Functional Expression of the Genomic DNA Sequences Encoding Mouse Na,K-ATPase α 1 Gene by Cotransfection of Overlapping Genomic DNA Segments

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The entire 33-kb coding region of the mouse Na,K-ATPase α 1 subunit gene was cloned in two overlapping cosmids which contain inserts of 40 kb. To assess the functional expression of the mouse α 1 gene, the two cosmids were cotransfected into ouabain-sensitive CV-1 monkey cells yielding an average of 64 resistant colonies per 10^6 cells per μ g of DNA. Analysis of the DNA transferred to the ouabain-resistant transformants by the two cosmids suggests that the generation of a functional gene can occur by homologous recombination between the two introduced segments, as demonstrated by generation of a novel diagnostic restriction fragment. The ability to reconstruct the intact mouse α 1 gene in a heterologous host cell and to monitor its functional expression with a selection protocol permits direct identification and isolation of regulatory sequences for the gene.

Na,K-ATPase is an integral membrane-bound enzyme responsible for the active transport of Na^+ and K^+ across the plasma membrane in animal cells. The electrochemical gradient produced serves as the primary energy source for active transport of other solutes and nutrients, for the regulation of cell volume, and for the maintenance of resting potential in excitable cells (8, 10). The enzyme is composed of two subunits (α and β) with molecular masses of approximately 112 and 35 kDa, respectively. In most animals, the α subunit is found as three isoforms (α 1, α 2, and α 3) whose genes are unlinked in both mice and humans (9, 23). Through high-affinity binding to a subset of α subunits, cardiac glycosides such as ouabain inhibit Na,K-ATPase activity (20, 21). Rodent and primate cell lines show extreme differences in ouabain sensitivity, a feature which has been exploited extensively in somatic cell genetics (1, 16). The transfer of ouabain resistance to ouabain-sensitive cells has been correlated with the presence of the rodent Na,K-ATPase α 1 subunit gene (6, 14). Gene transfer experiments using mouse and rat α 1 subunit cDNAs have also provided evidence that it is the expression of the rodent α 1 subunit that mediates ouabain resistance (5, 11, 18). Subsequent studies using chimeric interspecific cDNA constructs and site-directed mutagenesis have demonstrated that determinants of ouabain response reside within the amino-terminal portions of the rat and human α 1 subunits and that the presence of arginine and aspartic acid residues in the H1-H2 extracellular domain accounts for the ouabain resistance properties of the rat Na,K-ATPase (4, 18).

Na,K-ATPase mRNA and peptide levels are reported to be responsive to a variety of physiological agents, including thyroid and steroid hormones (19). To study the control of expression of the Na,K-ATPase α subunit genes, we have isolated genomic clones of the mouse α 1 subunit gene (*Atpa-1*). Utilizing transfection protocols to assess whether any single clone contained the regulatory and structural sequences necessary to confer ouabain resistance to primate

cells, we were unable to isolate a single cosmid clone carrying the entire intact mouse Na,K-ATPase α 1 gene. Previous studies have shown that an intact gene can be reconstructed in mammalian cells following transfection of a pair of overlapping but independent plasmids carrying the truncated genes (3, 26). We have used this approach to reconstruct the functional activity of cloned mouse α 1 genomic sequences. Two mouse α 1 genomic clones, with inserts of 40 kb, were found to span the entire coding region of the α 1 gene and to contain an overlapping 5-kb α 1 genomic sequence. Our data show that the cotransfection of these two overlapping mouse α 1 cosmids conferred ouabain resistance to ouabain-sensitive CV-1 cells and suggest that an intact mouse α 1 gene was generated by extrachromosomal homologous recombination.

MATERIALS AND METHODS

Construction and screening of mouse genomic library. The pseudodiploid mouse CAK (C57BL/6J $\times$ AKR/J) F_1 fibroblast cell line was grown to confluence, and high-molecular-weight genomic DNA was prepared by the procedure of Milbrandt et al. (17). Aliquots of high-molecular-weight CAK DNA were subjected to partial digestion with *Mbo*I restriction enzyme and were used to construct a genomic library in the cosmid vector pWE 15 as previously described (15, 29). Approximately 5×10^5 independent colonies were plated onto nitrocellulose filters, and replica filters were prepared and screened by filter colony hybridization by using a 3.0-kb *Eco*RI fragment derived from the full-length 3.6-kb mouse Na,K-ATPase α 1 cDNA (mba69) as a probe (11). Filters were hybridized at 42°C for 16 h in the presence of 50% formamide and were subjected to a final wash at 55°C in $0.1 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate. Radiolabeled cDNA probes were synthesized with the Klenow fragment of DNA polymerase I with random hexanucleotides (P-L Biochemicals) and [α - 32 P]dCTP (specific activity, 4×10^8 cpm/ μ g of DNA) (7).

Cell culture and DNA transfection. African green monkey CV-1 cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum. Transfections were

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FIG. 1. Restriction maps of two overlapping mouse genomic clones which hybridize with probes derived from the mouse Na,K-ATPase $\alpha 1$ cDNA. The mouse $\alpha 1$ subunit cDNA was used to screen a mouse genomic library constructed with the pWE 15 cosmid vector. Positive clones were analyzed by restriction enzyme digestions, and the identity and order of restriction fragments were determined by successively hybridizing the DNA to a series of eight labeled restriction fragments derived from the full-length mouse $\alpha 1$ DNA. The mouse $\alpha 1$ subunit cDNA fragments used as probes were as follows: B, *EcoRI-NcoI*, nucleotide (nt) -111 to -2; C, *NcoI-BamHI*, nt -1 to 594; D, *BamHI-XhoI*, nt 595 to 965; E, *XhoI-BamHI*, nt 966 to 1539; F, *BamHI-NcoI*, nt 1540 to 2199; G, *NcoI-EcoRI*, nt 2200 to 2824; H, *EcoRI-AatII*, nt 2825 to 3211; I, *AatII-EcoRI*, nt 3212 to 3411. The mouse $\alpha 1$ genomic sequences detected by the probes are represented by black boxes, and the adjacent thin lines illustrate the 5' and 3' flanking regions. The hatched boxes at each end indicate pWE 15 vector sequences. The unlabeled vertical lines indicate *EcoRI* sites. Boxed letters below the maps indicate cDNA probes to which the genomic fragments were shown to hybridize. N, The *NcoI* site which maps the translation initiation codon (ATG) of the mouse $\alpha 1$ gene; X, the *XhoI* site present in the overlapping $\alpha 1$ genomic sequences. The sizes of fragments are shown in kilobases.

performed essentially as described previously (11). CV-1 cells (1.5×10^6 cells) were exposed to a calcium phosphate precipitate of plasmid DNA containing the entire cDNA (10 μ g of pSV2 $\alpha 3.6$), cosmid DNA (10 μ g of either M α G 6 or M α G 9 or 5 μ g of M α G 6 and 5 μ g of M α G 9), or calcium phosphate without DNA. After 48 h, cells were subcultured at dilutions of 1 to 3, 1 to 10, and 1 to 50, and ouabain was added to a final concentration of 10^{-6} M. Ouabain-resistant colonies of transfected populations were picked at 3 weeks and expanded in the presence 10^{-6} M ouabain.

Southern blot hybridization. Cloned cosmid or cellular genomic DNA was digested to completion with restriction enzymes, fractionated on 1 or 0.7% agarose gels, and transferred in $10\times$ SSC to hybridization membranes (Zetabind; Bio Rad, Richmond, Calif.). The blots were then hybridized at 42°C in the presence of 50% formamide and washed to a final stringency of $0.1\times$ SSC-0.1% sodium dodecyl sulfate at 60°C.

RESULTS

Isolation and characterization of cosmid clones spanning the entire mouse Na,K-ATPase $\alpha 1$ gene. A mouse genomic library constructed in the cosmid vector pWE 15 (29) was probed with a full-length mouse $\alpha 1$ cDNA (mb $\alpha 69$) (11). Three independent clones were analyzed by restriction endonuclease mapping and shown to contain restriction fragments corresponding to mouse $\alpha 1$ genomic DNA sequences. Digested cosmid DNA was hybridized to a series of eight probes (Fig. 1) which encompass the entire coding region of the mouse $\alpha 1$ cDNA (S. L. Graw and R. B. Kent, unpublished data). Two of the three positive genomic clones, M α G 6 and M α G 9, contain inserts of approximately 40 kb, and together they span the entire transcription unit of the mouse $\alpha 1$ gene. The M α G 9 clone represents the 5' end of the $\alpha 1$ gene and contains approximately 22 kb of $\alpha 1$ genomic sequences and 20 kb of 5' flanking sequences. The 3' end of the $\alpha 1$ gene mapped within the M α G 6 clone, which carries approximately 17 kb of $\alpha 1$ genomic sequences and 21 kb of

3' flanking sequences (Fig. 1). The mouse $\alpha 1$ gene is estimated to be approximately 33 kb, and the two cosmids contain an overlapping 5-kb $\alpha 1$ genomic sequence. Limited DNA sequence analysis further mapped the translation initiation codon (ATG) and the entire 5' untranslated sequence of the mb $\alpha 69$ cDNA clone to the 8.4-kb *EcoRI* restriction fragment contained in the M α G 9 clone (Fig. 1). The remaining clone, M α G 11, has an insert of 20 kb, and the genomic sequences it represents are also contained in clone M α G 9. Preliminary studies of exon-intron boundaries of the mouse $\alpha 1$ gene, done by using direct sequencing of polymerase chain reaction products, revealed that the sequences contained in all of the exons examined are identical to those found in the mouse $\alpha 1$ cDNA clone (S. L. Graw, unpublished data).

Transfection of the two mouse $\alpha 1$ genomic clones with overlapping homologous sequences confers ouabain resistance to CV-1 cells. To determine whether the mouse $\alpha 1$ genomic clones could confer ouabain resistance to ouabain-sensitive CV-1 cells, the two cosmids, M α G 6 and M α G 9, were transfected either singly or together into CV-1 cells by the calcium phosphate coprecipitation procedure (11). Cells were selected for the ability to proliferate in 10^{-6} M ouabain, a concentration of drug that is cytotoxic to CV-1 parent cells. In two separate experiments, introduction of both M α G 6 and M α G 9 cosmids into CV-1 cells gave an average of 64 ouabain-resistant colonies per 10^6 cells per μ g of DNA within 2 weeks following selection (Table 1). Transfection of CV-1 cells with the plasmid pSV2 $\alpha 3.6$ (an expression vector containing the full-length cDNA insert [mb $\alpha 69$] encoding the mouse $\alpha 1$ gene) yielded an average of 392 ouabain-resistant colonies. Colonies were not observed in control CV-1 cells transfected either with M α G 6 or M α G 9 cosmid alone or with mock-transfected cells. These results suggest that the two cosmids together contain the entire genomic sequence encoding the ouabain-resistant mouse Na,K-ATPase $\alpha 1$ subunit.

Genomic DNA analysis of transfectants indicates integration

TABLE 1. Efficiency of transfection of mouse Na,K-ATPase $\alpha 1$ subunit genomic clones into ouabain-sensitive monkey CV-1 cells

DNA transfected	Colonies/ μ g of DNA/ 10^6 cells ^a		
	Expt 1		Expt 2
	A	B	
None	0	0	0
pSV2 $\alpha 3.6$	302	430	446
M α G 6	0	0	0
M α G 9	0	0	0
M α G 6 and M α G 9	44	42	107

^a CV-1 cells were exposed to a calcium phosphate precipitate of plasmid pSV2 $\alpha 3.6$, to cosmid M α G 6 or M α G 9, to cosmid M α G 6 and M α G 9, or to calcium phosphate without DNA. Forty-eight hours later, cells were subcultured at dilutions of 1 to 3, 1 to 10, and 1 to 50, and ouabain was added to the cells to a final concentration of 10^{-6} M. The data shown were derived from two separate experiments.

of mouse $\alpha 1$ genomic sequences into CV-1 cell chromosomes. To confirm that the ouabain-resistant colonies resulted from the introduction of mouse $\alpha 1$ genomic sequences, genomic DNA from individual CV-1 transfectant clones (A3, A4, A5, B3, B4, and B5) was analyzed by Southern blot analysis for the presence of exogenous mouse $\alpha 1$ genomic sequences by hybridizing to two probes representing the 3.6-kb full-length mouse $\alpha 1$ cDNA (mb α 69) (the 3.0-kb 5' *Eco*RI fragment and the 0.6-kb 3' *Eco*RI fragment). All of the *Eco*RI fragments representing the entire mouse $\alpha 1$ gene in the two cosmid M α G 6 and M α G 9 were detected in each of the ouabain-resistant CV-1 transfectants examined (Fig. 2). The 11.0-, 8.4-, and 2.5-kb DNA fragments were derived from M α G 9, whereas DNA fragments of 4.9, 3.6, 2.5, 2.4, and 1.2 kb originated from M α G 6. A similar hybridization pattern was seen with genomic DNA isolated from mouse CAK cells, the source of the cosmid library, but a distinctly different pattern was seen in the CV-1 parent. These results indicate that the expression of the ouabain resistance phenotype of the CV-1 transfectants is correlated with the introduction into CV-1 cells of genomic sequences spanning the entire coding region of the mouse $\alpha 1$ gene. Several additional DNA fragments of lower hybridization intensity were also detected in each transfectant. Some of these DNA fragments (5.0, 5.2, and 6.2 kb) represent the endogenous monkey $\alpha 1$ gene, whereas the other fragments may originate from the integration of truncated mouse $\alpha 1$ cosmid clones into the CV-1 cell chromosomes.

Genomic DNA analysis of transfectants reveals homologous recombination between overlapping mouse $\alpha 1$ genomic sequences. To investigate whether a functional mouse $\alpha 1$ gene had been generated by extrachromosomal homologous recombination, the $\alpha 1$ genomic segments in the ouabain-resistant recipient cells were restriction mapped and examined for a novel diagnostic fragment (Fig. 3). Double digestion with *Not*I and *Hind*III and hybridization with probe E (encompassing the 5-kb region of overlap) generates bands of 7.5 kb from M α G 6 and 12.0 kb from M α G 9. If a homologous recombination event has occurred within this overlapping region to yield a functional mouse $\alpha 1$ gene, then a novel *Hind*III restriction fragment of 14.5 kb hybridizing specifically with probe E is predicted. DNA isolated from all of the ouabain-resistant transfectants have this predicted 14.5-kb *Hind*III fragment (Fig. 4). Since most of the cosmids integrated into the CV-1 chromosomes without undergoing homologous recombination, the most intense hybridization is to the 12.0- and 7.5-kb *Hind*III-*Not*I nonrecombinant

FIG. 2. Southern blot analysis of genomic DNA from cells transfected with mouse $\alpha 1$ genomic clones. Genomic DNA (10 μ g) from CAK and CV-1 cells and transfectants selected in 10^{-6} M ouabain (A3, A4, A5, B3, B4, and B5) were digested with *Eco*RI. DNA fragments were electrophoresed through a 1% agarose gel and transferred to a Zeta-bind filter. The filter was hybridized to 10^7 cpm of two 32 P-labeled mouse cDNA probes spanning the entire mouse $\alpha 1$ cDNA (mb α 69). On the left, *Eco*RI fragments representing the entire mouse $\alpha 1$ gene as contained in the two cosmids, M α G 6 and M α G 9, are indicated by arrows. Molecular weight markers (in thousands) are shown on the extreme left.

bands. Additional DNA fragments were detected by probe E in all the transfectants, possibly derived from the endogenous monkey $\alpha 1$ gene or nonhomologous recombination events between the two cosmids. Nevertheless, the consistent presence of the 14.5-kb *Hind*III band in each of the ouabain-resistant clones suggests that the functional expression of the mouse $\alpha 1$ gene following cotransfection of two overlapping mouse $\alpha 1$ cosmid clones has been mediated by homologous recombination between the overlapping regions of the mouse $\alpha 1$ genomic sequences present in the two cosmids.

DISCUSSION

The results presented here show that transfection of cloned genomic sequences spanning the entire coding region of the mouse Na,K-ATPase $\alpha 1$ subunit gene confers ouabain resistance to ouabain-sensitive CV-1 cells. The data complement transfection studies using the mouse or rat $\alpha 1$ cDNA (5, 11, 18) and support the conclusion that the functional expression of the rodent Na,K-ATPase $\alpha 1$ gene mediates the ouabain-resistant properties of the rodent cells. Previous studies with somatic cell hybrids have assigned a gene responsible for ouabain resistance to mouse chromosome 3 (13). This observation agrees well with the subsequent localization of the Na,K-ATPase $\alpha 1$ gene (*atpa-1*) to mouse chromosome 3 and suggests that the *Atpa-1* locus on chromosome 3 encodes a ouabain-resistant mouse $\alpha 1$ subunit (12).

An intact and functional mouse Na,K-ATPase $\alpha 1$ gene appears to have been generated by extrachromosomal homologous recombination. Previous studies of extrachromosomal recombination in mammalian cells have utilized small DNA segments in plasmid or retroviral vectors as recombination substrates. These experiments demonstrate the feasibility of using cosmid clones, with their inserts of 40 kb of genomic sequences, as recombination substrates. The mouse gene is comparable in length (at least 33 kb) to the estimated 30 kb for the human gene (24, 25, 28). The

FIG. 3. Restriction map of overlapping regions of the two mouse $\alpha 1$ cosmid clones (MaG 6 and MaG 9) and the expected product formed by homologous recombination between the two cosmid. The restriction sites relevant to the analysis are shown. The sizes of fragments are shown in kilobases. The mouse $\alpha 1$ genomic sequences detected by probe E are represented by black boxes. The open boxes represent mouse $\alpha 1$ genomic sequences not labeled by probe E. The hatched boxes at each end indicate pWE 15 vector sequences, and dotted lines illustrate the 5' and 3' flanking regions.

recombination frequency observed in our studies between the two cosmid (16% of the frequency of an intact gene) is within the range reported by others for extrachromosomal recombination events (2). In experiments with the *tk* gene in Ltk⁻ cells, recombination frequencies of 1 to 20% have been obtained (22, 26). Similarly, in other studies using two defective *neo* gene segments, a frequency of 15% has been reported (27). Thus, the recombination frequency observed for the two cosmid is consistent with the Southern analysis, suggesting that the functional reconstitution of the mouse $\alpha 1$ gene is mediated by extrachromosomal homologous recombination between the two overlapping mouse $\alpha 1$ cosmid.

These procedures provide a direct approach to monitor the normal physiological expression of the Na,K-ATPase $\alpha 1$ gene. With the development of a direct assay for functional expression of the Na,K-ATPase gene, some of the difficulties associated with reporter gene assays for the identification of control sequences can be circumvented. This procedure is especially useful for identification of regulatory sequences outside of the 5' upstream region which are difficult to characterize by other methods. The system reported here should thus provide the basis for detailed analysis of the regulatory mechanisms involved in the control of expression of the mouse $\alpha 1$ gene.

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FIG. 4. Southern blot analysis of genomic DNA from cells transfected with mouse $\alpha 1$ genomic clones. Genomic DNA (10 μ g) from CAK and CV-1 cells and transfectants selected in 10^{-6} M ouabain (A3, A4, A5, B3, B4, and B5) were digested with *Hind*III and *Not*I. DNA fragments were electrophoresed through a 0.7% agarose gel and transferred to a Zeta-bind filter. The filter was hybridized to 10^7 cpm of 32 P-labeled probe E, which is a 573-bp *Xho*I-*Bam*HI restriction fragment derived from the mouse $\alpha 1$ cDNA. The expected diagnostic 14.5-kb fragment generated by homologous recombination is indicated by an arrow. On the left, *Hind*III-*Not*I DNA fragments detected by probe E as present in the two cosmid, MaG 6 and MaG 9, are indicated. Molecular weight markers (in thousands) are shown on the extreme left.

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