of CGS mRNA in *mto1-4* mutant plants was lower than in other *mto1* mutants (*10*), which suggests that the *mto1-4* mutation is leaky. The fact that the reporter activity of the *mto1-4* mutant construct in the presence of Met was also lower than that of the *mto1-1* mutant construct supports the idea that the reporter activity reflects the response to Met at the mRNA level.
17. The first four amino acids were included to provide the same context for the translational start site as the other constructs.
18. The MTO1 region is not necessary for enzyme activity (*23*).
19. Although CGS is transported to chloroplasts (*24*), synthesis of Met and most of its direct metabolites occurs in the cytosol (*5, 24*).
20. T. J. Yen, P. S. Machlin, D. W. Cleveland, *Nature* **334**, 580 (1988); N. G. Theodorakis and D. W. Cleveland, in *Control of Messenger RNA Stability*, J. Belasco and G. Brawerman, Eds. (Academic Press, San Diego, CA, 1993), pp. 219–238.
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28. The (full-length) CGS cDNA [GenBank accession no. X94756; L. Le Guen, M. Thomas, M. Kreis, *Mol. Gen. Genet.* **245**, 390 (1994)] was a gift from M. Thomas. The 5′ probe (nucleotide position −20 to 183 relative to the translation start site) and the 3′ probe (1693 to 1870) of CGS were prepared by PCR with primers 5′-CATTGAGAAACGAAACAACA-3′ and 5′-GATCCTGGAGGATAATCCAC-3′, and 5′-AAATGACACATCACACAAAAAC-3′ and 5′-GAACTAAACAGAATTTATTATATAGCACAAC-3′, respectively. The ubiquitin probe was the *UBQ5* gene of *Arabidopsis* [E. E. Rogers and F. M. Ausubel, *Plant Cell* **9**, 305 (1997)] (*21*). ^{32}P-labeled probes were prepared with a multiprime DNA labeling system (Amersham).
29. We determined CGS activity by phosphate release as described in (*25*) except that Mops replaced Tricine in the extraction buffer and the assay mixture contained 2 mM cysteine and 6 mM *O*-phosphohomoserine.
30. We prepared protoplasts from liquid callus cultures (*8*) as described in (*21*). Transfection of protoplasts by electroporation was carried out as described in (*21*) except that capacitance and voltage were 100 μF and 475 V cm^{-1}, respectively. Reporter activities were determined as described [Y. Sakata *et al., Biosci. Biotech. Biochem.* **58**, 2104 (1994)].
31. We followed standard procedures for DNA manipulations (*26*). Wild-type and *mto1* mutant exon 1 were amplified by PCR with respective genomic DNA and primers Ex1P1 (5′-CGAATCTAGAATGGCCGTCTC-3′) and Ex1P2 (5′-ATCTAGGATCCACCGGCATG-3′), which carried Xba I and Bam HI recognition sequences, respectively (mismatched bases are underlined). We constructed exon 1 with silent mutations as described in I. Mikaelian and A. Sergeant [*Nucleic Acids Res.* **20**, 376 (1992)]. The mutagenic primers used to construct G84G-1, G84G-2, S81S-1, S81S-2, S81S-3, and R77R-1 were 5′-CTGTAGCAACATCGGAGTTG-3′, 5′-CTGTAGCAACATCGGCGTTG-3′, 5′-AGAAACTGTCAAACATCGG-3′, 5′-AGAAACTGTCTAACATCGG-3′, 5′-AGAAACTGTCGAACATCGG-3′, and 5′-GCATTAAAGCCGGGAGAAAC-3′, respectively. The internal primer was 5′-GACGTAGTGAGGCTCCCATC-3′, and the external primers were Ex1P1 and Ex1P2. Synthetic oligonucleotides (5′-CTAGAATGGCCGTCTCAG-3′ and 5′-GATCCTGAGACGGCCATT-3′) were used to construct Δ5-183, which carries the first four amino acids of exon 1. To obtain plasmids carrying the GUS reporter gene [R. A. Jefferson, T. A. Kavanagh, M. W. Bevan, *EMBO J.* **6**, 3901 (1987)], we ligated the exon 1 DNA with Xba I– and Bam HI–digested pTF33 [T. Fujiwara, S. Naito, M. Chino, T. Nagata, *Plant Cell Rep.* **9**, 602 (1991)]. The control plasmid 221-LUC$^+$ carries the firefly luciferase (LUC) gene under control of the CaMV 35*S* RNA promoter (K. Hiratsuka, personal communication). To obtain plasmids carrying the LUC reporter, we excised the LUC coding region from pT3/T7-LUC (Clontech, Palo Alto, CA) by Bsm I and Sac I digestion and ligated it with Xba I– and Sac I–digested pTF33 along with exon 1 DNA. The exon 1 sequences were verified by sequencing.
32. J. D. Thompson, D. G. Higgins, T. J. Gibson, *Nucleic Acids Res.* **22**, 4673 (1994); http://www.genome.ad.jp/SIT/CLUSTALW.html. The gap open penalty was set at 3.
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Defective Thymocyte Maturation in p44 MAP Kinase (Erk 1) Knockout Mice

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The p42 and p44 mitogen-activated protein kinases (MAPKs), also called Erk2 and Erk1, respectively, have been implicated in proliferation as well as in differentiation programs. The specific role of the p44 MAPK isoform in the whole animal was evaluated by generation of p44 MAPK-deficient mice by homologous recombination in embryonic stem cells. The p44 MAPK$^{-/-}$ mice were viable, fertile, and of normal size. Thus, p44 MAPK is apparently dispensable and p42 MAPK (Erk2) may compensate for its loss. However, in p44 MAPK$^{-/-}$ mice, thymocyte maturation beyond the CD4$^+$CD8$^+$ stage was reduced by half, with a similar diminution in the thymocyte subpopulation expressing high levels of T cell receptor (CD3^{high}). In p44 MAPK$^{-/-}$ thymocytes, proliferation in response to activation with a monoclonal antibody to the T cell receptor in the presence of phorbol myristate acetate was severely reduced even though activation of p42 MAPK was more sustained in these cells. The p44 MAPK apparently has a specific role in thymocyte development.

Erk1 or p44 MAP kinase was the first mammalian MAPK to be characterized and cloned a decade ago (*1*). This MAPK together with its isoform p42 MAPK (Erk2) are commonly expressed in most, if not all, tissues and are activated through the small guanosine triphosphatase Ras and sequential activation of the protein kinases Raf and MEK upon stimulation of cells with a broad range of extracellular signals (*2*). This Ras-MAPK module appears to be as central to cellular signaling as the Krebs cycle and glycolysis are to energy metabolism. Indeed, the Ras-dependent MAPK signaling cascade functions in control of cell fate, differentiation, proliferation, and cell survival in various invertebrates and

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