Amplification of Sodium- and Potassium-activated Adenosinetriphosphatase in HeLa Cells by Ouabain Step Selection

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ABSTRACT A multistep selection for ouabain resistance was used to isolate a clone of HeLa S3 cells that overproduces the plasma membrane sodium, potassium activated adenosinetriphosphatase (Na\textsuperscript{+},K\textsuperscript{+}-ATPase). Measurements of specific \[^{3}H\] ouabain-binding to the resistant clone, C\textsuperscript{+}, and parental HeLa cells indicated that C\textsuperscript{+} cells contain 8–10 x 10\textsuperscript{6} ouabain binding sites per cell compared with 8 x 10\textsuperscript{5} per HeLa cell. Plasma membranes isolated from C\textsuperscript{+} cells by a vesiculation procedure and analyzed for ouabain-dependent incorporation of \[^{32}P\] phosphate into a 100,000-mol-wt peptide demonstrated a ten- to twelvefold increase in Na\textsuperscript{+},K\textsuperscript{+}-ATPase catalytic subunit. The affinity of the enzyme for ouabain on the C\textsuperscript{+} cells was reduced and the time for half maximal ouabain binding was increased compared with the values for the parental cells. The population doubling time for cultures of C\textsuperscript{+} cells grown in dishes was increased and C\textsuperscript{+} cells were unable to grow in suspension. Growth of C\textsuperscript{+} cells in ouabain-free medium resulted in revertant cells, C\textsuperscript{-}, with biochemical and growth properties identical with HeLa. Karyotype analysis revealed that the ouabain-resistant phenotype of the C\textsuperscript{+} cells was associated with the presence of minute chromosomes which are absent in HeLa and C\textsuperscript{-} cells. This suggests that a gene amplification event is responsible for the Na\textsuperscript{+},K\textsuperscript{+}-ATPase increase in C\textsuperscript{+} cells.

The plasma membrane provides the cell a variety of services at an economy of mass. Although integral proteins constitute half the weight of the plasma membrane, they represent only a few percent of total cell protein. The study of integral membrane proteins is complicated by their sparsity and their insolubility in aqueous solution. Technical advances, such as the expansion in choice and understanding of detergents (1), are reducing some of these problems. The discovery and employment of tissues and cells with unusual membrane properties can help overcome other difficulties imposed by the low copy number typical of integral membrane proteins. For instance, the human carcinoma cell line, A-431, has proved invaluable in the analysis of early events of hormone action. The A-431 cells bind 10–20 times more epidermal growth factor than normal cultured human cells (2) and contain 2.6 x 10\textsuperscript{6} receptors per cell (3). These cells have been used in structural studies of hormone binding and internalization (3, 4) and in chemical studies of the receptor and its activities (5, 6).

The overproduction of epidermal growth factor receptors by A-431 cells was discovered by screening a variety of non-selected cell lines with radiolabeled hormone (2). This paper describes the direct selection of variant cells that overproduce a different integral membrane protein, the sodium, potassium activated adenosinetriphosphatase (Na\textsuperscript{+},K\textsuperscript{+}-ATPase). The selection was attempted to establish a line bearing an increased amount of an integral membrane protein that is not a hormone receptor. Such cells would facilitate the study of ligand-induced clustering and endocytosis of nonreceptor integral proteins (7) in the same way that the A-431 cells are currently used in the study of the epidermal growth factor receptor. The selection scheme is based on previous work that employed a specific enzyme inhibitor to select for the overpro-

\textsuperscript{1} Abbreviation used in this paper: Na\textsuperscript{+},K\textsuperscript{+}-ATPase, sodium, potassium activated adenosinetriphosphatase.
dution of the cytoplasmic enzyme, dihydrofolate reductase (8, 9). Schimke and his co-workers have demonstrated that the overproduction is due to the amplification of the genes coding for this enzyme (10). Several more examples of protein overproduction through gene amplification selection have been reported (11, 12).

The present work utilizes ouabain, a specific inhibitor of Na⁺,K⁺-ATPase (13), in a multistep selection of the human carcinoma line, HeLa S₃. A clone of cells, referred to as C⁺, was isolated through three steps of increasing ouabain exposure. These cells have amplified the expression of the plasma membrane ATPase catalytic subunit ten- to twofold and thus contain ~10¹⁷ subunits per cell. The ouabain resistance of C⁺ cells is not stable and a revertant line, C⁻, was produced by growth of C⁺ cells in drug free medium. The increase in the Na⁺,K⁺-ATPase content of C⁺ cells is associated with alterations in the ouabain binding and growth properties, and with the appearance of minute chromosomes. The reversion of these alterations coupled with the loss of minute chromosomes in C⁻ cells strongly suggests that Na⁺,K⁺-ATPase overproduction in C⁺ cells is associated with gene amplification. It is likely that these cells will be useful in many studies, not only of the enzyme's structure and behavior, but for the broader field of membrane assembly, function, and genetics.

MATERIALS AND METHODS

Cell Culture: Two cell lines were used for selection: the human HT-1080 fibrosarcoma line (14) obtained from the American Type Culture Collection and the human carcinoma line HeLa S₃ (15), provided by Dr. Melvin Simon, University of California at San Diego. The cells were grown in a medium that consisted of 45% Dulbecco's modified Eagle's medium, 45% Ham's F12 medium, and 10% horse serum (Stirile Systems, Logan, UT; lot 30002789). This medium, designated D/F H10, was supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), Eagle's minimal essential medium nonessential amino acids, dextrose (1.35 g/liter), NaHCO₃ (2.45 g/liter), and, for the HT 1080 cells, asparagine (0.045 g/liter). For suspension culture conditions, the cells were grown on 10-cm diameter dishes rather than roller bottles because no bottle was found that would allow growth of C⁻ cells at 37°C in a humid 10% CO₂, 90% air atmosphere. When it was necessary to increase adhesion, the dishes were pretreated for 1 h with a 1 mg/ml solution of poly-L-ornithine hydrobromide (PORN, 1.5-5 × 10⁴ mol wt, Sigma Chemical Co., St. Louis, MO), rinsed with distilled water, and air dried. Clones were isolated from low density cultures using metal cloning rings. Ouabain killing curves were determined by plating low numbers of cells in 24-well cluster dishes (2-5 × 10⁵ cells/well) in various concentrations of ouabain. After several days growth and before the ouabain-free control wells became confluent, the wells were rinsed three times with phosphate buffered saline (PBS: 0.15 M NaCl, 0.01 M NaHCO₃, pH 7.4) and air dried. The protein in the wells was then solubilized by the addition of 0.1 ml 1 N NaOH followed in 30 min by 0.3 ml of Biuret reagent. The absorbance of the solutions measured at 550 nm was used to determine the relative amounts of protein in each well and absolute protein levels were determined by using known amounts of BSA as a standard.

Ultraviolet treatment of cells was accomplished by exposing cultures in a thin film of medium to an ultraviolet sterilizing lamp in the tissue culture hood. Ouabain binding curves were determined by placing low numbers of cells in 24-well plates (2-5 × 10⁵ cells/well) in various concentrations of ouabain. After several days growth and before the ouabain-free control wells became confluent, the wells were rinsed three times with phosphate buffered saline (PBS: 0.15 M NaCl, 0.01 M NaHCO₃, pH 7.4) and air dried. The protein in the wells was then solubilized by the addition of 0.1 ml 1 N NaOH followed in 30 min by 0.3 ml of Biuret reagent. The absorbance of the solutions measured at 550 nm was used to determine the relative amounts of protein in each well and absolute protein levels were determined by using known amounts of BSA as a standard.

Ultraviolet treatment of cells was accomplished by exposing cultures in a thin film of medium to an ultraviolet sterilizing lamp in the tissue culture hood at a distance of 55 cm. After 2 d of growth, cells were counted and the counts compared with those obtained from nonirradiated cultures to determine a dosage that reduced cell number by 30-50%.

Ouabain Binding: Cells were plated at 3-10 × 10⁶ cells/well in 24-well dishes and cultured for 48 hours in ouabain free medium. For assay, the medium was replaced with D/F H10 containing 25 mM HEPES buffer (Research Organics, Cleveland, OH) and 5 mM NaHCO₃, pH 7.4 at 37°C. For ouabain binding determination the medium contained 1 μC/ml [3H]ouabain (Amersham Corp., Arlington Heights, IL) and unlabeled ouabain (Sigma Chemical Co.) at concentrations from 1 × 10⁻¹⁰ to 10⁻⁷ M. Binding control wells contained 1 × 10⁻⁷ M unlabeled ouabain in addition to the radiolaabeled compound. 1 ml of medium was added to each well and the cluster dish incubated in a 37°C water bath. Incubation was stopped (after 90-120 min for fixed point assay) by rinsing a well four times with 2.0 ml of ice cold PBS and lysing the cells with 0.5 ml 0.1 NaOH. The lysate was then placed in minivials, 5 ml Biofluor (New England Nuclear, Boston, MA) was added, and radioactivity determined by counting in a Packard Tri-Carb scintillation counter (Packard Instrument Co., Inc., United Technologies, Downers Grove, IL). Counts from control wells were typically <3% of those from the experimental wells and the control counts were subtracted from the experimental values to determine the specific binding. Other wells were treated identically except that the radioactive ouabain was omitted. Cells were removed from these wells after rinsing by incubation with trypsin-EDTA and the cell number determined by titration with a Coulter cell counter (Coulter Electronics, Inc., Hialeah, FL). Alternatively, the cells were lysed with 0.2 ml 1 N NaOH and the protein determined by the Hartree modification (16) of the Lowry procedure (17) using BSA as a standard. Preliminary experiments utilized standard D/F H10 media with respect to ouabain concentration in the 10⁻⁸ to 10⁻⁶ M range. The water bath procedure utilizing the HEPES-buffered medium is more convenient and allows better control of temperature and pH.

Ouabain Step Selection: The effect of ouabain on cell growth was first assessed by constructing killing curves as described above. The level of ouabain that decreased growth to <1% (LD 1) was then used as a starting point for a more detailed analysis. Uncloned wild type cells were plated at 10⁶ per 10-cm dish at several ouabain concentrations above the LD 1. The concentration that allowed the growth of 5-20 colonies from the 10⁶ cells was determined and several more selective dishes were established at that concentration. The resulting colonies were picked and grown up in mass culture. Cells from each colony were tested for specific ouabain binding and colonies that demonstrated an increase in binding sites were carried on to the next step of selection which was again determined by finding the new LD 1. To reduce the number of ouabain binding assays, cells from selected colonies were first propagated and then the top two selections at the 10⁻⁸ to 10⁻⁷ times the ouabain selective concentration. Initial tests of some cells that could grow at these levels showed no increase in specific ouabain binding and all such colonies were subsequently discarded.

Growth and Reversion Analysis: Cells were trypsinized and plated in 60-mm dishes at 2.5 × 10⁵ cells per dish or 5 × 10⁴ per 10-cm dish. To measure the population doubling time, pairs of dishes were trypsinized and the cells counted daily for 4 d. To determine the stability of the ouabain resistant phenotype, normal D/F H10 medium was added every 4 d. At intervals the specific ouabain binding was determined for these cells as well as for parallel cultures of C⁺ cells in ouabain containing medium and HeLa cells in normal medium. A continuous record of cell counts was maintained to allow the determination of the population doubling number as the experiments progressed. For suspension culture cells were trypsinized and suspended in spinner medium at 1-2 × 10⁵ cells/ml. After a short lag period the HeLa and C⁻ cultures would typically begin growth, while the C⁺ behavior was more erratic as explained in Results.

Membrane Isolation and Analysis: The method of Cohen (18) which induces cells to shed membrane vesicles in Ca²⁺-free medium was used to isolate plasma membrane containing intact ATPase heavy chain. This method was developed for the isolation of epidural growth factor receptor protein which suffered proteolytic damage during other membrane purification procedures (18). Cohen's procedure was used with the following modifications: the pH of the vesiculation buffer was adjusted to 8.8 rather than 8.5 to increase membrane yield from HeLa cells and the cells were grown on 10-cm diameter dishes rather than roller bottles because no bottle was found that would allow the stable attachment of our cell lines. Purified membranes were suspended in 10 mM HEPES buffer and frozen at -70°C. Their protein content was determined by the Bradford method (18) following treatment with 0.1% SDS. BSA was used as a standard for this assay.

Membrane proteins were analyzed by SDS PAGE using the discontinuous buffer system of Laemmli (19) and slab gels modeled on those of Studer (20). A 4% stacking gel was poured on top of a 6 to 15% linear gradient separating gel. All buffers contained 0.1% SDS and the samples were denatured in 2% SDS and 2% mercaptoethanol. After electrophoresis at 20 W the gels were fixed and stained overnight in a solution of 25% 2-propanol, 10% acetic acid, and 0.01% Coomassie Brilliant Blue R-250. The gels were then destained with 10% methanol in 10% acetic acid and dried on filter paper, or on cellophane for scanning. Electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, CA) except for the SDS which was obtained from BDH Chemicals. The protein content of the gels was determined by the Lowry procedure (20). The pig kidney ATPase used as a gel marker was prepared from fresh medullae (21, 22) and other markers were obtained from Sigma Chemical Co.

The relative level of active Na⁺K⁺-ATPase in the membrane preparations was quantitated by the method of Resh (23). This method is based on the specific phosphorylation of the catalytic subunit by [³²P]phosphate in the absence of Na⁺ and the presence of Mg²⁺ and ouabain (24). Membrane samples
were thawed and resuspended in 100 vol of 100 mM HEPES, 5 mM MgCl₂ adjusted to pH 7.4 with solid Tris (Tris(hydroxymethyl)aminomethane) base. They were pelleted at 20,000 rpm in a Beckman JA 20 rotor (Beckman Instruments, Palo Alto, CA) resuspended in the HEPES-Mg²⁺ buffer, and the protein content redetermined. Assay mixtures of 135 µl in HEPES-Mg²⁺ buffer 10 µg of protein, 20 µM H₃PO₄, and either 15 µl of N,N-dimethylformamide or 10 mM ouabain in N,N-dimethylformamide. After incubation at 20°C for 20 min, 10 µl of HEPES-Mg²⁺ buffer containing 10 µCi of [³²P]phosphate (New England Nuclear) was added for an additional 20 min. The reaction was quenched by adding 50 µl of 1 mg/ml BSA followed by precipitation with 1 ml of ice cold 5% trichloroacetic acid.0.1 M H₃PO₄. After 5 min on ice the precipitates were collected by centrifugation for 2 min in a Brinkman microfuge (Brinkman Instruments, Inc., Westbury, NY) and the pellets washed three times with precipitation buffer. The final pellets were washed with 1 ml of 0.15 M H₂PO₄ and resuspended in 100 µl of sample buffer: 10% glycerol, 1% tetradecyltrimethylammonium bromide (Sigma Chemical Co.), 0.1 M KPO₄ (pH 4), 0.001% Pyronin Y, and freshly added 2-mercaptoethanol (2% final). Radioactivity in the samples was determined by counting aliquots in a liquid scintillation counter. The rest of the sample was analyzed in an acid slab gel system (25) with the addition of 1% glycerol to the separating gel and FeSO₄ and tetradecyltrimethylammonium at the stacking gel at the same concentrations as present in the separating gel. After electrophoresis at 20 W the acidic gels were soaked for 5 min in 1% glycerol, 10% 2-propanol in 10% acetic acid and then dried onto filter paper. The dried gels were then exposed to Kodak XAR-5 film at ~70°C.

Developed autoradiographs and Coomasie-stained gels were scanned with a Gilford multi media densitometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). The scans were analyzed with the microprocessor and software of a Gilford 2600 spectrophotometer.

Karyotype Analysis: Prior to harvesting for karyotype analysis, cells were trypsinized and plated at 10⁵ cells/100 mm dish. They were then incubated for a period of time sufficient for two population doublings. For each cell type, 0.1 ml colcemid (Gibco Laboratories, Grand Island, NY; # 170-5211) was added to the culture dish containing 10 ml of media (final concentration of colcemid 10⁻⁶ gm/ml) 4 h prior to completion of the second cell cycle. Upon completion of the second population doubling, the cells were harvested using a standard fibroblast technique (26). Cell suspensions were dropped onto clean, wet, chilled (4°C), microscope slides. Each slide was placed on a hot plate (60°C) for 1.5 min. Slides were stored a minimum of 24 h before staining with a giemsa or giemsa-trypsin technique (27).

Six representative cells from HeLa, C⁺ and C⁻ lines were photographed using a Zeiss microscope (planapo 63×/1.4 objective)/Contax RTS camera system and Kodak Technical Pan 2415, 35-mm film. Metaphases were analyzed for known, marker, and minute chromosomes. Metaphases were not studied if they were over or under spread or if they had poor chromosome morphology. These problems occurred infrequently.

RESULTS

The selection protocol is outlined in Materials and Methods and is based on Littlefield's step selection of amethopterine resistant hamster cells (9). Since the dihydrofolute reductase amplification selections involve several tenfold increases in inhibitor concentration (9,28) human cells rather than rodent cells were used for this study. Human cells are 10,000 times more sensitive than rodent cells to ouabain toxicity (29) which would allow greater freedom to increase ouabain concentration. The human fibrosarcoma line HT-1080 (15) was chosen for initial study because it contains a pseudodiploid karyotype. The selection protocol is outlined in Materials and Methods: [32p].

Growth and Phenotype Reversion of Clone C⁺

The stability of the ouabain resistance and high ouabain binding of the C⁺ clone was tested by growth of these cells in ouabain-free medium. As can be seen in Fig. 2, C⁺ cells began to lose their excess ouabain binding sites after four population doublings in normal medium and by 20 to 25 doublings they regained most of the binding observed for normal cells. Cells would often fail to reattach after trypsinization and other colonies would die out after many passages for no obvious reason. Of 30 original colonies selected several were followed and the best growing one, C9, was eventually taken through two more selective steps as diagrammed in Fig. 1.

The second step of selection was performed at 1×10⁻⁶ M ouabain. Resistant colonies would arise, become visible, and then disappear. This behavior was eventually corrected by treating the culture dishes with polyornithine which apparently increased the adhesion of these variant cells to the dish sufficiently to permit extensive growth. At the same time the effect of ultraviolet light treatment was explored as a means of increasing the frequency of amplification events (30). Such treatment increased the frequency of occurrence of fast growing colonies, but did not alter total survival as judged by visible colony formation. The effect of ultraviolet light treatment on the frequency of obtaining ouabain resistant HeLa cells has not yet been rigorously analyzed. The best growing colony selected at the second step was c9.31 which had four times the number of ouabain binding sites as the HeLa S₁ cells and absolutely required polyornithine treated culture dishes for attachment and growth.

Colonies were readily isolated from the c9.31 cells at 2.25×10⁻⁷ M ouabain without further ultraviolet treatment. Such colonies bound from 8–10 times as much ouabain as the parental cells. The best growing of these colonies was c9.31.1 which contained 8×10⁵ ouabain binding sites per cell and required polyornithine treated dishes for growth. The population doubling time of these cells was 36 h compared with 22 h for HeLa cells grown on untreated dishes.

The polyornithine treatment of the substrate was inconvenient since the c9.31.1 cells were difficult to remove from such dishes after short growth periods. For this reason cells were plated at low density on normal dishes which resulted in the selection of 15 clones that grew on untreated tissue culture plastic. The most rapidly growing of these clones was labeled C⁺ and characterized further as described below. Several additional selections of C⁺ cells failed to produce resistant cells that bind more ouabain, although many colonies were isolated that showed decreased ouabain binding. Recently, however, we have isolated clones of cells derived from C⁺ with 2×10⁷ ouabain binding sites per cell (Kalka, T., and J. F. Ash, unpublished observations).

![Figure 1](image-url)

Summary of the ouabain step selection of HeLa S₁ cells used to generate the C⁺ clone. The numbers under the arrows represent the ouabain concentrations used for each step and the numbers above the cell names indicate the average increase in specific ouabain binding per cell relative to HeLa of colonies selected at that step. UV indicates that the cells were treated with ultraviolet radiation and +PORN indicates that the substrate was treated with polyornithine as described in Materials and Methods.
returned to the level of HeLa S₁ cells. These revertant cells were designated C⁻ to indicate their origin from C⁺ and their loss of high ouabain binding. The sensitivity of HeLa, C⁺, and C⁻ cells to growth in ouabain is displayed by the killing curve (Fig. 3). The C⁺ cells grew well in ouabain-containing medium up to $5 \times 10^{-7}$ M and die off sharply above this level. HeLa S₁ and C⁻ cells suffer extensive killing between $3$ to $4 \times 10^{-8}$ M ouabain. The ouabain dose which reduces growth by 50% is shifted up approximately tenfold for the C⁺ cells which paralleled the increase in ouabain binding sites detected in these cells. The results of these experiments are reported on a per cell, rather than a per milligram protein, basis. The HeLa cells averaged 440 µg protein/10⁶ cells and C⁺ cells were usually 20–30% higher when measured at the same time. The protein content of these cells as measured by the Lowry or Bradford assays varied considerably over time and it proved to be more consistent to express the results on a per cell basis. Cook observed similar fluctuations in measurements of the protein content of HeLa cells (31).

The growth of these cells on dishes was analyzed by plating $2.5 \times 10^5$ cells per 60-mm dish and counting cells at 24-h intervals. The doubling time for C⁻ and HeLa cells averaged 22 h while the C⁺ doubling time was 31 h. The doubling time for the C⁺ cells is not rapidly altered by removing the ouabain from the growth medium as can be seen in Fig. 2 for the first two points for the C⁻ curve. It was only after the C⁻ cells were grown for more than 25 population doublings that their doubling time decreased to 22 h.

More dramatic growth differences are observed when these cells are placed in suspension culture. HeLa and C⁻ cells, when trypsinized and placed in spinner flasks at $1 \times 10^5$ cells/ml, quickly begin growth at identical doubling times of 24 h. The C⁺ cells appear to double once when placed in suspension and fail to show further growth with prolonged incubation and media changes. The C⁺ cells grow normally in the spinner medium when plated in tissue culture dishes to which they can attach. Reduction of the ouabain concentration to $1 \times 10^{-7}$ M does not improve growth in suspension and elimination of ouabain eventually produces rapidly growing cells which are probably revertants since they die when ouabain is reintroduced.

**Ouabain Binding**

The kinetics of radiolabeled ouabain binding to HeLa and C⁺ cells measured at $1 \times 10^{-4}$ M ouabain are displayed in Fig. 4. Half maximal binding occurred on HeLa cells at 10 min and on C⁺ cells at 25 min when the cells were grown on dishes. The early time course of binding has not been followed for cells in suspension culture but maximal binding occurred at 90 min in this case. HeLa cells show the same number of binding sites in suspension as on dishes; the C⁺ cells, however, bind less ouabain after 24 h in suspension ($5 \times 10^6$ sites vs. 8...
The binding affinities for the three cell types were measured in the experiments shown in Fig. 5. The binding is saturable between $10^{-5}$ and $2 \times 10^{-6}$ M ouabain. Scatchard analysis of this data indicates that ouabain binds to HeLa cells at $8 \times 10^5$ sites with a $K_d$ of $6 \times 10^{-8}$ while the $C^+$ cells have $1 \times 10^7$ binding sites with a $K_d$ of $3 \times 10^{-7}$. The values for $C^-$ cells fall on the HeLa curve and although more points must be collected before performing the Scatchard analysis for $C^-$ it is clear that the values for $C^-$ cells will be close to those for HeLa (Fig. 5).

**Plasma Membrane Alterations**

The membrane vesicle shedding procedure of Cohen (6) was the only method found useful for the preparation of plasma membranes from these cells containing intact Na\(^+\),K\(^+\)-ATPase catalytic subunits as judged by SDS PAGE. Proteins from membranes prepared by this procedure and analyzed on an SDS slab gel are shown in Fig. 6. There are slight alterations in staining intensities of co-migrating bands in the three cell types seen in different preparations, but the consistent and striking difference was a 7- to 8-fold increase in the staining intensity of a peptide in the $C^+$ cells which co-migrated with the catalytic subunit of pig kidney Na\(^+\),K\(^+\)-ATPase.

The identification of this increased peptide in $C^+$ cells was accomplished by Resh’s procedure of specifically labeling the Na\(^+\),K\(^+\)-ATPase catalytic subunits in these membranes with \(^{32}P\)phosphate in the presence of ouabain and Mg\(^{2+}\) (23). When such labeled membranes were subjected to electrophoresis on an acidic slab gel to maintain the phosphoprotein complex (25) the $C^+$ cells displayed a ten- to twelvefold increase in ouabain-dependent labeling of the catalytic subunit (Fig. 7). This phosphoprotein complex was base sensitive as indicated by electrophoresis of labeled membranes on standard SDS gels (23) which resulted in >90% loss of label from the peptides (results not shown). All membranes also displayed the labeling of a lower molecular weight peptide that was ouabain independent, but much reduced in $C^+$ and $C^-$ cells relative to HeLa (Fig. 7). Resh also observed this ouabain independent phosphorylation of a peptide of similar molecular weight in rat adipocyte plasma membranes (23). The identity of this phosphopeptide is not known, nor is the reason for its altered expression in $C^+$ and $C^-$ cells understood.

**Karyotype Analysis**

Table I summarizes the results of previous chromosomes studies performed in known and suspected HeLa cell lines (32–34). The karyotype of our HeLa cell line is consistent with the designation of HeLa S3 with a few modifications. It has long been known that HeLa cells exhibit cytogenetic

![Figure 6](image-url)
was variable, but not ouabain dependent (24). HeLa membranes contained a 70,000-mol-wt phosphorylated species whose labeling even numbered lanes and it is clear that the C⁺ cells contain more of this material (lane 3). There is also some material migrating at a higher molecular weight in the C⁺ ouabain treated membranes (lane 4) which could result from dimer formation (24). All membranes enhanced phosphorylation of peptides migrating at the 100,000-mol-wt position can be appreciated by comparing the odd and even numbered lanes and it is clear that the C⁺ cells contain more of this material (lane 3). There is also some material migrating at a higher molecular weight in the C⁺ ouabain treated membranes (lane 3) which could result from dimer formation (24). All membranes contained a 70,000-mol-wt phosphorylated species whose labeling was variable, but not ouabain dependent (24). HeLa membranes clearly contain much more of this material than either C⁺ or C⁻. Bottom, superimposed scans of an autoradiograph exposed for shorter time which allowed the quantitation of label in the 100,000-mol-wt bands from the three membranes labeled in the presence of ouabain. Comparison of the areas (calculated in arbitrary units) under the peaks labeled alpha indicated that C⁺ membranes contain 11-fold more phosphoprotein than HeLa and 13-fold more than C⁻ in this case. Other experiments yielded similar 10- to 12-fold differences between the C⁺ and other membranes. The labeling of these bands was >90% reduced in the absence of ouabain.

DISCUSSION

These results demonstrate the step selection amplification of an integral plasma membrane enzyme. The Na⁺,K⁺-ATPase expression is increased tenfold on C⁺ cell as measured by radiolabeled ouabain binding and specific phosphorylation of the catalytic subunit. This protein amplification is correlated with a tenfold increase in the resistance of the C⁺ cell to ouabain cytotoxicity. The lesser seven- to eightfold increase in the 100,000-mol-wt peptide observed on Coomassie-stained SDS acrylamide gels is likely due to interference from other peptides with similar mobilities. Assuming a molecular weight of 170,000 (36), the Na⁺,K⁺-ATPase represents ~0.3% of the total C⁺ cellular protein.

Not all features of this selection can be rationalized in a simple fashion. The mechanism of ouabain binding to, and inactivation of, the Na⁺,K⁺-ATPase is complex (37). However, if one assumes that a bimolecular interaction leads to an inactive enzyme-ouabain complex, then the results of Fig. 5 can be interpreted as a simple equilibrium situation. It would then appear from Fig. 3 that HeLa cells require approximately half of their Na⁺,K⁺-ATPase active to maintain growth. Thus, the doubling of ATPase content which allows survival of the first step colonies (Fig. 1) is reasonable, since this occurs at a ouabain concentration just below that which saturates half the HeLa enzyme. The third selection step, on the other hand, occurs at a ouabain concentration only four times the measured Kᵦ for the HeLa ATPase and results in a tenfold increase in Na⁺,K⁺-ATPase protein and fivefold increase in the apparent G for ouabain. The C⁺ cells can grow in a higher ouabain concentrations—up to 5–6 × 10⁻⁷ M (Fig. 3)—though it is not clear why so many enzyme molecules are required if their ouabain affinity has decreased as measured. It is probable that there is not a simple relationship between binding affinity and eventual cytotoxicity and it is also possible that the proximity of the Kᵦ and the first selective dose was fortuitous. Ouabain binding was measured over a 2-h period while cells require days to die in the selective medium. In fact, the mechanism of ouabain killing is not known, but it likely involves a cascade of failures as the cytoplasmic sodium and potassium ion concentrations leave their steady state values. The lack of a simple relationship between selective dose and amplification level is also seen, though in reverse, for dihydrofolate reductase selections where a hun-
Table 1

Summary of Marker Chromosomes in HEla and Suspected HEla Cell Lines

| Cell lines: | G11 | HBT3 | MA160 | 2563 | CLL74 | HeLa CCL2 | HeLa S2 | D98/ AH-2 | HeLa C+ | C- |
|-------------|-----|------|-------|------|-------|-----------|---------|-----------|--------|----|
| Nelson-Rees Studies* |     |      |       |      |       |           |         |           |        |    |
| 1 = F2      | +   | +    | +     | +    | +     | +         | +       | +         | +      | + |
| 2 = F1      | +   | +    | +     | +    | +     | +         | +       | +         | +      | + |
| 3 = F8      | +(2) | +(2) | +(2)  | +(5) | +(6)   | +(5)      | +(2)    | +(4)      | +(2)   | +(3) |
| 4 = F6      | +(2) | +(2) | +     | +    | +     | +         | +       | +         | +      | + |
| 5 = CY      | +   | +    | +     | +    | +     | +         | -       | -         | -      | - |
| 5 = SC      | ND* | ND   | ND    | ND   | ND    | ND        | ND      | +         | -      | - |
| 6           | -   | -    | -     | +    | +     | -         | -       | -         | -      | - |
| 7           | +   | +    | -     | -    | -     | -         | -       | -         | -      | - |
| 8 = CY      | +   | +    | +     | +    | +     | -         | -       | -         | -      | - |
| 8 = SC      | ND  | ND   | ND    | ND   | ND    | ND        | +       | +         | +      | + |
| 9           | -   | +    | -     | +    | -     | -         | -       | -         | -      | - |
| 10 = CY     | -   | +    | +     | +    | +     | -         | -       | -         | -      | - |
| 10 = SC     | ND  | ND   | ND    | ND   | ND    | ND        | +       | -         | +      | + |
| 11          | -   | -    | -     | +    | +     | -         | -       | +         | +      | + |
| 12          | +(2)| +    | +     | -    | -     | -         | -       | -         | -      | - |
| 13 = CY = 19 = SC | - | -   | -     | +    | -     | -         | -       | -         | -      | - |
| 14 = F4     | +   | +    | -     | -    | -     | -         | -       | -         | -      | - |
| 15          | +   | +    | -     | -    | -     | -         | -       | -         | -      | - |
| 16          | +   | +    | -     | -    | -     | -         | -       | -         | -      | - |
| 17          | -   | +    | -     | +    | +(2)  | -         | -       | -         | -      | - |
| 18          | -   | -    | -     | +    | -     | -         | -       | -         | -      | - |
| 19          | -   | +    | -     | +    | -     | -         | -       | -         | -      | - |
| 20 = CY     | -   | -    | -     | +    | +     | -         | -       | -         | -      | - |
| 20 = SC     | ND  | ND   | ND    | ND   | ND    | ND        | ND      | +         | -      | - |
| 21          | -   | -    | +     | -    | -     | -         | -       | -         | -      | - |
| 22          | -   | -    | +     | -    | -     | -         | -       | -         | -      | - |
| 23          | -   | -    | +     | -    | -     | -         | -       | -         | -      | - |
| 24          | +   | +    | -     | -    | -     | -         | -       | -         | -      | - |
| 25          | -   | -    | -     | -    | -     | -         | -       | -         | -      | - |
| 26          | -   | -    | -     | -    | -     | -         | -       | -         | -      | - |
| 27          | -   | -    | -     | -    | -     | -         | -       | -         | -      | - |
| 28          | +   | +    | -     | -    | -     | -         | -       | -         | -      | - |
| 29 = F12    | +   | -    | -     | -    | -     | -         | -       | -         | -      | - |
| 30 SC       | ND  | ND   | ND    | ND   | ND    | ND        | ND      | +         | -      | + |
| 31 SC       | ND  | ND   | ND    | ND   | ND    | ND        | ND      | +         | +      | + |
| Francke*    |     |      |       |      |       |           |         |           |        |    |
| 3           | ND  | ND   | ND    | ND   | ND    | ND        | ND      | +         | -      | - |
| 5           | ND  | ND   | ND    | ND   | ND    | ND        | ND      | +         | -      | - |
| 7           | ND  | ND   | ND    | ND   | ND    | ND        | ND      | +         | -      | - |
| 9           | ND  | ND   | ND    | ND   | ND    | ND        | ND      | +         | -      | - |
| 10          | ND  | ND   | ND    | ND   | ND    | ND        | ND      | +         | -      | - |
| 11          | ND  | ND   | ND    | ND   | ND    | ND        | ND      | +         | -      | - |
| Morgan**    |     |      |       |      |       |           |         |           |        |    |
| 1           | ND  | ND   | ND    | ND   | ND    | ND        | ND      | ND        | +      | + |
| 2           | ND  | ND   | ND    | ND   | ND    | ND        | ND      | ND        | +      | + |
| 3           | ND  | ND   | ND    | ND   | ND    | ND        | ND      | ND        | +      | + |
| 4           | ND  | ND   | ND    | ND   | ND    | ND        | ND      | ND        | +      | + |
| 5           | ND  | ND   | ND    | ND   | ND    | ND        | ND      | ND        | +      | + |
| Minutes     |     |      |       |      |       |           |         |           |        |    |
|             | ND  | ND   | ND    | ND   | ND    | ND        | ND      | ND        | +      | + |

* Nelson-Rees markers are derived from reference 32 (CY) and reference 33 (SC).
† Francke markers are derived from reference 34 (f).
‡ The number of copies of a specific marker chromosome in the particular cell line, designated within parentheses.
§ Marker chromosomes, noted in Nelson-Rees' two different publications, reported inconsistently in HeLa cell lines.
¶ Not discussed by author (ND).
** Two different marker chromosomes noted by Nelson-Rees but designated by the same number.
*** Markers found in this study.

A threefold increase in enzyme level confers a ten thousandfold increase in drug resistance (9, 28). It is possible that these amethopterine- or methotrexate-resistant cells simultaneously become impermeable to the inhibitor (38) as the target en- zyme is amplified. Ouabain binds to the ATPase outside the cell (39), which prevents protective permeability alterations and could explain the small step intervals used for these ouabain selections.
### Table II

**HELA Chromosomes, Markers, and Minutes Observed in the Present Study**

| Chromosome no. | 1  | 2  | 3  | 4  | 5  | 6  | 1  | 2  | 3  | 4  | 5  | 6  | 1  | 2  | 3  | 4  | 5  | 6  |
|---------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1            | 2  | 2  | 2  | 2  | 2  | 2  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| 2            | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 3            | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 4            | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  |
| 5            | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 6            | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  |
| 7            | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  |
| 8            | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 9            | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 10           | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 11           | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 12           | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 13           | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 14           | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  |
| 15           | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  |
| 16           | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 17           | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  | 3  |
| 18           | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 19           | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 20           | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 21           | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| 22           | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |

**Markers**

Nelson-Rees

| Markers | 1  | 2  | 3  | 4  | 5  | 6  | 1  | 2  | 3  | 4  | 5  | 6  | 1  | 2  | 3  | 4  | 5  | 6  |
|---------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Nelson-Rees | 32p+ | 33p+ |

**Minutes**

Single

| Minutes | 1  | 2  | 3  | 4  | 5  | 6  | 1  | 2  | 3  | 4  | 5  | 6  | 1  | 2  | 3  | 4  | 5  | 6  |
|---------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Single  | 13 | 11 | 7  | 5  | 13 | 4  | 13 | 4  |

Double

| Minutes | 1  | 2  | 3  | 4  | 5  | 6  | 1  | 2  | 3  | 4  | 5  | 6  | 1  | 2  | 3  | 4  | 5  | 6  |
|---------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Double  | 9  | 6  | 11 | 13 | 4  | 13 | 4  |

* See Table III.
* Nelson-Rees markers are derived from reference 32 (CY) and reference 33 (SC).
* Markers found in this study.

### Table III

**Structural Chromosomal Rearrangements**

| Chromosome no. | 2n = 67 | 67 | 66 | 66 | 62 | 66 | 63 | 63 | 61 | 64 | 64 | 62 | 51 | 64 | 63 | 64 | 66 | 63 |
|---------------|---------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Markers       |         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| Nelson-Rees: 30p+ |       |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

* Deletions to the short arm (p) or long arm (q).
* Additions to the short or long arm.
FIGURE 8  Chromosomes derived from a giemsa stained, C+ cell. Single and double minutes (arrows) appear in C+ cells but not HeLa or C− cells.

| Minute Chromosome Content of C+ Cells | Mean | Standard deviation | Range minimum-maximum |
|--------------------------------------|------|--------------------|-----------------------|
| DM                                   | 7.2  | 5.2                | 0-22                  |
| SM                                   | 17   | 18                 | 0-17                  |
| Total minutes (2 × DM + SM)          | 31   | 20                 | 5-88                  |

Twenty C+ metaphase spreads, such as seen in Fig. 8, were analyzed for double minute (DM) and single (SM) content. Spreads were selected randomly, but they were not counted if the metaphases were over or under spread or if they had poor chromosome morphology.

The alteration in the $K_d$ for ouabain of the ATPase on C+ cells could be due to the amplification of a variant form of the enzyme or due to a perturbation of the normal enzyme produced, for instance, by crowding in the C+ membrane. Unstable amplification of a variant dehydrofolate reductase associated with minute chromosomes in 3T6 cells has been reported (40). The apparent lack of two enzyme forms in C+ (Fig. 5), on the other hand, could argue for some modulation of all the Na+,K+-ATPase in these cells. In either case, such an alteration could extend to a reduction of the enzyme's ion transport function and result in the need to produce more ATPase for cell survival at $2 \times 10^{-7}$ M ouabain than one would predict from the behavior of HeLa cells. The increase in the half time of ouabain binding could also be a reflection of an alteration of the ATPase in these cells. Alternatively, since in ouabain-free medium the C+ cells do not pump $^{86}$Rb at a higher rate than HeLa (Moore, M., and J. F. Ash; manuscript in preparation), a delay required for recruiting active ATPase which could then bind ouabain would explain an increase in binding half time. Study of purified enzyme will be required to define the nature of the alterations of the C+ Na+,K+-ATPase.

The changes in growth properties observed here were unexpected and provided a major challenge for this selection. A thorough interpretation of these changes must await the independent selection of other ATPase amplified cells, as well as the amplification of different membrane proteins to the same levels. Variations in growth properties have been seen among nonselected HeLa clones (41), but it is clear that the growth rate alterations of C+ cells observed here on dishes quickly disappear with the loss of excess ouabain binding. The inability of the C+ cells to grow in suspension is also overcome with the reversion to normal ouabain binding. This implies that some features of the amplification of the Na+,K+-...
ATPase is closely correlated with the altered C+ cell growth. It is not clear if this is a result of the amplification of the ATPase enzyme, or a secondary effect of amplifying a membrane protein.

The elucidation of the mechanism responsible for increasing the Na\textsuperscript{+},K\textsuperscript{+}-ATPase is under investigation. Modulations of Na\textsuperscript{+},K\textsuperscript{+}-ATPase content have previously been observed under two conditions. The thermogenic response of some tissues to thyroid hormone involves an increase in the synthesis of the ATPase (42). Preliminary experiments indicate that triiodothyronine has little effect on the number of ouabain binding sites detected on HeLa S\textsubscript{3} or C\textsuperscript{+} cells (Kalka, T., and J. F. Ash; unpublished observations). Cook and his co-workers (43) have shown that HeLa cells grown in sublethal doses of ouabain or in low potassium medium increase the number of ouabain binding and \textsuperscript{86}Rb transport sites approximately two-fold. They have concluded that this results from a decrease in turnover of this enzyme rather than an increase in its synthesis (44). Their ouabain-induced ATPase increase apparently occurs in all treated cells and quickly disappears after the ouabain is removed from the medium.

The present example of Na\textsuperscript{+},K\textsuperscript{+}-ATPase increase differs from these previous cases by its greater amplitude and duration in the absence of external stimulus. The appearance of minute chromosomes in the ATPase amplified C\textsuperscript{+} cells and their loss in the revertant C\textsuperscript{−} cells argues strongly for the amplification of Na\textsuperscript{+},K\textsuperscript{+}-ATPase genes in the C\textsuperscript{+} cells as the mechanism of the enzyme overproduction (see references 12
FIGURE 10 Composite of giemsa-banded chromosomes derived from ouabain resistant or C+ cells, i.e., from metaphases 2 and 4 noted in Table II. Single SM and double minute (DM) chromosomes are shown at the bottom of the figure.

and 45 for a discussion of minute chromosomes and gene amplification). The other cytogenetic changes noted in C+ and C− cells (e.g., the loss of a chromosome 1, the loss of a chromosome 22, etc.) when compared with HeLa cells, as noted in Table II, may be artifactual. We are planning to reproduce entirely this part of the study to determine their significance.

The ATPase amplification in C+ cells is not the first example of membrane protein overexpression—the A-431 cells, which carry a 10- to 20-fold increase in epidermal growth factor receptors, have already been discussed (2, 3). This is also not the first example of a selection induced increase in a membrane protein. Ling and Thompson (46) selected Chinese hamster ovary cells to colchicine resistance and produced cells with an increase in a membrane glycoprotein (47). These cells became resistant to colchicine by virtue of a decrease in colchicine permeability which proved to extend to other drugs as well (47). The changes associated with drug impermeability in different cells have been found to be complex, involving alterations in levels of several other cell components (48–50).

The present work does provide an example of a selection specifically designed to increase the expression of a known...
membrane protein. The C* and related cells should prove useful in studies of membrane protein synthesis, recycling, and ligand-induced reorganizations by the expedient of providing a greater amount of a single species to follow through the flow of these processes. The amplification of the Na*,K+-ATPase in C* cells presents advantages for analysis of the assembly (51, 52) and structure (53) of this important enzyme and may facilitate the isolation of the ATPase genes (54). In addition, the unexpected changes induced in the C* cells by this selection will hopefully lead to an improved appreciation of the role played by the plasma membrane in the regulation of cell growth.

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FIGURE 11 Composite of giemsa-banded chromosomes derived from revertant or C- cells, i.e., metaphases 2 and 3 noted in Table II. Symbols as in Fig. 9.
