Functional expression of a human Na⁺/H⁺ antiporter gene transfected into antiporter-deficient mouse L cells

(intracellular pH regulation/Na⁺-H⁺ exchange/DNA-mediated gene transfer/somatic cell genetics)

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ABSTRACT To clone the gene for the human Na⁺/H⁺ antiporter, we first constructed a stable mouse LTK⁻ cell line (LAP1) lacking Na⁺/H⁺ antiport activity. Second, we devised a selective technique based on acid killing that specifically sorts out cells expressing low levels of Na⁺/H⁺ antiport activity from a population of antiporter-deficient cells (AP⁻). LAP1 cells (TK⁻ and AP⁻) were cotransformed with human genomic DNA and the thymidine kinase (TK) gene. TK⁺ transformants, first selected, were submitted to acid loading. The rare transformants that survived (frequency, 2–8 × 10⁻⁶) expressed Na⁺/H⁺ antiport activity (AP⁺). We found that: (i) transformation with mouse L1 DNA did not give rise to AP⁺ transformants; (ii) transformation of LAP1 cells with DNA from an altered Na⁺/H⁺ antiporter hamster variant led to AP⁺ transformants expressing the altered Na⁺/H⁺ antiporter of the DNA donor; (iii) human repeated sequences were present in all primary, secondary, and tertiary mouse AP⁺ transformants; (iv) six identical EcoRI human DNA fragments (55 kilobase pairs of the human genome) cosegregated with the Na⁺/H⁺ antiport activity in secondary and tertiary transformants. These results strongly suggest that we have stably expressed the structural gene for the human Na⁺/H⁺ antiporter in mouse cells.

The Na⁺/H⁺ antiporter that exchanges internal H⁺ for external Na⁺ is now recognized to play a fundamental role in the regulation of intracellular pH (pHᵢ) in virtually all vertebrate cells (reviewed in refs. 1–3). This membrane-bound system has aroused much interest because it is rapidly activated by a variety of external stimuli including sperm (4), insulin (5), growth promoting agents (6–12), chemotactic peptide (13), tumor promoters (14–18), etc., leading to a persistent cytoplasmic alkalization of the stimulated resting cells.

A low cytoplasmic pH is a common feature of resting prokaryotic and eukaryotic cells (19) that might be needed to maintain a low metabolic activity in spores, eggs, or quiescent cells. On the contrary, a rapid increase in pHᵢ appears to be a prerequisite for cell activation. Fibroblast mutants specifically defective in Na⁺-H⁺ exchange (20) have been very valuable in establishing the existence of a critical pHᵢ threshold, below which growth factor-stimulated DNA synthesis and growth are severely restricted (21, 22). For example, phosphorylation of ribosomal protein S6 is a pHᵢ-restricted step. Indeed, this growth factor-regulated event is tightly coupled to an increase in pHᵢ (23).

Although much has been learned about the physiology and mechanistic features of the Na⁺/H⁺ antiport system, nothing is known about its molecular structure. In this paper, we report the isolation of a stable Na⁺/H⁺ antiporter-defective mouse L cell line and its use as recipient for expression of a foreign Na⁺/H⁺ antiporter gene by DNA-mediated gene transfer. We show that human genomic DNA can restore the antiporter defective function of mouse L cells and that, in independent secondary and tertiary transformants, a common DNA-restriction endonuclease pattern of human repeated sequences cosegregates with the selected Na⁺/H⁺ antiport activity. This is the first step toward the molecular cloning of the amiloride-sensitive and growth factor-activable Na⁺-H⁺ exchange system.

MATERIALS AND METHODS

Cell Culture. Thymidine kinase-deficient mouse L cells (LTK⁻) originally established by Kit et al. (24) and derivatives (see Table 1) were cultured in a minimal essential medium supplemented with 10% (vol/vol) inactivated fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). Unless otherwise specified, transformed mouse L cells were continuously cultured in the presence of 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT). Culture conditions and properties of AR300, a stable variant derived from Chinese hamster lung fibroblasts which "overexpresses" an altered Na⁺/H⁺ antiporter activity, are described (25).

Isolation of LAP1 Cells Deficient in Na⁺-H⁺ Exchange Activity. LTK⁻ cells were mutagenized with ethyl methane-sulfonate (0.3 μl/ml for 18 hr), cultivated for 5 days, and submitted to the "proton suicide" selective isolation procedure described for hamster fibroblasts (20). After Li⁺-loading, cells were exposed for 30 min to the choline chloride saline medium (130 mM choline chloride/5 mM KCl/1 mM MgCl₂/2 mM CaCl₂/20 mM Mes-Tris, pH 5.5). Clones resistant to three consecutive H⁺ suicide tests were selected and analyzed for Na⁺-H⁺ exchange activity. One of them with 40% residual activity was grown and mutagenized a second time. Selected for resistance when the choline chloride saline, pH 5.5, was applied for 30 min, the cells were killed when the test was prolonged to 60 min. Further application of this H⁺ suicide selection three times over 2 weeks, led to the selection of few clones devoid of Na⁺-H⁺ exchange activity. LAP1 cells were recloned for further analysis.

Preparation of Cellular DNA. Human genomic DNA (from freshly isolated human lymphocytes) and mouse genomic DNA (from tissue culture cells) were prepared as described by Wigler et al. (26). DNA was treated with boiled RNase at 0.1 mg/ml for 3 hr at 37°C, phenol extracted, and dialyzed extensively into 10 mM Tris-HCl/1 mM EDTA, pH 7.8, with four changes of buffer. Supercoiled plasmid DNA TK⁺, pTK-x1 (27) was prepared according to the method of Maniatis et al. (28).

Transformation and Selection of Na⁺/H⁺ Antiporter Positive Transformants (AP⁺). Transformation of LAP1 cells was

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performed by the calcium phosphate precipitation technique (26, 29). L cells were plated at 5 × 10⁵ cells per 100-mm dish, incubated for 24 hr, and then exposed overnight to 10, 40, and 100 μg of RNase-treated genomic DNA mixed with 0.5-1 μg of plasmid TK + DNA. HAT was added 2 days later. After 6 days of culture, TK + colonies, which arose at a frequency ranging from 10⁻³ to 10⁻², were acid-load with the NH₄⁺-prepulse technique (1, 25) as follows: culture medium was aspirated and replaced by NH₄Cl-saline medium (50 mM NH₄Cl/70 mM choline chloride/5 mM KCl/1 mM MgCl₂/2 mM CaCl₂/5 mM glucose/15 mM Mops/Tris, pH 7.0). Unless otherwise specified, cells were incubated at 37°C for 30 min in this medium. Then, cells were quickly washed twice with a Na + -saline medium (120 mM NaCl/5 mM KCl/1 mM MgCl₂/2 mM CaCl₂/5 mM glucose/15 mM Mops, pH 7.0) to remove external NH₄Cl and incubated in the same medium for 60 min at 37°C. At the end of this period the Na + -saline medium was replaced with culture medium. This acid-load test that discriminated Na + /H + antiporter positive (AP +) transfectants from AP - cells (see Fig. 2) was applied twice a week.

DNA Analysis of Clonal Transformed Cells. DNA isolated from representative clones was subjected to Southern blot analysis (28) for detection of human repeated sequences. DNA (20 μg) was digested to completion with appropriate restriction endonucleases according to the conditions suggested by the supplier. Digested DNA was applied to 0.7% horizontal agarose gels, electrophoresed for 12 hr at 6 V/cm, and blotted to nitrocellulose filters. Baked nitrocellulose filters were preincubated for 4–6 hr at 42°C in 50% (vol/vol) formamide/5× SSC (750 mM NaCl/75 mM sodium citrate, pH 7.0)/5× Denhardt's solution/0.1% NaDodSO₄. Then filters were hybridized for 12–24 hr in the above buffer containing 10% (wt/vol) dextran sulphate, sonicated salmon sperm DNA at 100 μg/ml, and 32P-labeled human DNA at 10 ng/ml (1–2 × 10⁶ dpm/μg) as described by Kühn et al. (29). Total human DNA was prefered to Alu (Blur 8) as a hybridization probe because Alu sequences represent only one class of human repeated elements. Indeed, some restriction fragments that hybridized with the total human DNA probe were negative or weak with the human Alu probe Blur 8.

22Na⁺ Influx and pHï Measurements. Amiloride-sensitive Na⁺ influx and pHï determination with [14C]benzoic acid were conducted exactly as described (8, 20).

RESULTS

LAP1, a Mouse L Cell Line Defective in Na⁺/H⁺ Antiport Activity. Mouse LTK + cells were submitted to the proton suicide selection test that we developed for Chinese hamster fibroblasts (20). All the resistant clones analyzed had 30–40% residual Na⁺/H⁺ antiport activity. A second mutagenizing step followed by a more stringent "proton-suicide" test led to the isolation of another set of resistant clones completely lacking Na⁺/H⁺ antiport activity. The biochemical features of LAP1, a representative of these second step-H⁺-suicide resistant colonies, are shown in Fig. 1. When cells are loaded with Li + and rapidly incubated in a Na + - and Li + -free external medium at an extracellular pH of 5.5 (Fig. 1 Left), the antiporter operates in the reverse mode and leads to a rapid and strong H⁺ accumulation in the cytoplasm of LTK cells. In contrast, pHï of LAP1 cells slowly equilibrated with the extracellular H⁺ and remained at least 1 pH unit higher than in the parental LTK - cells. Even under conditions that maximally activate the antiporter such as an acid load (30), we did not detect any amiloride-sensitive Na⁺-influx in LAP1 cells (Fig. 1 Right). This LAP1 cell line, completely defective in Na⁺/H⁺ antiport activity, grew at the same rate as the parent in HCO₃⁻-buffered medium and remained stable after more than 50 generations in the absence of selective pressure.

![Graph of pH measurement](image)

**Fig. 1.** Comparison of H⁺ and Na⁺ influxes in mouse LTK + and its Na⁺/H⁺ antiporter-deficient derivative, LAP1. (Left) LAP1 (c) and LTK + (c) cells were submitted to proton suicide selection. First, cells were loaded with 120 mM LiCl for 2 hr, then washed quickly and exposed to 120 mM choline chloride saline, pH 5.5, that was Na + + Li + free (20). Intracellular pHï was monitored by the distribution of [14C]benzoic acid (8). (Right) LAP1 (c) and LTK + (c) cells were acid-load with the NH₄Cl-prepulse technique. First, cells were incubated with a Na + saline medium containing 50 mM NH₄Cl for 30 min, then washed quickly and exposed at time 0 to the 22Na⁺-uptake medium described (20). The amiloride-insensitive Na⁺-influx values that represent less than 10% of total Na⁺-uptake have been subtracted.

However, when selected for, spontaneous revertants were observed to arise at a frequency lower than 10⁻⁷.

A Specific Selection for Na⁺/H⁺ Antiporter Positive Transformants (AP⁺). Because of the toxicity of high cytoplasmic H⁺ concentration and the fact that the Na⁺/H⁺ antiporter plays a determining role in pH regulation, it was expected that a cell line lacking the antiporter would be extremely sensitive to an acid load. Fig. 2 validates this expectation. A severe acid-load, induced by an NH₄Cl prepulse (50 mM NH₄Cl for 60 min), kills almost 100% of LAP1 cells in less than 10 min (Fig. 2 Left). Under the same conditions, viability of the parental LTK + cells is not altered during the 30 min following the acute load. This rapid and powerful positive selection makes it possible to discriminate, in a large population of Na⁺/H⁺ antiporter deficient cells, only those that have regained the pHï-regulating system. Because we anticipated that genomic transfection would not restore 100% of the parental Na⁺/H⁺ antiport activity, we refined the selection test to discriminate cells having as little as 10–20% activity from cells with no activity. Fig. 2 Right shows the loss of cell viability when the intensity of the acid load is changed by varying the time of NH₄Cl-loading (0–60 min) in four simulated situations (cells with respectively 0, 15, 50, or 100% Na⁺/H⁺ antiport activity). The dashed line represents the conditions we have chosen for the selection of the Na⁺/H⁺ antiporter positive transformants (AP⁺). A 30-min period of NH₄Cl loading was found sufficient to kill with a high efficiency the recipient LAP1 cells (0% activity), whereas it allowed cells endowed with little as 15% of Na⁺/H⁺ antiport activity to partially escape the acid-load selection (25% survival as shown in Fig. 2 Right).

Human Genomic DNA Restores Na⁺/H⁺ Antiport Activity in LAP1 Cells. LAP1 cells deficient in thymidine kinase (TK⁻) and in Na⁺/H⁺ antiport activity (AP⁻) were cotransformed with a 40:1 ratio of high molecular weight human genomic DNA and plasmid DNA (pTK-xl) containing the herpes simplex virus thymidine kinase gene. TK + co-
transformants are shown in Fig. 3, and their origin is summarized in Table 1. In contrast, no acid-load-resistant colonies were observed when human DNA donor was replaced with high molecular weight LAP1 genomic DNA in parallel cotransfection experiments.

**Evidence that AP+ Transformants Express the Na+/H+ Antiporter Structural Gene of the DNA Donor.** The nature of the genetic lesion that has abolished the antipporter activity of LAP1 cells is unknown. It was, therefore, crucial to check that AP+ mouse transformants express the Na+/H+ antiporter gene of the human DNA donor. Pharmacologically, the mouse and human Na+/H+ antiporters are indistinguishable (31). To circumvent this difficulty, we transformed LAP1 cells with the DNA of a hamster variant (AR300), which expresses an altered Na+/H+ antiporter. The antiporter of this hamster cell line has the following two distinctive properties: (i) the binding affinity of the potent amiloride analog methylpropylamiloride (MPA) is decreased by a factor of 30 and (ii) the affinity for the substrates Li+ or Na+ has increased 2-fold (25). Fig. 4 compares the kinetic parameters of the mouse LTK– antiporter with those of TAR 8, a LAP1-transformant obtained with AR300 DNA as a donor. Interestingly, the two features of AR300 hamster cells, decreased affinity for methylpropylamiloride and increased affinity for Li+, are both expressed in the mouse cells TAR 8. We isolated and analyzed three additional independent transformants, all displaying the properties of TAR 8. This result strongly supports the idea that AP+ mouse transformants express the Na+/H+ antiporter of the DNA donor.

![Fig. 2](image)

**Fig. 2.** Sensitivity to an acute acid load as a function of Na+/H+ antiport activity. (Left) There is differential sensitivity between LTK– (○) and LAP1 (○) cells in response to an acid load. Cells (103 cells), plated 24 hr earlier in 35-mm dishes, were acid-loaded for 60 min with 50 mM NH4Cl, then rapidly washed with the 120 mM NaCl Na+/H+ saline medium (time 0), and incubated in this medium for various periods of time before culture medium was substituted. Cell viability was evaluated by counting the colonies 10–12 days later. (Right) Sensitivity as a function of the acid load and of the Na+/H+ exchange activity in LTK– cells. The acid loading was altered just by varying the time of exposure (0–60 min) to the NH4Cl saline medium (50 mM NH4Cl). The second phase of the test, corresponding to pH recovery in the Na+ saline medium was kept constant (60 min) but four parallel experiments were performed at the following NaCl concentrations: 0 mM (○), 10 mM (○), 25 mM (○), and 120 mM NaCl (●) with the isotonicity being maintained with choline chloride. Decreasing the extracellular concentration of Na+ of the pH-recovery phase, reduces the Na+/H+ exchange activity and mimics four discrete levels of Na+/H+ exchange activity (0, 15, 50, and 100%) in LTK– cells. The dashed line represents the conditions which have been chosen for the selection of AP+ transformants—a 30-min period of NH4Cl loading.

![Fig. 3](image)

**Fig. 3.** Na+/H+ antiport activity in the primary, secondary, and tertiary AP+ transformants relative to activity in the parental mouse L cells. Cells were acid loaded to maximally activate the Na+–H+ exchange activity, and amiloride-sensitive 22Na+ uptake was measured in 3 min as reported (20). PT, ST, and TT represent sets of primary, secondary, and tertiary independent transformants, respectively.

<table>
<thead>
<tr>
<th>Table 1. Origin of the cell lines and mouse transformants used in this study</th>
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<tr>
<td><strong>Cell line</strong></td>
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<td>LTK–</td>
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<td>LAP1</td>
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<td>AR300</td>
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<td>PT2, -11</td>
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<tr>
<td>ST31, -33</td>
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<td>ST34, -35, -37</td>
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<td>TT1, -3, -6</td>
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<td>TAR 8</td>
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The recipient cell for all transformants listed (PT2—TAR 8) was LAP1. +, 20–100%; + +, 300–900%; −, 0%.

*The Na+/H+ antiporter is overexpressed and has altered kinetic properties (see ref. 25).
Co-segregation of a Common Set of Human DNA Fragments with the Na\(^{+}/H\)^{+} Antiport Activity. To substantiate the latter point and to demonstrate that the serial transmission of the AP\(^+\) character from human DNA into AP\(^+\) mouse L cells is mediated by a human gene, we analyzed the presence of human repeated sequences in the DNA of various transformants. The first-cycle transformants PT2 and PT11 had a large number of DNA fragments that hybridized with \(^{32}\)P-labeled total human DNA. None of these fragments was detected in the LAP1 recipient cells. Interestingly, second-cycle transformants ST31, -33, -34, -35, and -37 issued from independent primary transformants (Table 1) lost most of the human repeated sequences, but retained the following common set of human EcoRI restriction fragments: 20, 14, 9, 8.5, 4, and 1 kilobase pairs. Similar results were obtained with HindIII digestion (data not shown). ST31 and ST37, which originated from two independent donors (PT2 and PT11), do not possess any extraneous human DNA fragments other than those reported above. We, therefore, used the DNA of ST31 to see if additional loss of human DNA could be obtained in third-cycle transformants. The results are shown in Fig. 5. The tertiary transfectant TT1 has retained the six common human repeated DNA fragments of ST31 and ST37, indicating that no further loss of human genome is compatible with the inheritance of the Na\(^{+}/H\)^{+} antiporter function. These results strongly suggest that the Na\(^{+}/H\)^{+} antiporter gene must be contained within these repeated sequences, encompassing at least 55 kilobase pairs of the human genome.

**DISCUSSION**

Human DNA fragments can be easily detected in a whole genomic murine background because of the presence of highly repeated sequences scattered throughout the human genome (32). This finding has made gene transfer techniques very valuable for cloning genes coding for protein of low abundance such as human oncogenes (33-36), cell surface antigens (37, 38), and membrane receptors (29). This cloning approach was particularly valuable for the Na\(^{+}/H\)^{+} antiporter because the only information available about this membrane transporter was its biological function. The lack of a specific molecular probe such as antibodies or a good ligand for affinity purification prompted us to develop the genetics of this system.

Most of the success in genomic transfection relies on the quality of the recipient cells and on the selectivity of the method used to isolate transformants arising at a low frequency. The LAP1 cell line that we isolated fulfills this requirement because it has kept the high transformability of mouse L929 cells (frequency of TK\(^-\) transformation ranged from 0.2 to \(1 \times 10^{-2}/\mu g\) of plasmid DNA) and because spontaneous reversion of the Na\(^{+}/H\)^{+} antiporter deficient mutation was estimated at a frequency lower than \(1 \times 10^{-7}\). A direct selection for AP\(^+\) transformants could have been performed. However, because the frequency of spontaneous revertants is of the same order of magnitude as the frequency for genomic transfection, it was much safer first to enrich the population in transformants by selecting for TK\(^-\). We calculated that, applying the AP\(^+\) selection when TK\(^-\) colonies reached a size of 10-50 cells per colony, the frequency of AP\(^+\) transformants was at least 100-fold higher than that for spontaneous reversion.

At the early stage of selection and during the expansion of clones that survived two successive acid-killing selections, it was clear that AP\(^+\) transformants were very unstable despite maintenance of TK\(^+\) selection. A similar observation has been reported with cotransformants expressing nonselectable human enzymes (39). Therefore, the pressure of selection for the AP\(^+\) character was maintained at least during all the phases of clonal expansion required to prepare enough DNA for analysis and transfection. The stability of two transfectants ST31 and TT1 was tested after they had been cultivated for 2 months under a constant and complete pressure of selection (TK\(^+\) and AP\(^+\)). Cells were grown for 50 generations under the following four conditions: (i) no selection, (ii) selection for TK\(^+\), (iii) selection for AP\(^+\), and...
(iv) selection for both TK⁻ and AP⁺. ST31 was found stable since it expressed 40% of Na⁺/H⁺ antipot activity after 50 generations in absence of any pressure of selection. In contrast, under the same growth conditions the Na⁺⁻H⁺ exchange activity of T71 segregated out (10% after 18 generations, 0.04% after 50 generations). However, TK⁻ selection in T71 was sufficient to maintain the stability of the nonselected AP⁺ character, suggesting that the TK⁻ and AP⁺ genes are integrated closely to each other.

Another important feature of the mouse recipient LAP1 cells is that the mutation(s) abolishing the expression of the resident antipoter genes does not seem to exert any trans-dominant effect on the expression of a transfected foreign Na⁺/H⁺ antipoter gene. This conclusion is based on the following observations: (i) primary mouse transformants arose at a frequency of 1 out of 2,000–10,000 TK⁻ transformants, a result consistent with that obtained by others; (ii) their expression, measured on 24 primary transformants, ranged from 20 to 40% of the parental LTK⁻ cells, a somewhat expected result considering gene dosage and some instability of the transformants; (iii) LAP1 cells overexpress (300% of the LTK⁻ corresponding activity) an altered Na⁺/H⁺ antipoter when transformed with the DNA of AR300, a hamster variant overexpressing an altered Na⁺/H⁺ antipoter (25). The four independent TAR transformants that we isolated had the properties of TAR 8 (Fig. 4 and Table 1). Therefore, LAP1 cells are adequate recipients in studying the expression and biochemical features of a transfected normal or mutated Na⁺/H⁺ antipoter gene.

The latter point was essential before we decided to go further with this cloning approach. Indeed the properties of TAR transformants indicated that AP⁺ colonies arose neither by reversion nor by a suppression mechanism leading to "reactivation" of a mouse silent antipoter gene. This result and the fact that the Na⁺/H⁺ antipot activity cosegregates in secondary and tertiary transformants with a common set of restricted human DNA fragments strongly suggest that these fragments (55 kilobase pairs of the human genome) contain at least part of the Na⁺/H⁺ antipoter human gene.

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