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Structure, Expression, and Chromosome Location of the Gene for the β Subunit of Brain-Specific Ca^{2+}/Calmodulin-Dependent Protein Kinase II Identified by Transgene Integration in an Embryonic Lethal Mouse Mutant

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The transgenic mouse strain CAT40 carries in its germ line one copy of a DNA construct consisting of the chloramphenicol acetyltransferase gene and the immunoglobulin heavy-chain enhancer. We show that transgene integration has resulted in a recessive lethal mutation that leads to death of homozygous CAT40 embryos shortly after implantation. The transgene has integrated adjacent to the 3′ end of the gene coding for the β subunit of the brain-specific Ca^{2+}/calmodulin-dependent protein kinase II (*Camk-2*). The complete cDNA sequence of the *Camk-2* gene and most of its exon/intron structure was determined. The deduced amino acid sequence is highly homologous to the previously described rat protein. The chromosomal location of the *Camk-2* locus was mapped by interspecific backcross analysis to the proximal region of mouse chromosome 11. This region lacks previously identified recessive embryonic lethal mutations. During embryonic development, *Camk-2*-specific transcripts are first seen in the head section of 12.5-day-old embryos, and in adult mice the gene is expressed almost exclusively in the brain. Although transcription of the *Camk-2* gene in heterozygous CAT40 mice is affected by transgene integration, it is unlikely that this gene is responsible for the mutant phenotype, since it is not expressed in blastocysts and the first transcripts during normal development are detected after the death of homozygous CAT40 embryos. Transgene integration is accompanied by a large deletion of cellular DNA; death is therefore most likely caused by the loss of a gene or genes that are important for early postimplantation development.

The analysis of spontaneous or experimentally induced mutations in mice has proved to be a powerful approach for understanding mammalian development at the molecular level (for a recent review, see reference 25). A frequently used method for obtaining experimentally induced developmental mutants is the introduction of foreign DNA into the germ line of mice by retroviral infection or microinjection (for a review, see reference 13). Since integration can occur at many chromosomal sites, the foreign DNA (transgene) can inactivate cellular genes by insertional mutagenesis. By using the transgene as a tag for molecular cloning, it is possible to isolate flanking cellular sequences and thus to identify the mutated gene. Well-studied examples that demonstrate the usefulness of transgenic mouse mutants for defining developmentally important loci are provirus integration into the α1(I) collagen gene of the Mov13 mouse strain (29) and transgene integration into the limb deformity locus (35, 36).

In this report, we describe the molecular analysis of the CAT40 mouse strain, which was derived by microinjection into fertilized eggs of a DNA construct consisting of the chloramphenicol acetyltransferase (CAT) gene and the immunoglobulin heavy-chain (IgH) enhancer (24). We show that transgene integration has resulted in a recessive lethal mutation that leads to the death of homozygous embryos shortly after implantation. The transgene has integrated next to the gene for the β subunit of the brain-specific Ca^{2+}/calmodulin-dependent (β CaM) protein kinase II (*Camk-2*), and insertion is accompanied by a large deletion. The structure, expression, and chromosomal location of the *Camk-2* gene are described. Although expression of the gene is affected by the transgene integration, it is unlikely that *Camk-2* is responsible for the mutant phenotype, as its transcription during development starts after the death of homozygous CAT40 embryos.

MATERIALS AND METHODS

Mice. All mice were bred in our mouse colony at the Heinrich-Pette-Institut. The origin of CAT40 mice has been described previously (24). Embryos were obtained from timed matings; the day of plug detection was counted as day 0.5 of gestation.

Genomic cloning. Genomic libraries were generated in the vector λ EMBL3 by partial digestion of mouse liver DNA with *Mbo*I. All recombinant DNA manipulations were done by standard procedures (27).

Isolation of cDNA clones. A cDNA library prepared from total brain RNA of adult BALB/c mice constructed in λgt10 was a gift from G. Grosveld, Erasmus University, Rotterdam, The Netherlands. About 5×10^5 plaques were screened with the genomic probe pC1-8 (see Results). Positive plaques were purified, and the DNA inserts were recloned in the Bluescript vector (Stratagene). To obtain cDNA clones representing the 5′ half of the *Camk-2* gene,

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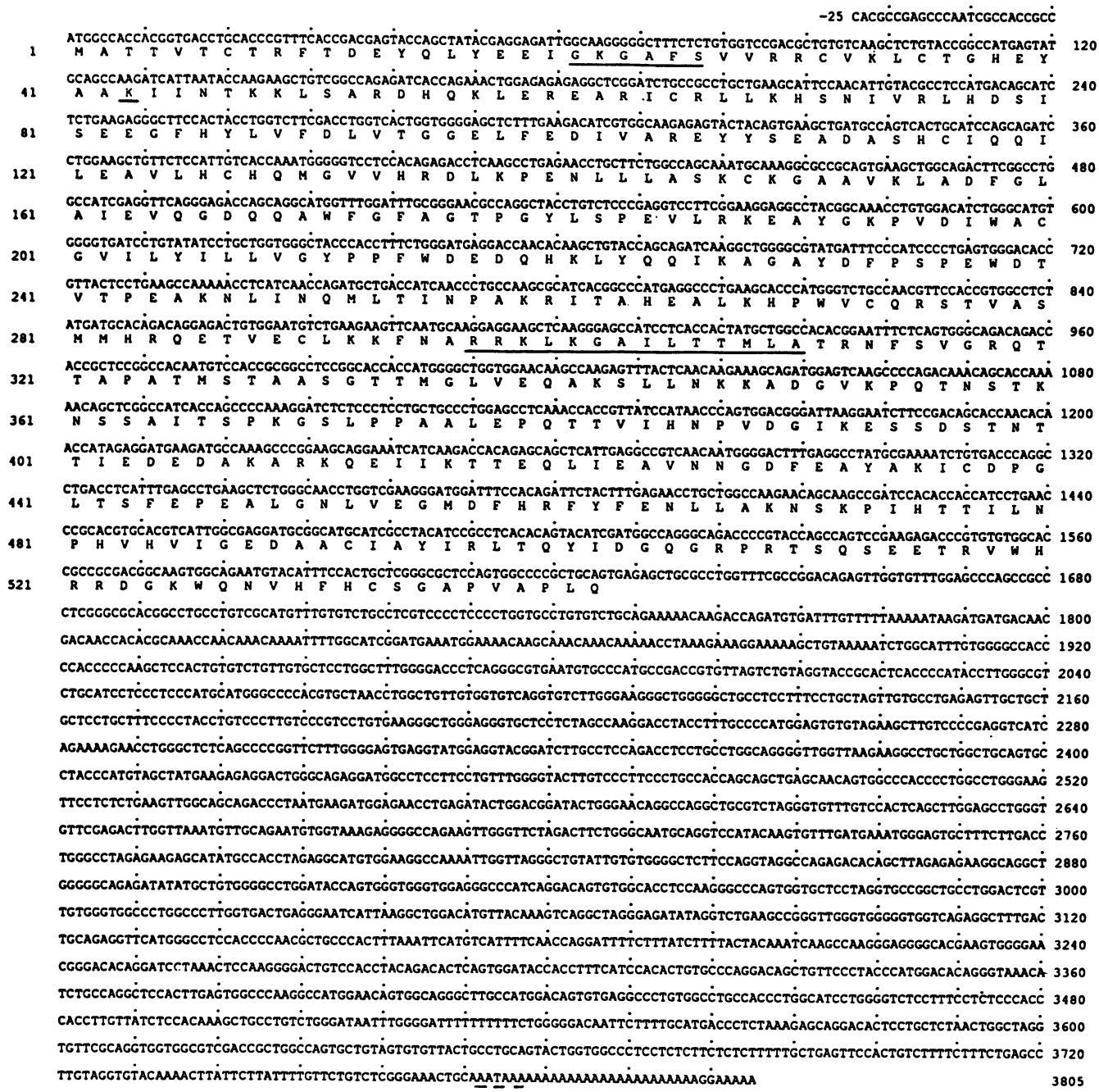

FIG. 1. Nucleotide sequence of *Camk-2* cDNA and deduced amino acid sequence of the protein. The residues most highly conserved in the family of type II CaM kinases are underlined. For details, see Results. The sequence AATAAA, potentially used as a polyadenylation signal, is indicated by a dashed line. Numbers at the left refer to amino acid positions, and those at the right refer to nucleotide positions.

total brain RNA from adult BALB/c mice was reverse transcribed and amplified by the polymerase chain reaction (PCR) as described below. The oligonucleotide CACCAAC TCTGTCCGGCGAA (positions 1663 to 1644; Fig. 1) was used as the primer for the reverse transcription and as the 3′ amplimer for PCR. The 5′ amplimer was derived from the published sequence of the gene for rat β CaM kinase II (3) and has the sequence CACGCCGAGCCCAATCGCCA (positions −25 to −6 in reference 3). Cleavage sites for *Xba*I and *Hin*dIII were added at the 5′ end of this amplimer. After PCR (30 cycles), the DNA was restricted with *Hin*dIII (cleavage site in the linker of the 5′ amplimer) and *Pst*I (cleavage site at position 1631 [Fig. 1] adjacent to the 3′ amplimer), purified by gel electrophoresis, and cloned in the Bluescript vector.

DNA sequencing. Nucleotide sequences of cDNA clones were determined by the dideoxy-chain termination method (28), using double-stranded DNAs in Bluescript vectors as templates and synthetic oligonucleotides as primers. Both strands were sequenced completely.

RNA preparation and analysis. Total cellular RNA was isolated by the method of Auffray and Rougeon (1) or by the guanidinium isothiocyanate procedure followed by centrifugation in cesium chloride (27). Blastocysts were flushed from the uterus at day 3.5 (counting the day of vaginal plug as day 0.5) as described previously (12). Total RNA from blastocysts was isolated either by a microadaption of the guanidinium isothiocyanate-CsCl procedure (23) or by phenol extraction (34). For Northern (RNA) blot analysis, RNA (15 μg per lane) was fractionated by electrophoresis in 1% agarose gels containing formaldehyde (27). After staining with ethidium bromide, rRNA bands were used to confirm the integrity and amount of RNA in each lane. RNA was transferred onto GeneScreen Plus membranes (Dupont) and hybridized in the presence of 50% formamide at 42°C for 18 to 20 h as described by the manufacturer. ^{32}P-radiolabeled probes were prepared by the random-priming procedure of Feinberg and Vogelstein (8). Final filter washes were done at 65°C with a solution of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 1% sodium dodecyl sulfate (SDS).

Specific RNA transcripts were amplified by reverse transcriptase-dependent PCR, essentially as described by Kawasaki (15). The conversion of RNA to cDNA was carried out in a final volume of 20 μl containing 1 μg of total RNA, 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 50 mM KCl, 5 mM dithiothreitol, 1 mM each nucleoside triphosphate, 40 U of RNAsin (Promega), 100 pmol of random hexamer primers (Pharmacia), and 8 U of avian myeloblastosis virus reverse transcriptase (Promega). Before the addition of RNAsin and reverse transcriptase, the reaction mixture was heated to 65°C for 5 min and cooled to room temperature. After 90 min at 42°C, the reaction was stopped by heating to 75°C for 10 min. Finally, 80 μl of H_2O was added, and the reaction mixture was stored at −20°C. Amplification of cDNA was carried out with *Taq* polymerase in a thermal cycler. Samples were prepared by combining 2.5 μl of the cDNA solution with 22.5 μl of PCR buffer containing 10 mM Tris (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 μM each nucleoside triphosphate, 100 ng of each gene-specific amplimer, and 1 U of *Taq* polymerase (Bethesda Research Laboratories). The following amplimers were used for detecting *Camk-2*-specific transcripts: at the 5′ site, AGCTCATTGAGGCCGTCAAC (positions 1259 to 1278, Fig. 1); and at the 3′ site, CACCAACTCTGTCCGGCGAA (positions 1663 to 1644; Fig. 1). Samples were overlaid with mineral oil, and the reaction was carried out in a thermocycler (Biometra) for 20 to 30 cycles consisting of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C. PCR products were visualized after separation by agarose gel electrophoresis in the presence of ethidium bromide under UV light. To confirm specificity and to detect low quantities, DNA was transferred to a nylon membrane and hybridized to a specific probe derived from *Camk-2* cDNA. As an internal standard, all cDNA samples were adjusted to yield relatively equal amplifications of β-actin.

Interspecific backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described previously (6). A total of 205 N_2 progeny were obtained; a random subset of these N_2 mice was used to map the *Camk-2* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described previously (14). All blots were prepared with Zetabind nylon membranes (AMF-Cuno). The CAT40 probe (pC1-8; Fig. 2) was labeled with [α^{32}P]dCTP by using a nick translation labeling kit (Boehringer Mannheim). Washing was done to a final stringency of 0.65× SSCP–0.1% SDS at 65°C. A 0.6-kb fragment was detected in *Pst*I-digested C57BL/6J DNA, and a 1.2-kb fragment was detected in *Pst*I-digested *M. spretus* DNA. The 1.2-kb *M. spretus*-specific fragment was monitored in this analysis. A description of the probes and restriction fragment length polymorphisms (RFLPs) for the *ErbB* proto-oncogene and *Rel* proto-oncogene loci has been reported previously (4). The probe for the leukemia inhibitory factor (*Lif*) locus was a 540-bp fragment of mouse cDNA kindly provided by Colin Stewart (Roche Institute, Nutley, N.J.). The *Lif* probe detected a 14.0-kb fragment in *Eco*RI-digested C57BL/6J DNA and a 7.2-kb fragment in *Eco*RI-digested *M. spretus* DNA. Recombination distances were calculated as described previously (10), using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

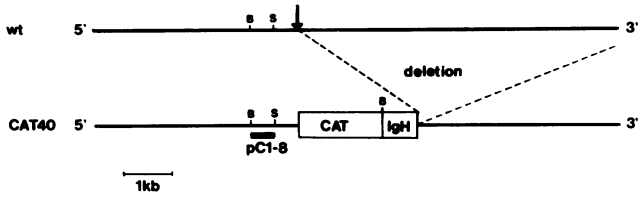

FIG. 2. Schematic representation of the CAT40 locus. Partial restriction maps of the wild-type (wt) and mutant (CAT40) alleles are shown. The arrow indicates the transgene integration site in wild-type DNA, and the open box represents the transgene consisting of a CAT-IgH construct. The deletion of wild-type sequences in the mutant CAT40 allele is indicated by dashed lines. The black bar represents the unique cellular probe pC1-8. Restriction sites for *Bam*HI (B) and *Sal*I (S) are indicated.

Nucleotide sequence accession number. The nucleotide sequence shown in Fig. 1 has been assigned EMBL accession number X63615.

RESULTS

Homozygosity at the CAT40 locus is lethal during embryogenesis. Previous results have shown that CAT40 mice heterozygous for the transgene integration carry one copy of the IgH-CAT construct in their germ line and that the foreign DNA does not interfere with normal development and postnatal life (24). In a screen of several transgenic mouse strains, the CAT40 insertion was found to be associated with several rarely cutting restriction sites in the vicinity of the transgene, suggesting the possibility that integration had occurred near an endogenous gene. Hence, to detect a possible recessive mutation caused by transgene integration, offspring were derived from heterozygous parents and analyzed for homozygosity at the CAT40 locus. Southern blots of tail DNAs were hybridized with the unique probe pC1-8 (black bar in Fig. 2), derived from sequences flanking the transgene, which distinguishes qualitatively between the different genotypes (data not shown). Crosses between parents heterozygous at the CAT40 locus failed to produce any homozygotes among 82 offspring analyzed. To determine at what stage of development homozygous CAT40 embryos die, embryos resulting from double heterozygous crosses were isolated between days 10.5 and 16.5 of gestation (counting the day of vaginal plug as day 0.5) and genotyped by Southern blots. Of a total of 72 embryos analyzed, 58

were heterozygous and 14 were wild type, while homozygous embryos were missing. These results indicate that homozygous CAT40 embryos die before day 10.5 postcoitus. Therefore, uteri were removed from pregnant females at days 6.5 and 7.5 postcoitus, and the implantation sites were dissected to determine the presence or absence of an embryo. Of the 160 implantation sites analyzed in the heterozygous intercrosses, a total of about 30% were retarded, abnormal, or resorbed. In control experiments, 102 implantation sites from crosses of nontransgenic animals of the same genetic background were examined. Only 3% were abnormal or resorbed. After correction for these 3%, the frequency of abnormal development or resorption in the heterozygous matings is 28%. The observation that approximately one-quarter of the deciduae produced in the heterozygous intercrosses contained resorbed embryos at day 6.5 postcoitus suggests that homozygous CAT40 embryos are capable of triggering a decidual response in the uterus and thus are viable through the preimplantation stage. Developmental arrest, therefore, seems to occur shortly after implantation.

Molecular cloning of the CAT40 locus and *Camk-2* cDNA. To identify the gene or genes mutated by the transgene integration, flanking cellular sequences were molecularly cloned, using the CAT gene as a probe, and unique subclones were derived. The preintegration site of the CAT40 locus was cloned by probing a genomic library of BALB/c DNA with the unique subclone pC1-8. To study the sequence arrangement of the CAT40 locus, DNAs from wild-type mice and CAT40 mice heterozygous for the transgene were compared by restriction analysis and Southern blotting, using unique fragments from the genomic clones and the CAT gene as probes. The results showed that no major rearrangements detectable by restriction enzyme digestion had occurred within about 12 kb 5′ of the transgene. Analysis of the cellular sequences 3′ to the transgene, however, clearly showed that integration of the foreign DNA had resulted in a major deletion, which, on the basis of pulsed-field gel electrophoresis, was estimated to be around 80 kb (data not shown). A schematic map summarizing the sequence arrangement of the CAT40 locus from wild-type and mutant mice is shown in Fig. 2.

When subclone pC1-8 was used as a probe on Northern blots containing total RNAs from several tissues of embryos and adult mice, a 4.2-kb band was detected in brain RNA only (data not shown). To further characterize this transcript, a brain λgt10 cDNA library was screened with pC1-8, resulting in the isolation of several partial cDNA clones. The sequence of the longest cDNA clone is represented by nucleotides 1619 to 3805 of the sequence shown in Fig. 1. A search for similarities with DNA sequences collected in the EMBL data base revealed a region of homology (nucleotides 1620 to 1778) with the β CaM kinase II from rat brain (3). The homology comprises the very 5′ end of our cDNA sequence and the very 3′ end of the published rat sequence. These results suggest that our cDNA clone represents the 3′ half of the mouse *Camk-2* gene. Further sequence comparison between the two cDNAs was not possible, as only the 5′ region, not the 3′ end, of the rat cDNA has been published. To obtain the 5′ end of the mouse *Camk-2* cDNA, this region of the RNA was amplified by PCR, using a 3′ oligonucleotide from our mouse cDNA sequence and a 5′ oligonucleotide from the 5′ end of the published rat cDNA sequence (see Materials and Methods for details). After cloning, the nucleotide sequence was determined and is shown in Fig. 1 (nucleotides −25 to +1725).

The complete sequence of the mouse *Camk-2* cDNA as determined from the different cDNA clones described above is 3,805 bp long (Fig. 1), about 400 nucleotides shorter than the *Camk-2*-specific RNA detected on Northern blots of total brain RNA, which is estimated to be about 4.2 kb long. The difference in length can be explained partly by the absence of the poly(A) tail but, in addition, might be due to the fact that the 5′ end of the transcript is not fully represented in the cDNA. It is unlikely that sequences from the 3′ end are missing, as nuclease S1 mapping experiments confirmed that the 3′ end of the cDNA clone is identical with the 3′ end of the RNA (data not shown). Therefore, the sequence AATAAA from positions 3772 to 3777 (underlined in Fig. 1) most likely serves as a polyadenylation signal. The cDNA sequence contains a major open reading frame of 1,626 nucleotides beginning with a potential ATG initiator codon at nucleotide +1 and ending with a TGA terminator codon at position 1626. At the 3′ end is a rather long untranslated region of about 2,170 nucleotides. The length of the 5′ untranslated region is not known. The encoded protein contains 542 amino acids and has a calculated molecular mass of about 60 kDa. The predicted amino acid sequence shows the three-domain structure typical for multifunctional CaM kinases (31). The catalytic domain at the N-terminal end contains the ATP-binding region consisting of the sequence G-21xG-23xxS-26 and K-43 (underlined in Fig. 1). The regulatory domain adjacent to the catalytic domain is distinguished by the calmodulin-binding region. Underlined in Fig. 1 is the core CaM-binding sequence (residues 297 to 310) as defined previously (11). The remaining C-terminal region constitutes the third domain and is thought to be an association domain involved in the assembly of subunits into the holoenzyme (31).

The sequence of the coding region of the mouse *Camk-2* cDNA is very similar to the published rat sequence (3). Of a total of 58 changes at the nucleotide level, only 2 result in changes of amino acids. The amino acids Glu (residue 19) and Arg (residue 522) of the mouse sequence have been changed to Asp and Pro, respectively, in the rat protein. With respect to the 3′ untranslated region, only 148 nucleotides (residues 1630 to 1778; Fig. 1) can be directly compared, as this is all of the sequence information published so far for the rat cDNA (3). Within this region there is only one nucleotide change. Indirect evidence from Southern blot hybridization experiments suggests that the remaining part of the 3′ untranslated region is also highly conserved between the two species (data not shown).

Genomic organization of the *Camk-2* gene and position of the transgene in the mutant CAT40 allele. To map the transgene integration site relative to the transcriptional unit of the cellular gene, the exon/intron structure of *Camk-2* was determined. Genomic clones from the CAT40 locus from BALB/c mice were isolated as described above and partially sequenced, using oligonucleotide primers deduced from the cDNA sequence. The gene is split into at least 17 exons (Fig. 3). The longest exon is at the 3′ end and comprises the total length of the 3′ untranslated region. The other 15 exons that have been analyzed so far vary in length between 38 and 233 bp. As genomic clones from the 5′ end were not available, the exon/intron structure of the 5′ untranslated region and the remaining 341 nucleotides of the coding region were not analyzed. Exons I to XVI and their intervening sequences comprise a total of about 28 kb of cellular sequences. Of particular interest is exon VI, coding for 15 amino acids. These same 15 amino acids are deleted in the β′ subunit, a minor 58-kDa subunit found in most brain regions

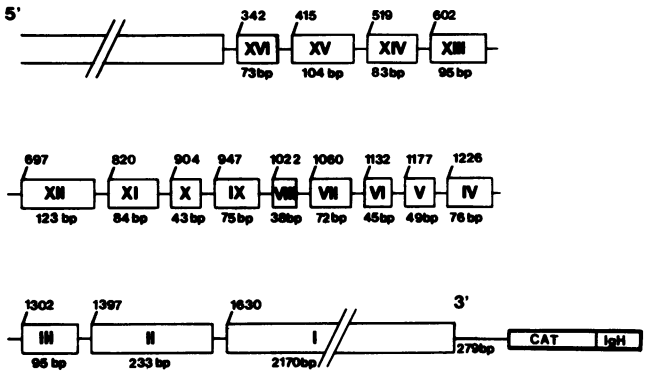

FIG. 3. Schematic representation of the exon/intron structure of the *Camk-2* gene and position of the transgene in the mutant CAT40 allele. Open boxes indicate exons. The introns are not drawn to scale and are indicated by a line connecting the exons. Numbers at the top indicate the 5′ ends of exons and refer to nucleotide residues of the sequence shown in Fig. 1. The position of the transgene (CAT-IgH) in the mutant CAT40 locus and its distance (279 bp) from the 3′ end of the *Camk-2* gene are indicated.

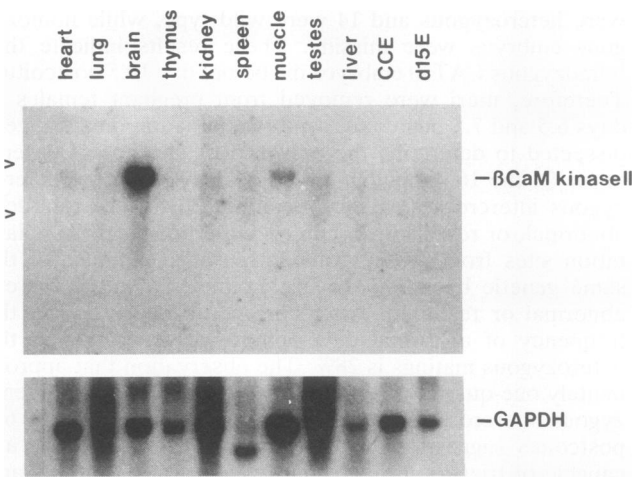

FIG. 4. Expression of the *Camk-2* gene in different organs of adult mice, total 15-day-old embryos (d15E), and the stem cell line CCE. Arrowheads indicate positions of 18S and 28S rRNAs. The blot was subsequently rehybridized with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe.

of the rat and identical to the β subunit except for the deletion (5). The results presented in Fig. 3 strongly suggest that the β′ transcript is generated by alternative splicing of the transcript of the β gene, resulting in the deletion of exon VI.

Restriction analysis showed that the transgene in the mutant CAT40 allele mapped close to the 3′ end of the *Camk-2* gene. For a more detailed analysis, subclones of this region were isolated and sequenced. The results showed that the transgene was located 279 bp 3′ from the poly(A) addition site (Fig. 3). The only difference in this region between the wild-type and mutant alleles was an insertion of 5 bp near the site of transgene integration in the mutant allele (data not shown).

Analysis of *Camk-2* expression in wild-type and CAT40 mice. When RNAs from different tissues of adult BALB/c mice, whole 15.5-day-old embryos, and the embryonal stem cell line CCE were analyzed on Northern blots, *Camk-2*-specific transcripts were found predominantly in the brain and, at much lower levels, in the skeletal muscle and 15.5-day-old embryos (Fig. 4). Expression during embryonal development was studied by using a more sensitive approach based on the amplification of transcripts by PCR. For the amplification, oligonucleotides were used that, according to the published sequences from rat, are specific for the β transcript and are not present in the cDNA sequence of the α CaM kinase II gene (3, 5, 19). The results presented in Fig. 5A show that the mRNA molecules of the β gene are found for the first time during development in the head of 12.5-day-old embryos. As homozygous CAT40 embryos die shortly after implantation, blastocysts were also analyzed for expression of *Camk-2* but were shown to be negative (Fig. 5B). Taken together, the results seem to indicate that expression of *Camk-2* correlates with fetal brain development.

To test whether the transgene integration affected the level of transcription of the *Camk-2* gene, RNA from the brains of 3-week-old offspring from the same litter of heterozygous CAT40 parents was analyzed by Northern blot analysis (Fig. 6). The offspring were genotyped by blot analysis of liver DNA. Autoradiograms were scanned with a densitometer, and the amount of RNA in each lane was normalized by rehybridizing the blots with a β-actin probe. This analysis showed the level of the transcript to be 1.7 (±0.2)-fold lower in heterozygous than in wild-type animals, indicating that the transgene integration interferes with transcription of the gene.

Chromosomal mapping of the *Camk-2* locus. The mouse chromosomal location of the *Camk-2* locus was determined by interspecific backcross analysis, using progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 800 loci that are well distributed among all the autosomes as well as the X chromosome (6). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative RFLPs, using subclone pC1-8 as probe. The 1.2-kb *M. spretus*-specific *Pst*I RFLP (see Materials and Methods) was used to monitor the segregation of the *Camk-2* locus in backcross mice. The mapping results indicated that *Camk-2* is located in the proximal region of mouse chromosome 11 linked to *Lif*, *ErbB* and *Rel*. Although 131 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 7), up to 172 mice were typed for some markers. Each locus was analyzed in pairwise combinations for recombination frequencies, using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are centromere–*Camk-2*–0/172–*Lif*–5/133–*ErbB*–6/158–*Rel*. The recombination frequencies (expressed as genetic distances in centimorgans ± standard error) are (*Camk-2*, *Lif*)–3.8 ± 1.6–*ErbB*–3.8 ± 1.5–*Rel*. The fact that no recombination between *Camk-2* and *Lif* was observed in 172 mice typed in common suggests that the two loci are within 1.7 centimorgans (upper 95% confidence limit).

DISCUSSION

Embryonic lethal mutation. Transgene integration into the CAT40 locus has resulted in a recessive lethal mutation. At day 10.5 of gestation, the earliest time at which embryonic material could be isolated in an amount sufficient to allow genotyping, no homozygous embryos were found among the

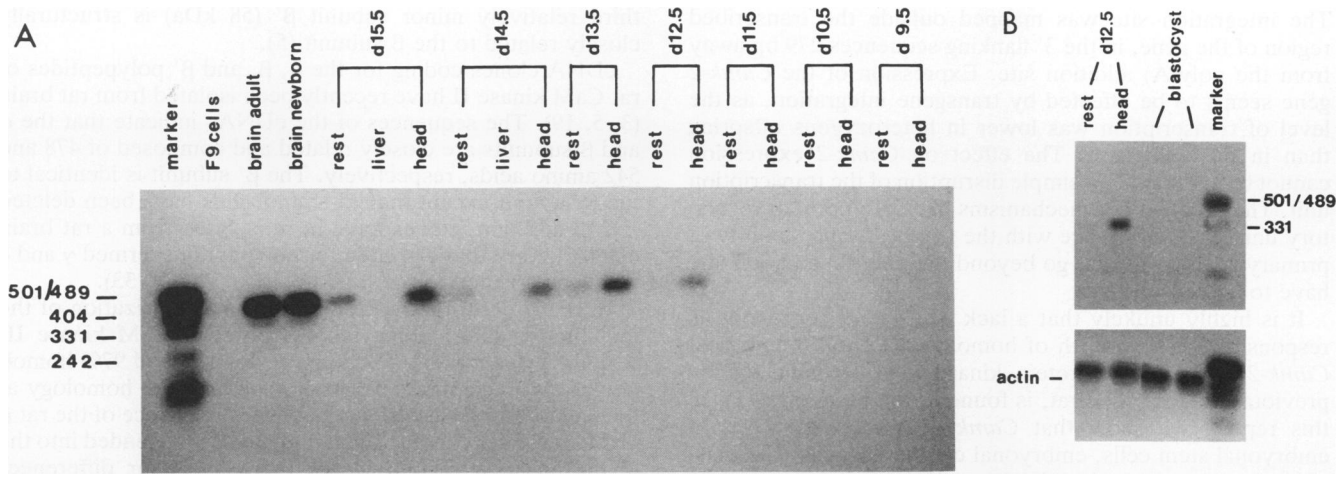

FIG. 5. Expression of the *Camk-2* gene during embryonal development. Total cellular RNA from the head, the liver, and the rest of the embryo at the indicated days of development, from the brains of adult and newborn animals, and from F9 cells (A) and from two independent blastocyst preparations (B) were analyzed by reverse transcription PCR as described in Materials and Methods. In panel A, each PCR reaction mixture contained cDNA equivalent to 25 ng of total RNA. In panel B, PCR reaction mixtures for 12.5-day-old embryos contained cDNA equivalent to 10 ng of total RNA, and mixtures for blastocysts contained cDNA equivalent to total RNA of about 10 embryos. As a control, expression of the β-actin gene was measured in parallel experiments. *Hpa*II-digested plasmid vector pUC9 end labeled with Klenow polymerase and [^{32}P]dCTP was used as a length marker.

normal embryos. About one-quarter of the implantation sites analyzed at day 6.5 contained resorbed embryos. This finding suggested that the resorbed embryos represent the missing class of homozygous offspring. Embryos homozygous for transgene integration at the CAT40 locus, therefore, develop normally during the preimplantation stage and die shortly after implantation in the uterus.

The transgene has integrated adjacent to a gene which was identified to be coding for β CaM protein kinase II (*Camk-2*).

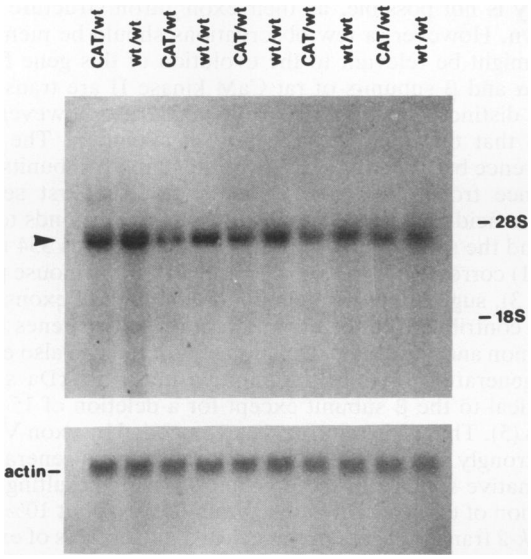

FIG. 6. Distribution of *Camk-2* transcripts in wild-type and heterozygous CAT40 mice. Total RNAs from brains of wild-type (wt/wt) and heterozygous (CAT40/wt) 3-week-old offspring from the same litter of heterozygous CAT40 parents were analyzed by blot hybridization with a *Camk-2* cDNA clone representing the 3′ untranslated exon. The positions of 28S and 18S rRNAs are indicated. The blot was rehybridized with a β-actin cDNA probe.

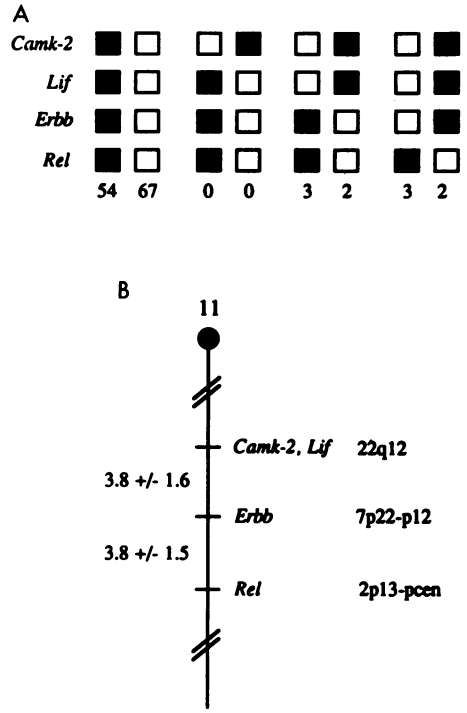

FIG. 7. Position of the *Camk-2* locus on mouse chromosome 11. *Camk-2* was placed on mouse chromosome 11 by interspecific backcross analysis. (A) Segregation patterns of *Camk-2* and flanking genes in 131 backcross animals that were typed in common for *Camk-2*. For individual pairs of loci, more than 131 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of an *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed below each column. (B) Partial chromosome 11 linkage map showing the location of *Camk-2* in relation to linked genes. Recombination distances between loci in centimorgans are shown at the left, and the positions of all loci except *Camk-2* in human chromosomes are shown at the right.

The integration site was mapped outside the transcribed region of the gene, in the 3′ flanking sequences 279 bp away from the poly(A) addition site. Expression of the *Camk-2* gene seems to be affected by transgene integration, as the level of transcription was lower in heterozygous offspring than in the wild type. The effect on *Camk-2* expression cannot be explained by simple disruption of the transcription unit. Therefore, other mechanisms like disruption of regulatory units or interference with the synthesis and stability of primary transcripts that go beyond the poly(A) addition site have to be considered.

It is highly unlikely that a lack of *Camk-2* expression is responsible for the death of homozygous CAT40 embryos. *Camk-2* codes for a protein kinase that, as indicated by previous studies in the rat, is found in the brain only (2). In this report, we show that *Camk-2* is not transcribed in embryonal stem cells, embryonal carcinoma cells, and blastocysts, strongly suggesting that it is not expressed in preimplantation embryos. During development, transcripts are found for the first time in the head of 12.5-day-old embryos, much later than the time of death of homozygous CAT40 embryos. It is therefore unlikely that *Camk-2* is causally related to the death of the embryos. We rather think that the deletion of about 80 kb of cellular sequences 3′ to the transgene has resulted in the loss of a gene or genes that are important for early postimplantation development. Attempts to clone this region from wild-type DNA have so far been unsuccessful, as DNA inserts in cosmids or phage λ vectors are extremely unstable. Deletion and rearrangements are often associated with integration of foreign DNA by microinjection of zygotes (7, 22).

Chromosomal mapping studies have shown that *Camk-2* is located in the proximal region of mouse chromosome 11. We have reported previously a comparison of our interspecific mouse backcross map of chromosome 11 with a composite mouse linkage map compiled by M. T. Davisson, T. H. Roderick, A. L. Hillyard, and D. P. Doolittle (4). These studies indicate that the two maps are generally colinear and that *Camk-2* has been placed in a region that lacks previously identified recessive embryonic lethal mouse mutations similar in phenotype to mice homozygous for the CAT40 transgene. The fact that no recombination was observed between *Camk-2* and *Lif* suggests that these two genes are closely linked. Therefore, we have tested whether the *Lif* gene is deleted in the mutant CAT40 locus. However, Southern blot analysis gave no evidence for such a deletion (data not shown). Finally, the *Lif* locus has been assigned to human chromosome 22, band q12. The close linkage between *Lif* and *Camk-2* in the mouse suggests that *CAMK-2* resides on human chromosome 22 as well.

Structure and expression of *Camk-2*. Molecular analysis of the CAT40 locus has led to the identification of the mouse gene for β CaM protein kinase II. Type II CaM kinases are a group of closely related serine/threonine kinases that phosphorylate several substrate proteins in different tissues (for reviews, see references 18, 30, and 31). One isozyme of type II CaM kinase is expressed specifically in the brain and constitutes about 1% of total brain protein in the rat (2, 9). It appears to be concentrated in forebrain postsynaptic densities (16) and acquires a Ca^{2+}-independent activity when it phosphorylates itself, suggesting that it may be involved in producing long-lasting changes in neuronal function in response to transient calcium signals (17, 20). The enzyme purified from rat brain is a multimeric protein, composed of structurally similar α (54-kDa) and β (60-kDa) subunits (2). A third relatively minor subunit β′ (58 kDa) is structurally closely related to the β subunit (5).

cDNA clones coding for the α, β, and β′ polypeptides of rat CaM kinase II have recently been isolated from rat brain (3, 5, 19). The sequences of the cDNAs indicate that the α and β subunits are closely related and composed of 478 and 542 amino acids, respectively. The β′ subunit is identical to the β subunit except that 15 amino acids have been deleted (5). In addition, clones have been isolated from a rat brain cDNA library that can code for polypeptides termed γ and δ which are related to the α and β subunits (32, 33).

Here we describe the cloning and characterization of the first mouse gene coding for a subunit of CaM kinase II. Sequence analysis of the coding region showed 97% homology at the nucleotide level and close to 100% homology at the amino acid level with the published sequence of the rat β CaM kinase II gene (3). The homology also extended into the 3′ untranslated region, where there are clear differences between the four rat genes, leaving no doubt that we have indeed isolated the mouse homolog of the β CaM kinase II gene. At the amino acid level, the two proteins are virtually identical, as one of the residues by which they differ is a conservative exchange (Glu versus Asp), leaving only a difference in a single amino acid at the carboxy-terminal end (Arg versus Pro). The extraordinary high sequence conservation between two species which diverged in evolution about 5 to 10 million years ago emphasizes the vital role of this kinase in eukaryotic cells and suggests that residues outside the well-defined sequence motifs in the catalytic and regulatory domains are essential for the proper function of the enzyme. It will be of interest to determine whether other members of the CaM kinase II family show the same high degree of sequence conservation.

The genomic organization of *Camk-2* seems to be rather complex. The 3′ untranslated region and about four-fifths of the coding region which has been analyzed so far consist of 16 exons distributed along 28 kb of cellular DNA (Fig. 3). A direct comparison with other members of the CaM kinase II family is not possible, as their exon/intron structure is not known. However, a few observations should be mentioned that might be relevant to the evolution of this gene family. The α and β subunits of rat CaM kinase II are transcribed from distinct genes. Their strong similarity, however, suggests that they diverged recently in evolution. The major difference between the cDNAs of the α and β subunits is the absence from α of two segments (5). The first segment (amino acid residues 316 to 339; Fig. 1) corresponds to exon IX and the second segment (amino acid residues 354 to 392; Fig. 1) corresponds to exons VI and VII of the mouse β gene (Fig. 3), suggesting that deletion or insertion of exons might have contributed to the evolution of these two genes from a common ancestral gene. Deletion of an exon can also explain the generation of the β′ subunit, a minor 58-kDa subunit identical to the β subunit except for a deletion of 15 amino acids (5). These 15 amino acids are encoded by exon VI (Fig. 3), strongly suggesting that the β′ transcript is generated by alternative splicing of the β gene transcript, resulting in the deletion of exon VI. In mouse brain RNA, about 10% of the *Camk-2* transcripts are missing the 45 nucleotides of exon VI and therefore code for the β′ subunit (11a).

A fairly well defined domain within the CaM-dependent protein kinases is the calmodulin-binding site. In the β CaM kinase II, as in many other CaM-binding proteins, this domain consists of a basic α helix which, in a helical wheel projection, forms a basic and a nonpolar surface (for a review, see reference 21). The core sequence of the CaM-

binding domain of the β subunit is underlined in Fig. 1. The bipartite character of this domain is emphasized by the fact that it is split into two exons in such a way that the basic part (R-297RKLK-301) is in one exon and the nonpolar part (G-302AJLTTMLA-310) is in another exon.

The *Camk-2* gene is expressed during embryonic development, and transcription is first seen in the head of 12.5-day-old embryos, that is, around the time when neurodifferentiation in the mouse begins (26). The observation that *Camk-2*-specific transcripts are not detected in the rest of the embryo after removal of the head indicates that the gene is not expressed in the spinal cord. However, more detailed analysis by in situ hybridization is needed to examine the cell type and exact stage of *Camk-2* activation. In the adult mouse, the *Camk-2* gene is expressed more or less exclusively in the brain. Minor transcripts seen in the skeletal muscle might be due to the fact that the probe used cross-hybridizes with a skeletal muscle CaM kinase II. The expression pattern in the adult mouse is in agreement with previously described results for the rat (5, 32). Furthermore, as in the rat, the transcription level of *Camk-2* in mouse brain is very high and comparable to levels of the neurofilament genes (data not shown). This observation has led to the speculation that *Camk-2* has a structural function in addition to its enzymatic function (30). The cloning and characterization of the mouse gene described here will allow us to test this hypothesis by targeted mutagenesis of the gene in embryonal stem cells and by establishing transgenic mouse strains that express no *Camk-2* or an altered form of *Camk-2*.

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REFERENCES

1. **Auffray, C., and F. Rougeon.** 1980. Purification of mouse immunoglobulin heavy-chain messenger RNA's from total myeloma tumor RNA. Eur. J. Biochem. **107**:303–314.
2. **Bennett, M. K., N. E. Erondu, and M. B. Kennedy.** 1983. Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. J. Biol. Chem. **258**:12735–12744.
3. **Bennett, M. K., and M. B. Kennedy.** 1987. Deduced primary structure of the β subunit of brain type II Ca^{2+}/calmodulin-dependent protein kinase determined by molecular cloning. Proc. Natl. Acad. Sci. USA **84**:1794–1798.
4. **Buchberg, A. M., E. Brownell, S. Nagata, N. A. Jenkins, and N. G. Copeland.** 1989. A comprehensive genetic map of murine chromosome *11* reveals extensive linkage conservation between mouse and human. Genetics **122**:153–161.
5. **Bulleit, R. F., M. K. Bennett, S. S. Molloy, J. B. Hurley, and M. B. Kennedy.** 1988. Conserved and variable regions in the subunits of brain type II Ca^{2+}/calmodulin-dependent protein kinase. Neuron **1**:63–72.
6. **Copeland, N. G., and N. A. Jenkins.** 1991. Development and applications of a molecular genetic linkage map of the mouse genome. Trends Genet. **7**:113–118.
7. **Covarrubias, L., Y. Nishida, and B. Mintz.** 1986. Early postimplantation embryo lethality due to DNA rearrangements in a transgenic mouse strain. Proc. Natl. Acad. Sci. USA **83**:6020–6024.
8. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137**:266–270.
9. **Goldenring, J. R., B. Gonzales, J. S. McGuire, and R. J. DeLorenzo.** 1983. Purification and characterization of a calmodulin-dependent kinase from rat brain cytosol able to phosphorylate tubulin and microtubule-associated protein. J. Biol. Chem. **258**:12632–12640.
10. **Green, E. L.** 1981. Genetics and probability in animal breeding experiments, p. 77–113. Oxford University Press, New York.
11. **Hanley, R. M., A. R. Means, B. E. Kemp, and S. Shenolikar.** 1988. Mapping of calmodulin-binding domain of Ca^{2+}/calmodulin-dependent protein kinase II from rat brain. Biochem. Biophys. Res. Commun. **152**:122–128.

11a. **Harbers, K.** Unpublished data.

12. **Hogan, B., F. Constantini, and E. Lacy.** 1986. Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Jaenisch, R.** 1988. Transgenic animals. Science **240**:1468–1474.
14. **Jenkins, N. A., N. G. Copeland, B. A. Taylor, and B. K. Lee.** 1982. Organization, distribution, and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of *Mus musculus*. J. Virol. **43**:26–36.
15. **Kawasaki, E. S.** 1990. Amplification of RNA, p. 21–27. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols. Academic Press, Inc., San Diego, Calif.
16. **Kelly, P. T., T. L. McGuinness, and P. Greengard.** 1984. Evidence that the major postsynaptic density protein is a component of a Ca^{++}/calmodulin dependent protein kinase. Proc. Natl. Acad. Sci. USA **81**:945–949.
17. **Kennedy, M. B.** 1989. Regulation of synaptic transmission in the central nervous system: long-term potentiation. Cell **59**:777–787.
18. **Kennedy, M. B., M. K. Bennett, N. E. Erondu, and S. G. Miller.** 1987. Calcium/calmodulin-dependent protein kinases, p. 61–107. *In* W. Y. Cheung (ed.), Calcium and cell function, vol. VII. Academic Press.
19. **Lin, C. R., M. S. Kapiloff, S. Durgerian, K. Tatemoto, A. F. Russo, P. Hanson, H. Schulman, and M. G. Rosenfeld.** 1987. Molecular cloning of a brain-specific calcium/calmodulin-dependent protein kinase. Proc. Natl. Acad. Sci. USA **84**:5962–5966.
20. **Miller, S. G., and M. B. Kennedy.** 1986. Regulation of brain type II Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation: a Ca^{2+}-triggered molecular switch. Cell **44**:861–870.
21. **O'Neil, K. T., and W. F. DeGrado.** 1990. How calmodulin binds its targets: sequence independent recognition of amphiphilic α-helices. Trends Biochem. Sci. **15**:59–64.
22. **Overbeek, P. A., S. P. Lai, K. R. Van Quil, and H. Westphal.** 1986. Tissue specific expression in transgenic mice of a fused gene containing RSV terminal sequences. Science **231**:1574–1577.
23. **Rappolee, D. A., C. A. Brenner, R. Schultz, D. Mark, and Z. Werb.** 1988. Developmental expression of PDGF, TGF-α, and TGF-β genes in preimplantation mouse embryos. Science **241**:1823–1825.
24. **Reik, W., G. Williams, S. Barton, M. Norris, M. Neuberger, and M. A. Surani.** 1987. Provision of the immunoglobulin heavy chain enhancer downstream of a test gene is sufficient to confer lymphoid-specific expression in transgenic mice. Eur. J. Immunol. **17**:465–469.
25. **Reith, A. D., and A. Bernstein.** 1991. Molecular basis of mouse developmental mutants. Genes Dev. **5**:1115–1123.
26. **Rugh, R.** 1990. The mouse, its reproduction and development. Oxford University Press, Oxford.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.

29. **Schnieke, A., K. Harbers, and R. Jaenisch.** 1983. Embryonic lethal mutation in mice induced by retrovirus insertion into the α1(I) collagen gene. Nature (London) **304:**315–320.
30. **Schulman, H.** 1988. The multifunctional Ca^{2+}/calmodulin-dependent protein kinase. Adv. Second Messenger Phosphoprotein Res. **22:**39–112.
31. **Schulman, H., and L. L. Lou.** 1989. Multifunctional Ca^{2+}/calmodulin-dependent protein kinase: domain structure and regulation. Trends Biochem. Sci. **14:**62–66.
32. **Tobimatsu, T., and H. Fujisawa.** 1989. Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. J. Biol. Chem. **264:**17907–17912.
33. **Tobimatsu, T., I. Kameshita, and H. Fujisawa.** 1988. Molecular cloning of the cDNA encoding the third polypeptide (γ) of brain calmodulin-dependent protein kinase II. J. Biol. Chem. **263:** 16082–16086.
34. **Watson, C. J., and J. McConnell.** 1987. Construction of cDNA libraries for pre-implantation mouse embryos, p. 183–197. *In* M. Monk (ed.), Mammalian development: a practical approach. IRL Press, Oxford.
35. **Woychik, R. P., R. L. Maas, R. Zeller, T. F. Vogt, and P. Leder.** 1990. 'Formins': proteins deduced from the alternative transcripts of the *limb deformity* gene. Nature (London) **346:**850–855.
36. **Woychik, R. P., T. A. Stewart, L. G. Davis, P. D'Eustachio, and P. Leder.** 1985. An inherited limb deformity created by insertional mutagenesis in a transgenic mouse. Nature (London) **318:**36–40.