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Genetics

Molecular cloning of the mouse ouabain-resistance gene

(DNA transfection/ouabain resistance/phage cloning/Na^+,K^+-ATPase)

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ABSTRACT DNA prepared from ouabain-resistant mouse cells was able to transform ouabain-sensitive CV-1 cells to ouabain resistance after DNA-mediated gene transfer. The murine DNA fragment responsible for ouabain resistance was detected on the background of CV-1 DNA by virtue of a repetitive DNA sequence element that reacts positively with a mouse repeat DNA clone. CV-1 DNA is nonreactive with this probe. Southern analysis of several independently derived ouabain-resistant transformants indicates that the mouse *oua^R* gene is located on a 6.5-kilobase *Eco*RI restriction fragment. The 6.5-kilobase DNA fragment was initially isolated from a λ phage library made from a ouabain-resistant secondary transformant and subsequently was subcloned in the plasmid vector pAT153. This plasmid was able to transform wild-type CV-1 cells to ouabain resistance at a frequency of about 10 cells per ng of DNA.

Recent developments in gene transfer and recombinant DNA techniques have led to significant advances in the ability to isolate specific eukaryotic genes. In particular, the method of DNA-mediated gene transfer has been successfully applied to the purification of the mouse dihydrofolate reductase gene (1), the avian thymidine kinase gene (2), the hamster adenine phosphoribosyltransferase gene (3), and the human bladder carcinoma gene (4). In this paper, we describe the use of DNA-mediated gene transfer as an experimental approach for isolating the murine ouabain-resistance (*oua^R*) gene.

The cardiac glycoside ouabain is a specific inhibitor of the plasma membrane Na^+,K^+-ATPase (5), the enzyme primarily responsible for regulating the active transport of Na and K ions across cell membranes (6). Cell lines of different species differ considerably with respect to ouabain sensitivity, primate cells being quite sensitive to ouabain as compared to mouse cells (7). Because ouabain cytotoxicity is attributable in all cases to inhibition of Na^+ and K^+ transport, the variation in ouabain resistance is presumably due to species-specific differences in the ouabain binding site of the Na^+,K^+-ATPase (7).

We took advantage of the species difference in ouabain sensitivity to develop a gene-transfer system for isolating a mouse *oua^R* gene. Previous experiments have demonstrated that ouabain resistance can be transferred by DNA-mediated gene transfer (8). We find that when CV-1 African green monkey cells, which are highly sensitive to ouabain, are exposed to DNA from mouse cells, which are relatively resistant to ouabain, a small subpopulation is transformed to the ouabain-resistant phenotype (oua^R) by virtue of the stable incorporation and expression of the donor *oua^R* gene. This phenotype can be transferred through a series of transformations to segregate the murine sequences associated with the oua^R phenotype from all other mouse DNA sequences. The DNA fragment carrying the *oua^R* gene has been detected in secondary transformants by using a cloned mouse repetitive sequence element, suggesting that the homologous repeat sequence can be used as a biological marker for cloning the *oua^R* gene. The *oua^R* gene has been isolated from a phage library prepared from the DNA of a secondary transformant and subcloned in the plasmid vector pAT153. When applied to CV-1 cells, this plasmid is highly active in transforming CV-1 cells to ouabain resistance.

The isolation of the *oua^R* gene may prove extremely useful for understanding the biochemical basis of ouabain resistance and for elucidating the structure and function of the Na^+,K^+-ATPase.

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MATERIALS AND METHODS

Cell Culture. Friend cells (line 745-PC-4) were maintained in α medium supplemented with 13% fetal calf serum. All other cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. The HT1080 trans mouse oua^R-F cell line was provided by T. Gross and R. Baker. This cell line was derived by the transformation of human HT1080 fibrosarcoma cells with metaphase chromosomes prepared from oua^R mouse fibroblasts (T. Gross and R. Baker, personal communication).

Preparation of Genomic DNA and DNA Transfection. Genomic DNA was prepared from confluent cultures of cells by essentially using the method of Shih and Weinberg (4). DNA transformation of CV-1 cells was carried out by the calcium phosphate precipitation technique of Graham and van der Eb (9) as modified by Wigler *et al.* (10). Two days after transformation, cells were trypsinized, replated at lower density ($\approx 5 \times 10^5$ cells per plate) and selected in medium containing either 1 or 0.1 μm ouabain (Sigma). Cells were grown in selective media for 3–5 days and then were grown under nonselective conditions for 3–5 days. Cells then were reexposed to drug and maintained under selective conditions. After about 2 wk, surviving colonies were picked by using cloning cylinders and grown into mass populations.

Plasmids and Nick Translation. The plasmid pMR81 was used as a probe to screen transformants. The derivation of pMR81 has been described (11). This plasmid contains a 520-base-pair *Sau*3A fragment of mouse repeat sequence DNA inserted into the *Bam*HI site of pBR322. This repeat sequence is represented about 10,000 times in the mouse genome (unpublished data). Nick-translations were carried out essentially as described (12). The specific activity of the probe was routinely $2\text{–}4 \times 10^8$ cpm/μg of DNA.

Blot Hybridization. Electrophoresis of DNA fragments on agarose gels and transfer of DNA to nitrocellulose was es-

Abbreviation: kb, kilobase(s).

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sentially as described by Southern (13). Hybridizations were performed for 18 hr at 42°C in 50% formamide containing 5× SSCPE (1× SSCPE is 150 mM NaCl/15 mM sodium Citrate/13 mM KH_2PO_4/1 mM EDTA), 1× Denhardt solution (14), 200 μg of denatured herring sperm DNA per ml, and 10% dextran sulfate. After hybridization, blots were washed three times at RT for 15 min in 1× SSCPE/0.1% $NaDodSO_4$ and three times at 65°C for 1 hr and then were exposed to Kodak XAR-5 film at −80°C with a Cronex (DuPont) intensifying screen.

Isolation of 6.5-Kilobase (kb) *Eco*RI Fragment. DNA (100 μg) from an oua^R secondary transformant (HT1080-2) was cleaved to completion with *Eco*RI and fractionated on a 10–40% sucrose gradient essentially as described (4). Fractions showing positive hybridization to pMR81 on Southern blots were pooled, concentrated by precipitation with ethanol, and resuspended in 10 mM Tris chloride/1 mM EDTA, pH 7.5.

Creation and Screening of Bacteriophage Library. The isolated 6.5-kb *Eco*RI fragment was ligated to *Eco*RI-cleaved calf intestinal phosphatase-treated phage λ Charon 16A DNA and packaged *in vitro* by the method of Grosveld *et al.* (15). An estimated 1 × 10^6 plaque-forming units were plated and screened by the method of Benton and Davis (16). Positive phage were plaque-purified and grown to large scale in culture, and DNA was extracted essentially as described by Shih and Weinberg (4).

Subcloning into Plasmid pAT153. *Eco*RI-cleaved phage clone DNA was electrophoresed through a 0.6% agarose gel, and the 6.5-kb insert was isolated from the gel by a modification of the glass powder method (17) as described (18). DNA was incubated with calf intestinal phosphatase-treated, *Eco*RI-cleaved pAT153 in the presence of T4 DNA ligase (Bethesda Research Laboratories). This DNA was used to transform *Escherichia coli* strain HB101 by the procedure of Cohen *et al.* (19). The large scale preparation of plasmid subclone DNA was carried out by published procedures (4).

RESULTS

Experimental Design. The strategy adopted for the isolation of the murine *oua^R* gene is outlined in Fig. 1. DNAs from several cell lines resistant to ouabain were used to transform ouabain-sensitive CV-1 cells to ouabain resistance (oua^R phenotype) by the calcium phosphate precipitation method (10). Transforming DNAs were prepared from Friend (murine erythroleukemia) cells, which are resistant to 0.5 mM ouabain and HT1080 trans mouse oua^R-F cells, a primary transformant derived from the transfer of metaphase chromosomes from oua^R mouse cells into human HT1080 fibrosarcoma cells (T. Gross and R. Baker, personal communication). This cell line is resistant to 3 mM ouabain. Wild-type CV-1 cells, on the other hand, are much more sensitive to ouabain (50 nM). Thus, transformants expressing the oua^R phenotype can be selected at intermediate levels of ouabain (1 μM).

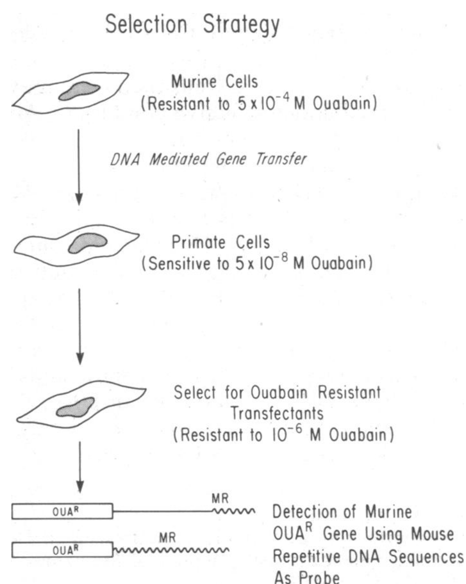

FIG. 1. Strategy for transfer and detection of the mouse *oua^R* gene. For explanation see text. MR, mouse repetitive DNA sequences (∿∿∿).

DNA from primary transformants was serially passaged by means of a second round of transfection into CV-1 cells. The mouse *oua^R* gene can then be detected in secondary transformants by sequence hybridization to a cloned mouse repetitive DNA sequence element. This analysis is made possible because of the fact that the homologous mouse repeat sequence is physically linked to the *oua^R* gene and, thus, can serve as a biological marker. Sequences homologous to this repetitive sequence element are not detectable in CV-1 DNA.

Construction of Oua^R Transformants and Preliminary Analysis of Transforming Sequences. In an initial series of experiments, the DNAs from the cell lines listed in Table 1 were used to transfect CV-1 cells in a primary round of transformation. A cDNA clone representing the entire poliovirus genome, which is infectious when applied to CV-1 cells (18), was used as a positive control. As shown in Table 1, CV-1 or HT1080 donor DNA yielded no transformants. No colonies were observed in a control culture of untreated CV-1 cells. Friend cell DNA yielded transformants at a rate of 0.2 transformants per μg of donor DNA. Oua^R transformants survived selection only when ouabain selection was applied intermittently. No drug-resistant transformants were observed when continuous ouabain-selection conditions were used. The molecular basis for this response is not as yet understood.

DNAs from three independently derived primary transformants (FCV-1A, -1C, and -1D) were used to transform CV-1 cells in a secondary round of transfection. These donor DNAs yielded secondary transformants at a rate of 0.05–0.15 per μg of donor DNA (Table 1). When DNA from HT1080 trans mouse oua^R-F was used, 0.3 transformants per μg of DNA was obtained. HT1080 trans mouse oua^R-F DNA consistently yielded secondary transformants at a higher and more reproducible rate than any of the FCV lines tested.

Table 1. Transfer of ouabain resistance

Donor DNA	Colonies, no.	
	per μg of DNA	per dish
Primary transfection of CV-1 cells		
Friend	0.2	6, 8, 8
CV-1	0	0
HT1080	0	0
None	0	0
Polio cDNA	—	12 plaques
Secondary transfection of CV-1 cells		
FCV-1A	0.05	2, 2, 0
FCV-1C	0.05	1, 2, 1
FCV-1D	0.1	3, 3, 4
HT1080 trans mouse oua^R-F	0.3	12, 12, 9

Genomic DNAs were sheared three times through a 21-gauge needle. Aliquots of DNA (40 μg) were then applied to CV-1 cells. Each dish contained about 2 × 10^6 cells. For ouabain selection, each dish was trypsinized and replated at one-third density in medium containing 1 μM ouabain. The total number of oua^R colonies on three plates represents the transformation efficiency for the amount of added DNA.

Preliminary experiments designed to characterize the sequences carrying the *oua*^{R} gene were carried out by subjecting transforming DNA to digestion with one of several restriction endonucleases prior to transfection. DNAs from the primary transformants HT1080 trans mouse oua^R-F and FCV-1D were treated with the restriction enzymes *Eco*RI, *Hin*dIII, *Bam*HI, or *Hin*fI and then applied to CV-1 cells. The biological activity of transforming DNA was spared by the enzymes *Eco*RI, *Hin*dIII, and *Bam*HI; however, *Hin*fI treatment inactivated transforming activity (Table 2). These findings suggest that the *oua*^{R} gene is contained within a fragment of DNA encompassed by either *Eco*RI, *Hin*dIII, or *Bam*HI restriction sites and that the transforming sequence is most likely contained within a relatively small DNA fragment.

Detection of the Transforming DNA Segment. Initial experiments designed to detect the DNA sequences containing the *oua*^{R} gene were carried out by using secondary transformants derived from the transfer of HT1080 trans mouse oua^R-F DNA into CV-1 cells. When total mouse DNA was used to probe secondary transformant DNA, this probe was found to preferentially hybridize to a background of mouse satellite DNA (unpublished results). Secondary transformant DNAs were then screened with several unique mouse repetitive-sequence-element probes that had been cloned in pBR322 (11). It was hoped that one of these probes would react specifically with mouse repetitive DNA sequences that were closely linked to the *oua*^{R} gene. One probe, designated pMR81, appeared to fulfill this criteria.

When DNAs from two independently derived secondary transformants were digested with one of several restriction enzymes and screened with pMR81, a characteristic pattern of hybridizing bands, including an *Eco*RI fragment of about 7 kb was observed (Fig. 2). DNAs from four other independent transformants also exhibited an *Eco*RI fragment of about 7 kb when probed with pMR81 (data not shown). We wished to ascertain whether transforming activity and the repetitive sequence element were both contained on this *Eco*RI fragment. To do this, we treated DNA of a secondary transformant with *Eco*RI and then separated the DNA fragments by sucrose gradient fractionation. DNA from gradient fractions that failed to hybridize with pMR81 yielded no oua^R colonies upon transfection into CV-1 cells (Table 3). *Eco*RI fragments of 6.5–7 kb that gave positive signals in Southern blots with pMR81 probe (data not shown) were able to transform CV-1 cells to oua^R upon transfection. This result suggests that the *oua*^{R} gene lies within one or more of these *Eco*RI fragments and that a mouse repetitive sequence homologous to pMR81 is closely linked to the *oua*^{R} gene.

Molecular Cloning of the *oua*^{R} Gene. The sucrose gradient fractions that contained transforming activity (described above) were pooled together and ligated to *Eco*RI-cut, calf intestinal phosphatase-treated phage λ Charon 16A DNA and packaged *in vitro* (15). Approximately 10^6 plaque-forming units were plated, and the resulting plaques were screened with pMR81. Of the 40 positively hybridizing plaques that were identified in the initial round of screening, 12 were identified as true positives after four rounds of screening and plaque purification. DNA was prepared from three independent phage stocks, digested with *Eco*RI, and analyzed by Southern blotting with pMR81 probe. DNA from one recombinant phage contained a 6.5-kb insert (Fig. 3, lane 1). DNA from a second phage stock contained a 6.5-kb insert and several additional bands (Fig. 3, lane 3), while DNA from the third phage stock contained a 5-kb insert (Fig. 3, lane 2). Phage DNA containing the 6.5-kb insert (lane 1) was used to transfect CV-1 cells. One microgram of phage DNA was mixed with 10 μg of CV-1 DNA and applied to CV-1 cells. After 4 days of selection, about 50 colonies were observed, whereas no colonies were visualized in control cultures in which phage λ Charon 16A DNA alone was used as donor DNA. These oua^R transformants proved to be highly unstable when reselected with ouabain. Only one oua^R transformant survived under selective pressure. When the DNA of this transformant was analyzed, it did not appear to contain

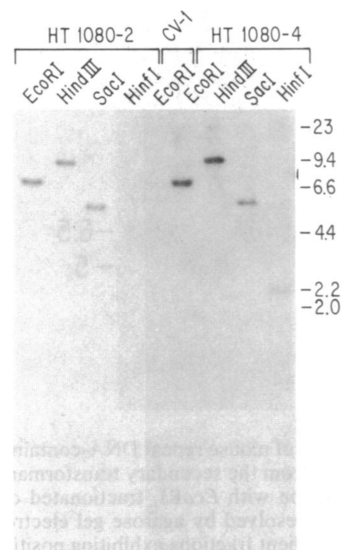

FIG. 2. Detection of murine repeat DNA containing fragments in transformed cells. DNA was prepared from two independent transformants, HT1080-2 and HT1080-4. These DNAs were digested with *Eco*RI, *Hin*dIII, *Sac*I, or *Hin*fI, and 10 μg of DNA from each digest was resolved by electrophoresis through a 1% agarose gel. *Eco*RI-digested CV-1 DNA (10 μg) was run in a separate lane. DNA fragments were transferred to nitrocellulose filters, and a mouse repetitive DNA sequence clone (pMR81) was used as probe. Sizes are shown in kb.

Table 2. Effect of restriction enzymes on transfection of DNA from a primary transformant

Donor DNA	Colonies per dish, no.				
	No enzyme	*Eco*RI	*Hin*dIII	*Bam*HI	*Hin*fI
HT1080 trans mouse oua^R-F	10, 12	11, 10	12, 9	6, 7	0
FCV-1D	4, 3	3, 3	2, 3	2, 1	0

Genomic DNA from two independent oua^R primary transformants was digested to completion with the restriction enzymes listed in the table. Transformations were carried out with 40 μg of DNA as described in Table 1, except that each dish was replated at one-half density prior to selection.

Table 3. Transfection of sucrose gradient fractions

Fraction no.	Hybridization to pMR81	Colonies per dish, no.
2–10	−	0
14–16	+	9, 11
18–26	−	0
HT1080-2 (unfractionated)		10, 11

DNA from the secondary transformant, HT1080-2, was digested to completion with *Eco*RI and fractionated on a 10–40% sucrose gradient as described. Fractions were resolved by electrophoresis through a 1% agarose gel, and the DNA fragments were transferred to nitrocellulose filters and probed with pMR81. Fractions exhibiting positive and negative hybridization were pooled and used to transfect CV-1 cells. DNA (30 μg) from fractions 2–10 and 18–26 was used. DNA from fractions 14–16 (2 μg) was mixed with 28 μg of CV-1 DNA and cotransfected into CV-1 cells.

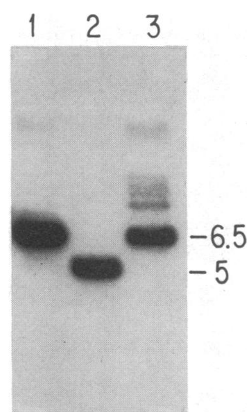

FIG. 3. Detection of mouse repeat DNA-containing sequences in phage clones. DNA from the secondary transformant HT1080-2 was digested to completion with *Eco*RI, fractionated on a 10–40% sucrose gradient, and resolved by agarose gel electrophoresis as described. Sucrose gradient fractions exhibiting positive hybridization to pMR81 were pooled and used to construct a library of recombinant phage in the phage λ cloning vector, Charon 16A, as described. DNA was prepared from three independent phage clones, digested with *Eco*RI, resolved by electrophoresis through a 0.8% agarose gel, and transferred to a nitrocellulose filter. pMR81 was used as a probe in sequence hybridization analysis. Five hundred nanograms of DNA were applied to each lane. Sizes are shown in kb.

the 6.5-kb *Eco*RI mouse DNA fragment (data not shown). Recent data has suggested that phage DNA may act to inhibit mammalian cell transformation when mixed with eukaryotic DNA (20). Therefore, we subcloned the *Eco*RI 6.5-kb fragment purified from phage DNA into the plasmid vector pAT153. Several recombinant clones containing the 6.5-kb insert were isolated. DNA from one of these subclones, pRLoua^R-8 was used to transfect CV-1 cells. This subclone has a high degree of biological activity upon transfection (Table 4). One hundred nanograms of plasmid DNA yielded oua^R transformants at a rate too numerous to count, whereas 10 ng of DNA yielded about 100 oua^R colonies. This result strongly suggests that the *oua^R* gene is contained within the 6.5-kb *Eco*RI segment of inserted DNA.

Restriction Map of *oua^R* Gene. A preliminary restriction map of the inserted DNA of pRLoua^R-8 was constructed by a series of single and double digestions (Fig. 4). A more detailed analysis of the DNA segment containing the *oua^R* gene is necessary. It will be of particular interest to determine the position of the repetitive sequence element(s) and to localize the region essential for biological activity.

DISCUSSION

In this study we took advantage of species differences in ouabain sensitivity to design a selection system for isolation

Table 4. oua^R Transformation efficiency of pRL oua^R-8

Donor DNA	Plasmid DNA added, ng	Transformants per plate*	Colonies per ng of plasmid DNA, no.
pRLoua^R-8 + 10 μg CV-1	100	>1000	>10
pRLoua^R-8 + 10 μg CV-1	10	≈100	≈10
CV-1 (10 μg)	—	0	0
pAT153 + 10 μg CV-1	100	0	0

About 10 μg of DNA was applied in all cases. CV-1 DNA was sheared prior to transfection as described in Table 1.
*Number of oua^R transformants.

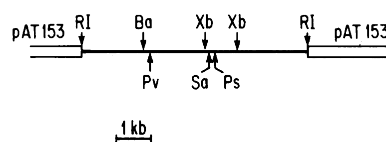

FIG. 4. Restriction map of DNA sequence containing the *oua^R* gene. Mapping of the plasmid pRLoua^R-8 was constructed by a series of single and double digestions. Ba; *Bam*HI; Sa, *Sac* I; Pv, *Pvu* II; Xb, *Xba* I; Ps, *Pst* I.

of the mouse *oua^R* gene. The physical linkage of a mouse repetitive DNA sequence to the *oua^R* gene has provided a biological marker for detecting the *oua^R* gene in secondary transformants and in a library of a recombinant phage constructed from the DNA of these secondary transformants. The ability of a plasmid subclone containing inserted DNA to transform CV-1 cells to ouabain resistance provides direct biological evidence that the inserted DNA fragment contains within its *Eco*RI termini the *oua^R* gene. It should be pointed out that the donor cells used in these experiments (HT1080 trans mouse oua^R-F) may contain both the mutant murine allele responsible for increased levels of ouabain resistance and the wild-type murine allele. Either gene could confer ouabain resistance on CV-1 cells after DNA transfection. It will be of interest to determine which gene has been cloned in the experiments reported here.

It is generally assumed that the α subunit of the Na^+,K^+-ATPase is the principal, if not the sole, ouabain-binding protein in eukaryotic cells (6). It seems likely, therefore, that species variations in ouabain sensitivity may be due to differences in the structure of the Na^+,K^+-ATPase α subunit and, hence, in the nucleotide sequence of the gene encoding this protein. These considerations raise the issue of whether the *oua^R* gene we have cloned does in fact code for the α subunit of the Na^+,K^+-ATPase.

In several systems, the α subunit of the Na^+,K^+-ATPase has an apparent M_r of ≈100,000 (6) and exhibits a high degree of evolutionary conservance (6). Thus, a protein of this size would contain about 1000 amino acids, although the exact number is unknown. Therefore, about 3000 bases of DNA would be required to encode the complete amino acid sequence of the α subunit.

The coding regions of most eukaryotic genes are interrupted by one or more intervening sequences (21), which in sum total may be much larger than the coding region itself (21). In view of this, it is somewhat surprising that the *oua^R* gene is entirely contained within a genomic fragment of only 6.5 kilobase pairs. The fact that this fragment also contains one or more repetitive sequence elements (of unknown size) means that the gene itself is even smaller than 6.5 kb.

Our results on the effect of restriction endonuclease digestion on genomic DNA from primary transformants (Table 2) suggests that *Bam*HI spares the biological activity of the *oua^R* gene. The restriction map of the *oua^R* gene (Fig. 4) indicates that there is one *Bam*HI site located 1.5 kb from one of the *Eco*RI ends of the cloned insert. If *Bam*HI does in fact spare the biological activity of the gene, we would predict that transfection of *Bam*HI-cut pRLoua^R-8 into CV-1 would yield transformants at a rate equivalent to undigested plasmid. Although this experiment has not yet been performed, if *Bam*HI digestion preserved biological activity, it would now place the *oua^R* gene within a 5-kb fragment. Thus if the *oua^R* gene codes for the α subunit of the Na^+,K^+-ATPase, any intervening sequences would be small.

It is possible that the *oua^R* gene could code for a protein other than the α subunit of the Na^+,K^+-ATPase. For instance, it is possible that the β subunit of the ATPase could affect ouabain binding and, hence, alter sensitivity to ouabain. Modifying enzymes also might affect ouabain binding

of the ATPase. Direct sequence determination of the gene (cDNA) and/or use of an expression vector to analyze the gene product should be illustrative of whether the 6.5-kb *oua*^{R} gene does code for the α subunit of the Na^+,K^+-ATPase. Answers to these questions should establish the biochemical basis of ouabain resistance and may provide important insights into the structure and function of the Na^+,K^+-ATPase.

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