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# Amplification of DNA Sequences Coding for the Na,K-ATPase α-Subunit in Ouabain-Resistant $C^+$ Cells

JANET RETTIG EMANUEL,[1] SUSAN GARETZ,[1] JAY SCHNEIDER,[2] JOHN F. ASH,[3] EDWARD J. BENZ, JR.,[2] AND ROBERT LEVENSON[1]*

*Department of Cell Biology[1] and Department of Human Genetics,[2] Yale University School of Medicine, New Haven, Connecticut 06510, and Department of Anatomy, University of Utah School of Medicine, Salt Lake City, Utah 84132[3]*

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**We have studied the mechanism of cellular resistance to cardiac glycosides in $C^+$ cells. $C^+$ cells were resistant to ouabain and overproduced plasma membrane-bound Na,K-ATPase relative to parental HeLa cells. Overexpression of Na,K-ATPase in $C^+$ cells correlated with increased ATPase mRNA levels and amplification (~100 times) of the ATPase gene. Growth of $C^+$ cells in ouabain-free medium resulted in a marked decline in ATPase mRNA and DNA levels. However, when cells were reexposed to ouabain, they proliferated and ATPase mRNA and DNA sequences were reamplified. Restriction analysis of $C^+$ and other human DNA samples revealed the occurrence of rearrangements in the region of the Na,K-ATPase gene in $C^+$ cells. Furthermore, $C^+$ cells expressed an ATPase mRNA species not found in HeLa cells. These results suggest that amplification of the gene coding for Na,K-ATPase results in overproduction of Na,K-ATPase polypeptides. Amplification of the ATPase gene or the expression of new ATPase mRNA sequences or both may also be responsible for acquisition of the ouabain-resistant phenotype.**

The plasma membrane Na,K-ATPase is the enzyme primarily responsible for regulating the active transport of Na and K ions across cell membranes. The enzyme is composed of two subunits, α and β. The known functional characteristics of the enzyme appear to reside solely in the α subunit, a polypeptide of $M_r$ 100,000 which contains the binding site for ATP (3). The β subunit is a glycosylated polypeptide of $M_r$ 55,000 whose function is unknown.

An important feature of the Na,K-ATPase is its sensitivity to the cardiac glycoside ouabain, a steroid compound which appears to bind to the α subunit of the enzyme (14). Treatment of cells with ouabain results in inhibition of $Na^+$ and $K^+$ transport, arrest of proliferation, and progressive loss of viability (2). Cell lines of different species vary considerably with respect to ouabain sensitivity, primate cells being quite sensitive to the drug compared with rodent cells (2, 9, 11). Although ouabain cytotoxicity is attributable in all cases to inhibition of the Na,K-ATPase, the molecular basis for the species-specific variation in drug sensitivity remains unknown.

Cell lines resistant to levels of ouabain which are lethal to wild-type cells have been isolated in several laboratories (1, 2, 11, 13, 18). One such cell line, $C^+$, was found to express a 10-fold-higher level of Na,K-ATPase than parental HeLa cells (1, 12). The increase in Na,K-ATPase content of $C^+$ cells was correlated with the appearance of minute chromosomes, a hallmark of gene amplification (15). The reversion of $C^+$ cells to ouabain sensitivity, coupled with a decrease in Na,K-ATPase and a loss of minute chromosomes (1, 12), suggested that Na,K-ATPase overproduction in $C^+$ cells was associated with gene amplification.

In this study we have examined the relationship between ouabain resistance and Na,K-ATPase DNA and RNA levels in $C^+$ cells. Using a human Na,K-ATPase α-subunit cDNA as probe, we find that the levels of both α subunit DNA and mRNA sequences are amplified ~100-fold in $C^+$ cells compared with parental HeLa cells. $C^+$ cells exhibit rearrangements in the region of the α-subunit gene and overproduce ATPase mRNA. $C^+$ cells also appear to express an ATPase mRNA species which is not detectable in HeLa cells. These findings suggest that amplification of the ATPase gene is associated with overproduction of Na,K-ATPase polypeptides. Amplification of the ATPase gene, coupled with the expression of a new ATPase mRNA species, may also play an important role in the acquisition of the ouabain-resistant phenotype.

## MATERIALS AND METHODS

**Cell lines and culture conditions.** HeLa and $C^+$ cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Armour). $C^+$ cells were maintained in medium containing 1 μM ouabain (Sigma Chemical Co.). $C^-$ cells are $C^+$ cells grown in the absence of ouabain for the times indicated in each experiment. For example, $C^-$ 7.5 weeks were $C^+$ cells grown in ouabain-free medium for 7.5 weeks.

**DNA and RNA blot hybridization.** High-molecular-weight DNA was prepared from cells by the method of Gross-Bellard et al. (7). DNA was digested with restriction enzymes, fractionated by agarose gel electrophoresis, and transferred to Zetabind filters (AMF) essentially as described by Southern (19). Blots were prewashed according to the manufacturer's instructions and then prehybridized, hybridized, and washed as described previously (9, 16). Blots were exposed to X-ray film (Kodak XAR-5 or Kodak XRP) at −80°C with a Cronex (DuPont Corp.) intensifying screen, For reprobing, blots were washed two times at 95°C for 15 min in 0.1× SSCPE (15 mM NaCl, 1.5 mM sodium citrate, 1.3 mM $KH_2PO_4$, 100 μM EDTA)–0.1% sodium dodecyl sulfate.

RNA was isolated from cells essentially by the guanidinium isothiocyanate method of Chirgwin et al. (4). Total

* Corresponding author.

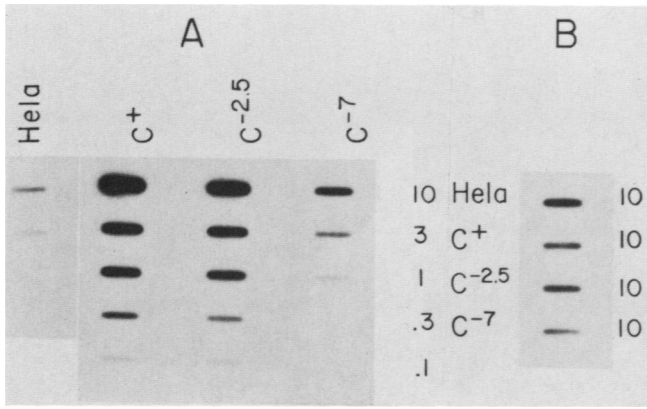


FIG. 1. Amplification of Na,K-ATPase α-subunit DNA sequences in $C^+$ cells. DNA was prepared from HeLa, $C^+$, $C^-$ 2.5-week, and $C^-$ 7-week cells. DNA samples were denatured, passed through a slot blot manifold, and immobilized on a Zetabind filter. An autoradiograph of the blot was scanned with a Joyce-Loebl microdensitometer. (A) Serial dilutions (in micrograms) of total cellular DNA from HeLa, $C^+$, $C^-$ 2.5-week, and $C^-$ 7-week cells were hybridized with $5 \times 10^6$ cpm of the pHANK cDNA probe. (B) A 10-μg portion of DNA from each cell line was reacted with $6 \times 10^6$ cpm of the human gamma actin probe.

cellular RNA was denatured by heating at 65°C for 10 min in 50% (vol/vol) formamide and was fractionated by electrophoresis through a 1% agarose formaldehyde gel (6). The RNA was transferred to Zetabind, prewashed, and then prehybridized and hybridized as described by Thomas (20), except for the addition of single-stranded poly(A), poly(I), and poly(C) ribonucleotides (each at 100 μg/ml; Boehringer Mannheim Biochemicals) to the hybridization buffer (16).

For slot blot analysis, total cellular DNA or RNA was denatured, passed through a slot blot manifold (Schleicher & Schuell), and immobilized on a Zetabind filter. Conditions for prewashing, prehybridization, hybridization, and washing of the filters were as described above. Autoradiographs of slot blots were scanned with a Joyce-Loebl microdensitometer.

**cDNA probes.** The human Na,K-ATPase α-subunit probe (pHANK) was isolated from a λ phage Charon 16A human neuroblastoma cDNA library prepared from the NB-16 cell line. This library was kindly provided to us by Fred Alt (Columbia University). The library was screened with the rat Na,K-ATPase α-subunit cDNA clone rb5 (16). Positive phage clones were plaque purified and the cDNA inserts were characterized by *Eco*RI endonuclease cleavage, agarose gel fractionation, and hybridization to rb5 on Southern blots. One clone, λHANK, contained a 2.4-kilobase (kb) cDNA insert. This cDNA insert was subcloned into the plasmid cloning vector pAT153 and amplified in the bacterial host strain DH-1. Restriction endonuclease mapping, DNA sequence analysis (Schneider et al., manuscript in preparation), and comparison with the rat brain (16) and sheep kidney (17) α-subunit cDNA sequences indicated that pHANK corresponded to bases ~600 to 3,000 of Na,K-ATPase α-subunit cDNA.

To control for loading artifacts when quantitating ATPase DNA and mRNA levels, blots were hybridized with a human gamma actin cDNA probe, pHF γA. This cDNA clone was generously provided by Larry Kedes (Stanford University School of Medicine).

cDNA probes were labeled to high specific activity, using the Klenow fragment of *Escherichia coli* DNA polymerase I (Boehringer Mannheim) and oligodeoxynucleotide primers (Pharmacia P-L Biochemicals) according to the method of Feinberg and Vogelstein (5).

## RESULTS

**Amplification of Na,K-ATPase α-subunit DNA and mRNA sequences in ouabain-resistant $C^+$ cells.** The human Na,K-ATPase α-subunit cDNA probe pHANK was used in slot blotting experiments to quantitate sodium pump α-subunit DNA and mRNA levels in HeLa, $C^+$, and $C^-$ cells. Serial dilutions of total cellular DNA or RNA from these cell lines were passed through a slot blot manifold and immobilized on Zetabind filters. The filters were reacted with radiolabeled pHANK, and the intensity of hybridization in each sample was quantitated by microdensitometry.

A comparison of the levels of ATPase genomic DNA sequences in HeLa, $C^+$, and $C^-$ cells is shown in Fig. 1A. DNA sequences reactive with the ATPase cDNA probe exhibited a significant degree of amplification in $C^+$ cells compared with parental HeLa cells and $C^-$ 7-week cells. Quantitation of an autoradiograph of the filter by microdensitometry (Table 1) indicated that ATPase α-subunit genomic DNA sequences were amplified ~100 fold in $C^+$ cells compared with HeLa cells. In $C^-$ 2.5-week and $C^-$ 7-week cells, ATPase sequences were amplified ~80- and ~4-fold, respectively, over the level in HeLa cells. In the same experiment, 10 μg of DNA from each cell line was spotted onto the filter and probed for the single-copy human gamma actin gene. Each sample reacted to approximately the same extent with the actin probe (Fig. 1B). Thus, the differences in sequence content of ATPase genes in $C^+$ and $C^-$ cells versus HeLa cells appear to be due to amplification of these sequences rather than to any loading artifact. These results show that the Na,K-ATPase α-subunit gene is amplified in $C^+$ cells and that α-subunit gene copy number declines when $C^+$ cells are cultured in the absence of drug.

Slot blot comparison of ATPase α subunit mRNA levels in

TABLE 1. Relative abundance of Na,K-ATPase α-subunit DNA and mRNA sequences in HeLa, $C^+$, and $C^-$ cells[a]

| Cells | Na,K-ATPase α-subunit sequence abundance (arbitrary units) | | Quabain concn (μm) |
|---|---|---|---|
| | Genomic DNA | mRNA | |
| HeLa | 1 | 1 | 0 |
| HeLa $C^+$ | 100 | 130 | 1.0 |
| HeLa $C^-$ | | | |
| 2.5 wk | 80 | | 0 |
| 3.0 wk | | 40 | 0 |
| 7.0 wk | 4 | | 0 |
| 7.5 wk | | 5 | 0 |
| HeLa $C^-$ 11 wk[b] | | | |
| A | 4 | 5 | 0 |
| B | 20 | 10 | 0.1 |
| C | 100+ | 70 | 1.0 |

[a] Total cellular DNA and RNA were prepared from the cell lines described in Table 1 and the legends to Fig. 1 and 2. DNA and RNA samples were denatured, passed through a slot blot manifold, and immobilized on a Zetabind filter. Blots were hybridized with radiolabeled pHANK or actin cDNA probes, and autoradiographs of the filters were scanned with a Joyce-Loebl microdensitometer. DNA or RNA content of each cell line was normalized to the actin control.

[b] $C^-$ 11-week cells were grown for an additional 10 days under the conditions described above and then were harvested for DNA and RNA preparation.

HeLa, $C^+$, and C-cells is shown in Fig. 2A. ATPase mRNA sequences reactive with pHANK also appear to be amplified in $C^+$ and $C^-$ cells versus HeLa cells. Microdensitometer analysis of an autoradiograph of the filter (Table 1) indicated that ATPase mRNA sequences were amplified ~130-fold in $C^+$ cells compared with HeLa cells. In $C^-$ 3-week and $C^-$ 7.5-week cells, ATPase mRNA levels were amplified ~40- and ~5-fold, respectively, over ATPase mRNA levels in HeLa cells. Hybridization of RNA from each cell line with the actin cDNA probe indicated that approximately equal amounts of RNA were loaded in each sample set (Fig. 2B). That there appears to be a linear relationship between the abundance of ATPase α-subunit DNA and mRNA sequences suggests that the expression of elevated levels of sodium pump mRNA in $C^+$ cells probably does not result from changes in the transcriptional activity of ATPase genes.

**Characterization of Na,K-ATPase DNA and mRNA sequences in $C^+$ cells.** pHANK cDNA was used in Southern blotting experiments to probe genomic DNA prepared from HeLa, $C^+$, and $C^-$ cells, as well as from normal human lymphocytes. All genomic DNA fragments reactive with the probe were amplified in $C^+$, $C^-$ 2.5-week, and $C^-$ 7-week cells compared with HeLa cells and normal human lymphocytes (Fig. 3). (In the blot shown here, we loaded 10 μg of HeLa and normal human DNA and 2 μg of $C^+$ and $C^-$ DNA so that the restriction fragments in $C^+$ cells were not obscured when visualizing DNA fragments in HeLa and normal human cells.) *Eco*RI digestion generated a series of restriction fragments reactive with pHANK cDNA which were common to all cell lines. Two additional DNA fragments of >8 kb were also generated by *Eco*RI digestion of $C^+$ and $C^-$ DNAs. These results suggest that rearrangements occur in the region of the α-subunit gene during amplification of ATPase DNA sequences in $C^+$ cells.

Using the cDNA insert of pHANK as a probe, we analyzed the RNA products of Na,K-ATPase α-subunit gene expression in HeLa, $C^+$, and $C^-$ cells by hybridization to total cellular RNA on Northern blots. The probe hybridized to a 5-kb mRNA in HeLa, $C^+$, and $C^-$ cells (Fig. 4A). The size for this ATPase mRNA in HeLa cells is consistent with previous experiments (16) which demonstrated that the

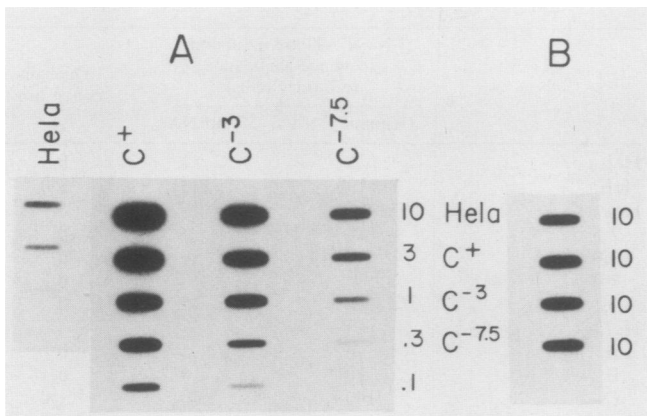


FIG. 2. Overexpression of Na,K-ATPase mRNA sequences in $C^+$ cells. Total RNA prepared from HeLa, $C^+$, $C^-$ 3-week, and $C^-$ 7.5-week cells was slot blotted onto a Zetabind filter. An autoradiograph of the blot was scanned by microdensitometry as in the legend to Fig. 1. (A) Serial dilutions of RNA (in micrograms) from HeLa, $C^+$, $C^-$ 3-week, and $C^-$ 7.5-week cells were hybridized with $5 \times 10^6$ cpm of the pHANK probe. (B) A 10-μg portion of RNA from each cell line was hybridized with $6 \times 10^6$ cpm of the human gamma actin probe.

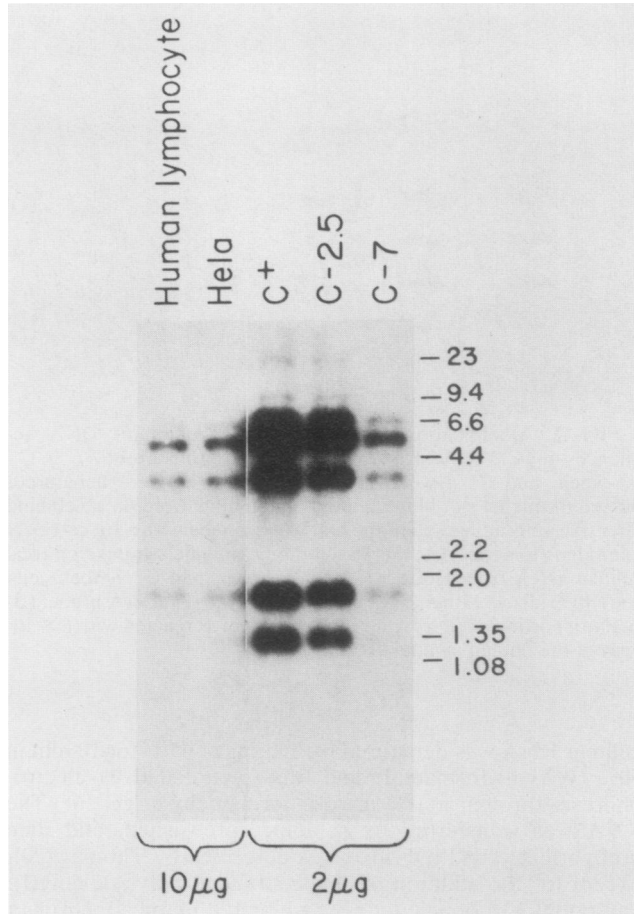


FIG. 3. Southern blot analysis of amplified Na,K-ATPase genomic DNA fragments in $C^+$ cells. Total cellular DNA was extracted from normal human lymphocytes, HeLa, $C^+$, $C^-$ 2.5-week, and $C^-$ 7-week cells. DNA was digested with *Eco*RI and the fragments were electrophoresed through a 0.8% agarose gel. A 10-μg amount of lymphocyte and HeLa cell DNA was run per lane, whereas 2 μg of DNA from each of the other cell lines was loaded. The DNA fragments were transferred to a Zetabind filter which was then hybridized with $10^7$ cpm of radiolabeled pHANK cDNA. Molecular weight markers are shown at the right.

Na,K-ATPase α-subunit gene encodes a 5-kb mRNA in several different rat tissues. An additional, less reactive RNA species of 2.4 kb was also visualized in HeLa, $C^+$ and $C^-$ cells. A third RNA species, ~3 kb in length, was observed only in $C^+$ and $C^-$ cells. This RNA was not detectable in parental HeLa cells, even after prolonged exposure of the blot.

The RNA species reactive with the ATPase probe were clearly distinguishable from the 28S and 18S markers and were not visualized when the same blot was probed with human actin cDNA. Therefore, the RNA sequences we observe apparently do not result from nonspecific hybridization to the ATPase probe. The RNAs reactive with ATPase cDNA appear to be overproduced in $C^+$, $C^-$ 3-week, and $C^-$ 7.5-week cells compared with HeLa cells. When the same blot was probed with human actin cDNA (Fig. 4B), an RNA species which hybridized with approximately equal intensity was observed in all four lanes. Thus, the differences in sequence content of RNAs which hybridize to the ATPase probe appear to be due to an increase in the abundance of these sequences in $C^+$ and $C^-$ cells relative to HeLa cells.

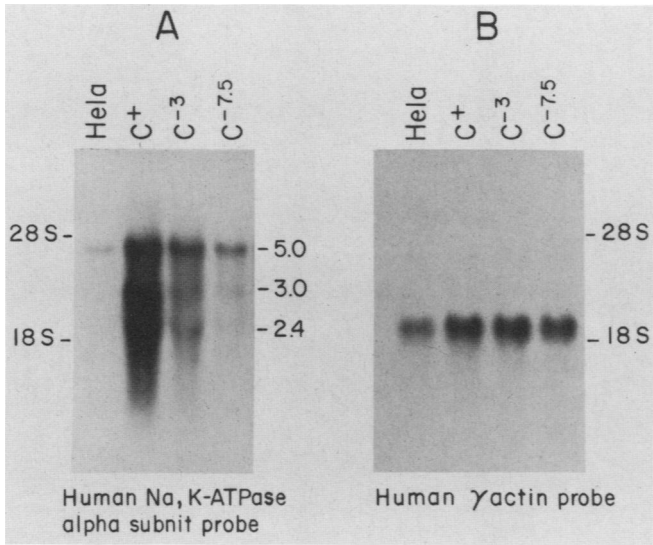


FIG. 4. Northern analysis of Na,K-ATPase α-subunit gene expression in $C^+$ cells. RNA was prepared from HeLa, $C^+$, $C^-$ 3-week, and $C^-$ 7.5-week cells. A 20-μg portion of total cellular RNA was run in each lane. RNA was electrophoresed through a 1% formaldehyde agarose gel and then transferred to a Zetabind filter. (A) The filter was reacted with $10^7$ cpm of the radiolabeled pHANK cDNA probe. The positions of 28S and 18S RNA markers are shown on the left. The positions of the RNA species which hybridize to the probe are shown on the right. (B) The same filter as in (A), washed free of probe and then hybridized with $10^7$ cpm of the human actin cDNA probe. The positions of the 28S and 18S RNA markers are shown on the right.

**Amplification of Na,K-ATPase DNA and mRNA in $C^-$ cells challenged with ouabain.** To determine whether $C^-$ cells were capable of expressing the drug-resistant phenotype when challenged with ouabain, $C^-$ cells were grown for 11 weeks in the absence of ouabain and then were divided into four groups. DNA and RNA were prepared from one group of cells for hybridization analysis. The second group was exposed to 1.0 μM ouabain, the third was treated with 0.1 μM ouabain, and the fourth was grown in the continued absence of drug.

The growth properties of HeLa, $C^+$ and $C^-$ 11-week cells are shown in Fig. 5. HeLa, $C^+$, and $C^-$ 11-week cells all appeared to proliferate with a doubling time of ~24 h. After treatment with 0.1 μM ouabain, $C^-$ 11-week cells exhibited a lag of ~48 h before cell growth was resumed. Thereafter, the cells proliferated with a doubling time similar to that of control HeLa and $C^-$ 11-week cells. After 3 days of exposure of $C^-$ 11-week cells to 1.0 μM ouabain, the cell number was reduced by ~50%, indicating a significant degree of cytotoxicity. After 8 days of ouabain treatment, however, the doubling time of these cells again approximated that of control cultures. Since $C^+$ cells are normally resistant to treatment with 1.0 μM ouabain, the cytotoxicity that results when $C^-$ cells are reexposed to this concentration of drug suggests that a portion of $C^+$ cells reverted to the ouabain-sensitive phenotype during growth in ouabain-free medium.

To characterize Na,K-ATPase DNA sequences in $C^-$ cells challenged with ouabain, Southern hybridization analysis was carried out with genomic DNAs from HeLa, $C^+$, $C^-$ 11-week, and $C^-$ 11-week cells grown in the presence of 0.1 or 1.0 μM ouabain for 10 days. A Southern blot containing this panel of DNAs digested with *Eco*RI and fractionated by electrophoresis was screened with the pHANK cDNA probe. For this blot, 10 μg of HeLa DNA and 2 μg of each of the other DNAs were loaded onto the gel and electrophoresed. *Eco*RI digestion generated a series of DNA fragments which were common to all cell lines (Fig. 6). The additional *Eco*RI fragments (>8 kb in size) present in $C^+$ cells can also be visualized in $C^-$ 11-week cells reexposed to ouabain. All genomic DNA fragments hybridizing to the ATPase cDNA probe appear to be amplified in $C^+$ and $C^-$ 11-week cells treated with ouabain relative to HeLa and $C^-$ 11-week cells grown in the absence of ouabain. Slot blot analysis of these samples (Table 1) indicated that, in $C^-$ 11-week cells exposed to 0.1 μM ouabain, ATPase genomic DNA sequences were amplified ~20-fold versus HeLa cells. In $C^-$ 11-week cells exposed to 1.0 μM ouabain, on the other hand, ATPase DNA sequences were amplified ~100-fold compared with HeLa cells, a level of amplification normally exhibited by $C^+$ cells. These results are consistent with the view that amplification and rearrangement of the gene coding for the Na,K-ATPase are associated with expression of the ouabain-resistant phenotype.

RNAs prepared from the same panel of cells were analyzed by hybridization to pHANK cDNA on Northern blots. An mRNA of 5 kb hybridized to the probe in HeLa cells and human kidney (Fig. 7). Weak hybridization also occurred to an RNA species migrating at ~2.4 kb. In all $C^+$-derived cell lines, RNAs of 5, 3, and 2.4 kb hybridized to pHANK cDNA. Each of these RNAs were amplified in $C^+$ cells, and each RNA species was reduced in abundance in $C^-$ 11-week cells. Slot blot analysis demonstrated that RNA sequences reactive with the probe were amplified ~5-fold in $C^-$ 11-week cells compared with HeLa cells (Table 1). The mRNAs appeared to be amplified ~10-fold in $C^-$ 11-week cells treated with 0.1 μM ouabain and ~70-fold in $C^-$ 11-week cells grown in 1.0 μM ouabain. These data indicate that Na,K-ATPase mRNA sequences increase in abundance

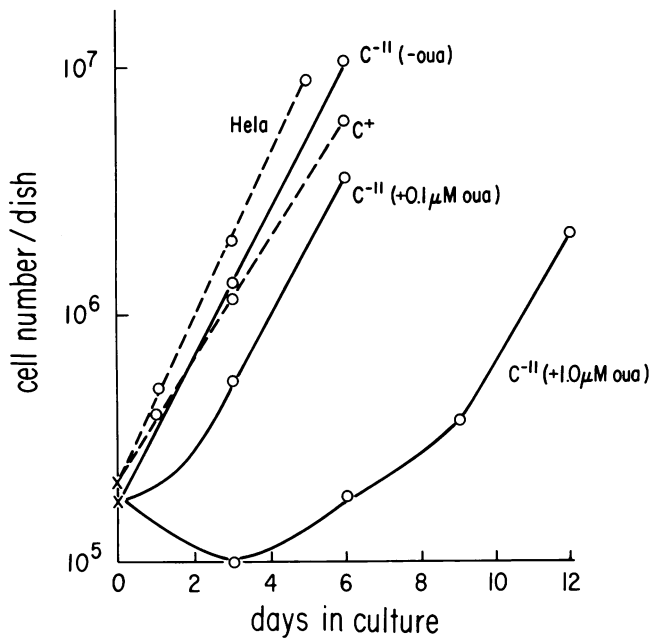


FIG. 5. Effect of ouabain (oua) dose on proliferation of $C^-$ cells. $C^-$ cells were maintained in drug-free medium for 11 weeks. The culture was then divided into three parallel subcultures, and growth was continued either in the presence of 1.0 or 0.1 μM ouabain or in the absence of the drug. At the times indicated, cell density in each culture was determined with an automated cell counter.

when $C^-$ cells are reexposed to drug. The level of expression of these sequences appears to be correlated with both the degree of Na,K-ATPase gene amplification and the degree of drug resistance.

## DISCUSSION

The data reported in this paper are consistent with the following conclusions. (i) Expression of the ouabain-resistant phenotype in $C^+$ cells is associated with overproduction of Na,K-ATPase mRNA. (ii) The increase in Na,K-ATPase mRNA production is correlated with amplification of the Na,K-ATPase gene. (iii) The level of Na,K-ATPase gene amplification is proportional to the concentration of ouabain. (iv) Ouabain resistance is associated with rearrangements in the region of the Na,K-ATPase gene. Drug resistance may also involve expression of variant Na,K-ATPase mRNAs.

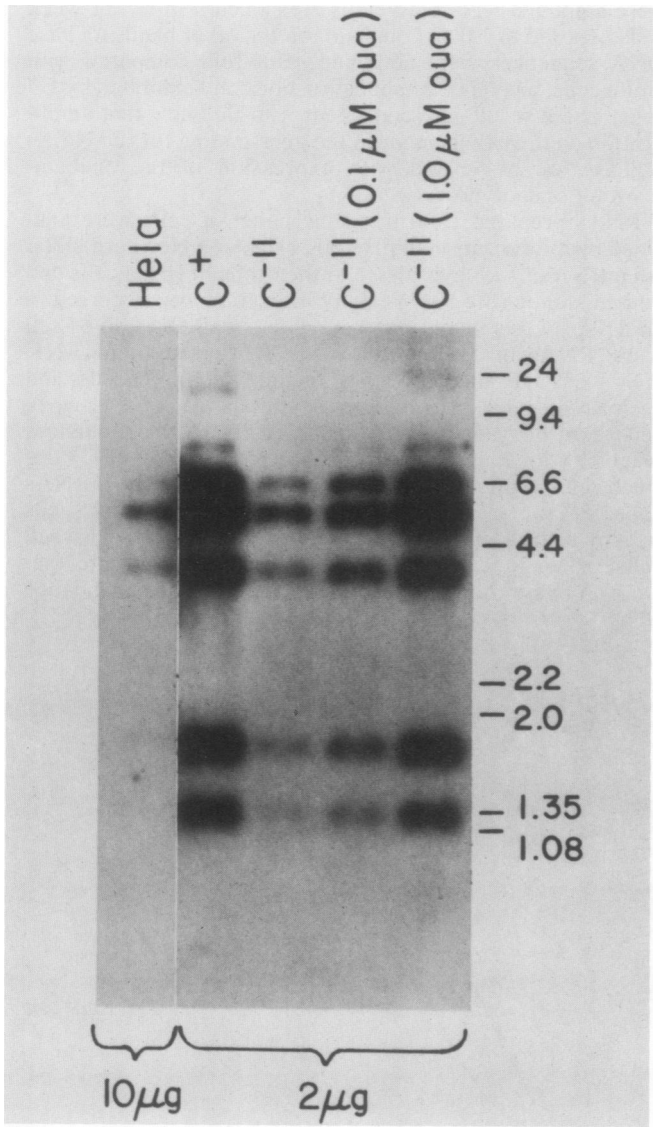


FIG. 6. Amplification of Na,K-ATPase genomic DNA seqences in $C^-$ cells reexposed to ouabain. $C^+$ cells were grown in the absence of ouabain for 11 weeks. The culture was then divided into three parallel subcultures as described in the legend to Fig. 5. After 10 days of growth, total cellular DNA was extracted from each subculture as well as from control HeLa and $C^+$ cells and digested with *Eco*RI. A 10-μg portion of HeLa DNA was loaded on the gel, whereas 2 μg of DNA from all other cell lines was loaded. The DNA frgments were electrophoresed through a 0.8% agarose gel and then transferred to a Zetabind filter. The filter was hybridized with $10^7$ cpm of the radiolabeled pHANK probe. Molecular weight markers are shown at the right.

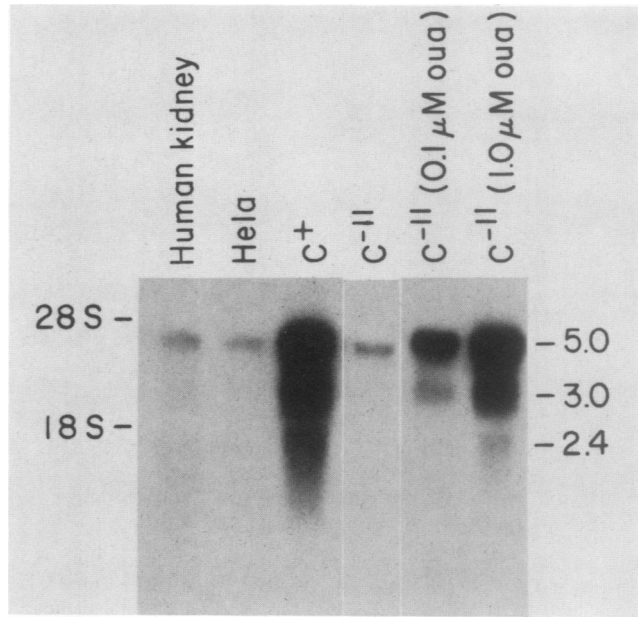


FIG. 7. Expression of Na,K-ATPase mRNA in $C^-$ cells reexposed to ouabain. Cells were grown under the conditions described in the legends to Fig. 5 and 6. RNA was extracted from each subculture 10 days after the addition of ouabain. A 20-μg portion of total cellular RNA was run in each lane; the source of RNA in each lane is indicated. RNA was electrophoresed through a 1% formaldehyde-agarose gel and then transferred to a Zetabind filter. The filter was reacted with $6 \times 10^6$ cpm of the pHANK cDNA probe. The positions of the 28S and 18S RNA markers are shown at the left.

Even though amplification and corresponding overexpression of the gene encoding the Na,K-ATPase are the most likely mechanisms responsible for ouabain resistance in $C^+$ cells, other genes may be involved in the establishment of drug resistance. An RNA 2.4 kb in length which hybridizes to the ATPase probe is overexpressed in $C^+$ cells relative to HeLa and $C^-$ cells. This RNA is too small to code for the mature Na,K-ATPase α subunit, since previous studies have shown that the coding region of α-subunit mRNA is ~3 kb in length (8, 17). $C^+$ cells also express a 3-kb RNA which is undetectable in parental HeLa cells. This RNA species is overproduced in $C^+$ versus $C^-$ cells and is large enough to code for a polypeptide the size of the ATPase α subunit. It is possible that the 3- and 2.4-kb RNAs represent specific breakdown products of the 5-kb ATPase mRNA. These RNAs may also arise via alternative processing of the α-subunit gene or may represent homologous products of other genes. Alternative processing of amplified genes has been found to occur in other systems. For example, the amplified EGF receptor gene appears to encode at least three different overproduced EGF receptor mRNAs in A431 cells (10). It remains to be determined, however, whether expression of alternative forms of the Na,K-ATPase is essential for establishment or maintenance, or both, of ouabain resistance in $C^+$ cells.

The idea that the Na,K-ATPase, and in particular the α

subunit of the enzyme, plays an important role in the mechanism of ouabain resistance bears some consideration. Previous studies have shown that a murine gene which is unrelated to Na,K-ATPase can confer ouabain resistance to ouabain sensitive cells after DNA-mediated gene transfer (9). Furthermore, in a ouabain-resistant MDCK cell line (18), the Na,K-ATPase α-subunit gene does not exhibit amplification (R. Levenson, J. R. Emanuel, S. Garetz, and J. W. Schneider, *in* J. Patrick and S. Heinemann, ed., *Molecular Neurobiology*, in press). Thus, there appear to be several mechanisms by which ouabain resistance can be acquired by cultured cells: (i) amplification of the sodium pump gene; (ii) alterations in the gene coding for the ATPase; and (iii) expression of other gene products.

Our results strongly suggest that the mechanism of ouabain resistance in $C^+$ cells involves overexpression of Na,K-ATPase α-subunit mRNA and its polypeptide product. It is not clear at this time what role, if any, the Na,K-ATPase β subunit plays in this process, although preliminary experiments suggest that this protein is overproduced in $C^+$-derived cells (12). It also remains to be determined whether the phenotype of ouabain resistance in $C^+$ cells is due to overexpression of the unaltered endogenous α-chain gene or whether changes in the nucleotide sequence of the α-chain gene followed by overproduction of its altered product are also essential. Transfection of cDNA clones complementary to $C^+$ cell ATPase mRNAs could resolve this issue.

The $C^+$ cell line used in this study represents a valuable system to study the molecular basis of ouabain resistance. Further analysis of Na,K-ATPase DNA and mRNA sequences in this cell line should allow us to characterize the structure and mechanism of the cardiac glycoside binding site and better understand the relationship between Na,K-ATPase structure and function.

## ACKNOWLEDGMENTS

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