Chromosome-Mediated Transfer of the Murine Na,K-ATPase Alpha Subunit Confers Ouabain Resistance

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We transferred murine NIH 3T3 metaphase chromosomes into monkey CV-1 cells to investigate the different ouabain sensitivities of rodent and primate cells. In 16 ouabain-resistant transfectants, the mouse Na,K-ATPase \( \alpha_1 \) subunit gene was detected, suggesting that structural differences between the rodent and primate \( \alpha_1 \) subunits determine the different ouabain sensitivities.

The Na,K-ATPase is the enzymatic activity responsible for the maintenance of high internal K and low internal Na levels characteristic of most animal cells. The enzyme has been shown to consist of two subunits. The \( \alpha \) subunit, approximately 100 kilodaltons in size, contains the catalytic site for ATP hydrolysis. The function of the \( \beta \) subunit, a glycosylated polypeptide of approximately 55 kilodaltons, has not yet been established (2). The cardiac glycoside ouabain is a specific inhibitor of the Na,K-ATPase (1). This drug is believed to bind to the \( \alpha \) subunit of the enzyme (13).

The rodent Na,K-ATPase is relatively resistant to ouabain. Most rodent cell lines proliferate actively in concentrations of ouabain as high as 100 \( \mu \)M, while primate cell lines are killed by exposure to 10 \( \mu \)M ouabain (1, 6, 9). For this reason, resistance to ouabain has long been used as a selectable marker in primate-rodent cell fusion experiments (6). Using somatic cell hybrids, ouabain resistance has been assigned to mouse chromosome 3 (5). Chromosome-mediated transfer of the murine ouabain resistance phenotype to human cell lines has also been achieved (8). However, the molecular basis for the species-specific difference in ouabain sensitivity has not been clearly explained.

In an initial series of experiments, we isolated a murine gene which can confer ouabain resistance to primate cells (7). However, this gene does not encode either the \( \alpha \) or \( \beta \) subunit of the Na,K-ATPase. Moreover, cDNA probes for three distinct isoforms of the rat Na,K-ATPase \( \alpha \) subunit have been described (15; R. B. Kent, D. A. Fallows, E. Geissler, T. Glaser, J. R. Emanuel, P. A. Lalley, R. Levenson, and D. E. Houman, Proc. Natl. Acad. Sci. USA, in press). A cDNA clone of the rat Na,K-ATPase \( \beta \) subunit has also been characterized (10). To further investigate the molecular basis of the difference between rodents and primates in ouabain sensitivity, we carried out a series of chromosome-mediated gene transfer experiments. This technique is less likely to discriminate against genes of large size than is DNA-mediated gene transfer. We prepared metaphase chromosomes from murine NIH 3T3 cells and introduced them into African green monkey CV-1 cells by the calcium phosphate coprecipitation procedure (4, 11). Confluent cultures of NIH 3T3 cells grown in Dulbecco modified Eagle medium (DME; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum, penicillin, and streptomycin were exposed to colcemid for 16 h at a concentration of 1 \( \mu \)g/ml. Mitotic cells (1.3 \times 10^7 cells) were harvested by trypsinization, lysed in an ice-cold solution of 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.1)–3 mM CaCl_2 (15 min at 0°C), and then mechanically disrupted by repeated passage through a 22-gauge needle. Chromosomes were recovered by serial centrifugations and suspended in 6.0 ml of transfection buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 5.5 mM glucose, 1 mM Na_2HPO_4 [final pH 7.10]). A chromosome-calcium phosphate precipitate was allowed to form for 20 min at 20°C after the addition of CaCl_2 to a final concentration of 117 mM. Recipient CV-1 cells were plated in DME at a density of 8 \times 10^4 cells per 60-mm dish 16 h before transfection. A total of 4.8 \times 10^8 CV-1 cells (12 dishes) were exposed for 15 min at 20°C to the chromosome-calcium phosphate precipitate. Fresh medium was then added to each plate, and the cells were incubated at 37°C. After 15 h, the cells were shocked with 15% glycerol in DME, washed once, fed fresh medium, and further incubated at 37°C. After 24 h, the cells were subcultured 1 to 9 into 100-mm dishes. Two-thirds of the cells from each transfection plate were subjected to the following selection procedure originally used to isolate the mouse ouabain resistance gene (7). The cells were not further subcultured during selection.

After 48 h of incubation, the cells were 80 to 90% confluent and were fed fresh medium containing 1 \( \mu \)M ouabain (Sigma Chemical Co., St. Louis, Mo.), incubated a further 48 h, and then incubated 48 h in medium lacking ouabain. Two further cycles of 48-h incubation, one in 1 \( \mu \)M ouabain and a second in ouabain-free medium, were performed in selecting for the ability to proliferate in medium containing 1 \( \mu \)M ouabain. The cells were then constantly maintained in 1 \( \mu \)M ouabain. Culture of the chromosome precipitate alone yielded no colonies, indicating that viable NIH 3T3 cells were not present in the chromosome preparation. In culture dishes containing CV-1 cells which had been transfected with murine chromosomes, ouabain-resistant colonies were first

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TABLE 1. Chromosome transfer of the a1 isomorph of the mouse Na,K-ATPase a subunit into CV-1 cells correlates with acquisition of ouabain resistance

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Hybridization (+) to mouse DNA fragment:</th>
<th>Tu96</th>
<th>a1</th>
<th>a2</th>
<th>a3</th>
<th>b</th>
<th>oua'</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH 3T3</td>
<td>---</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CV-1</td>
<td>---</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Transferents</td>
<td>A1, A2, B1, C1, C2, C3, D1, D2, G1, G2, G3, H1, I1, I2, K1, K1'</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L1</td>
<td>---</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

detected 4 weeks after the beginning of drug selection. In one such experiment, 24 colonies were obtained, corresponding to a transfer efficiency of 1 ouabain-resistant colony per $1.3 \times 10^7$ cells. No colonies were observed when $1.3 \times 10^7$ mock-transfected CV-1 cells were similarly maintained in selection for 5 weeks at 37°C. Similar efficiencies were obtained in other experiments (data not shown). From nine original transfection dishes, 17 clones were selected and expanded for further characterization. Clones arising in the same transfection plate were given the same letter designation. A difference in letter designation between two clones therefore denotes their independent derivation.

High-molecular-weight DNA was prepared from the various cell lines by standard proteinase K treatment and successive phenol and chloroform extractions. Electrophoresis, transfer, and hybridization of EcoR1-digested DNA samples were performed as previously described (3). DNA from the ouabain-resistant colonies was initially screened for the presence of mouse DNA sequences. Genomic DNA samples from each clone were hybridized to a diagnostic mouse-specific probe Tu96. This 1-kilobase (kb) DNA probe hybridizes to an interspersed repeat DNA family which is present at approximately 30,000 copies per genome (12). The results are summarized in Table 1. Of the 17 clones, 16 contained mouse DNA sequences which hybridized to the probe. We next hybridized the genomic DNA from these clones to separate cDNA probes for the three isoforms of the rat a subunit: clone rb5 (1.2-kb rat cDNA encoding a portion of the a1 subunit (14), clone rb2 (3.2-kb rat cDNA encoding a portion of the a2 subunit), and clone rb13c (3.0-kb rat cDNA encoding a portion of the a3 subunit) (Kent et al., in press). We also hybridized the genomic DNA to a cDNA for the rat b subunit (1.2-kb rat clone rb19 encoding a portion of the b subunit) (10) and to the 900-base-pair Xba1 fragment probe SPOX isolated from the mouse ouabain resistance gene (7). Results of these experiments are given in Table 1 and Fig. 1. In each of the clones which showed hybridization to the mouse-specific repeat, four characteristic DNA fragments homologous to the a1 isomorph probe were detected. These DNA fragments correspond in position to hybridizing DNA fragments characteristic of mouse a1 DNA sequences. The data suggest that in each of 16 transfereents, corresponding to at least eight independent chromosome transfer events, the transfer of the mouse a1 subunit gene is responsible for the ouabain-resistant phenotype of the cells. The mouse genes encoding the a2 and a3 subunits were not detected in any of the transfereents. It therefore appears that these two genes are either incapable of conferring ouabain resistance on primate cells in this protocol or do so at a frequency substantially lower than does the a1 gene. The absence of the coding sequences for the murine b subunit in the transfereents leads to similar conclusions regarding the possible role of the rodent b subunit gene in conferring ouabain resistance to primate cells. The fact that the ouabain resistance gene previously characterized by our group did not appear in any of the transfereents cannot be attributed to its inability to convey ouabain resistance in this protocol.

We must therefore conclude that the frequency with which ouabain resistance is transferred by this gene is considerably lower than the frequency for the a1 subunit gene. In the one clone in which mouse DNA was not detected by using the Tu96 repeat probe, no mouse gene associated with Na,K-ATPase function was detected either. It is possible that no mouse DNA sequences were transferred to this clone and that mutation in an endogenous monkey gene conferred ouabain resistance. The appearance of such a mutation reflects a spontaneous mutation frequency of 1 in $3 \times 10^8$ cells.

To assess the extent of ouabain resistance in transfereents containing murine Na,K-ATPase a1 subunit DNA sequences, we measured the survival of four independent transfereents at various concentrations of ouabain. Duplicate 0.2-ml cultures containing 2,000 cells were plated in 96-well plates in DME-10% fetal calf serum with 1 $\mu$M, 10 $\mu$M, 100 $\mu$M, and 1 mM ouabain and without ouabain. Cell lines tested for survival were the independent transfereents A2, C1, D1, G2, the spontaneous mutant clone L1, the donor NIH 3T3 line, and the recipient CV-1 line. After 7 days of incubation at 37°C in 5% CO2, the cells were fixed in methanol for 15 min and stained with 2% crystal violet in ethanol for 5 min. The absorbance of stained cells in each well was measured by using a 492-nm filter in a Titertek Multiscan densitometer (Fig. 2). The survival of each of the four transfereent clones at each ouabain concentration tested was the same as that of the original mouse donor line, indicating that these clones are true transfereents for murine ouabain resistance. CV-1 cells did not survive at any ouabain concentration used in these experiments. The spontaneous mutant clone L1 was as ouabain resistant as NIH 3T3 cells were, a phenotype not previously reported for spontaneous

FIG. 1. Southern analysis of genomic DNA from chromosome transfereents. A Southern blot was prepared by using EcoR1-digested DNA (7 $\mu$g) from 17 chromosome transfereents, mouse NIH 3T3 cells, and monkey CV-1 cells. The blot was probed with cDNA probe rb5, a 1.2-kb fragment that encodes a portion of the rat Na,K-ATPase a1 subunit (14).
mutants arising from murine chromosome transfer into human cells (8).

Chromosome transfer experiments thus provide one route to defining the DNA sequences involved in the expression of the ouabain resistance phenotype. Previous studies using fusion of somatic cells localized a gene responsible for ouabain resistance to mouse chromosome 3 (5). It is of interest that the $\alpha_1$ subunit gene maps to mouse chromosome 3, while all of the other genes tested in this study map to other mouse chromosomes (Kent et al., in press). The level of ouabain resistance of the Na,K-ATPase derived by expression of the rodent $\alpha_2$ and $\alpha_3$ subunit genes is currently unknown. It is not possible, therefore, to distinguish at this time whether the failure to detect transfer of these genes in our protocol is due to the inherent ouabain sensitivity of these genes or to other aspects of the transfer protocol.

The data presented here indicate that the murine $\alpha_1$ subunit is capable of associating with the monkey $\beta$ subunit to form a functional Na,K-ATPase. This result suggests that the insertion of the $\alpha_1$ subunit gene into an appropriate expression system will permit the transfer of ouabain resistance to primate cells by a direct DNA transfer procedure.

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LITERATURE CITED


