Stable Gene Amplification and Overexpression of Sodium- and Potassium-Activated ATPase in HeLa Cells

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Cell lines stably resistant to ouabain were isolated from an unstably resistant HeLa line after growth in nonselective medium. Stable resistant lines bound ouabain at levels 10-fold higher than did HeLa cells and at similar levels to those bound by the unstable C⁺ line previously described (J. F. Ash, R. M. Fineman, T. Kalka, M. Morgan, and B. Wire, *J. Cell Biol.* 99: 971-983). Expression and synthesis of the Na⁺,K⁺-ATPase alpha chain showed a similar amplification over that for HeLa cells by Western blots and [³⁵S]methionine pulse-labeling. In addition, a glycoprotein labeled with [³H]fucose and comigrating with the Na⁺,K⁺-ATPase beta chain was eight- to ninefold amplified in stably resistant lines. Dot blots with a cDNA clone specific for Na⁺,K⁺-ATPase alpha chain gene sequences confirmed the amplification of this gene. Karyotyping suggested that the amplification is associated with an expanded, abnormal banded region on the long (q) arm of one chromosome 17.

Gene amplification with subsequent overproduction of a target enzyme is now a well-established mechanism for the acquisition of drug resistance during step selection procedures (13, 15). A several-hundredfold amplification of the dihydrofolate reductase gene has been demonstrated in response to selection with methotrexate, with corresponding increases in the levels of dihydrofolate reductase.

A variant of the HeLa S₃ cell line which expressed 10- to 12-fold more ouabain-binding sites was recently selected with the sodium- and potassium-activated ATPase (Na⁺,K⁺-ATPase) inhibitor ouabain (1). The unstable ouabain-resistant phenotype was correlated with the presence of minute chromosomes, and amplification of the gene for Na⁺,K⁺-ATPase was proposed as the mechanism leading to drug resistance. Gene amplification in our HeLa S₃ ouabain-resistant line has recently been confirmed in another laboratory (14).

In the present work, we analyzed reversion of the unstable ouabain-resistant phenotype by correlating minute chromosome number with ouabain-binding sites. In this study of the reversion process, we isolated clones which are stably resistant to ouabain. Characterization of these stable lines has demonstrated coordinate amplification of ouabain-binding sites, ATPase alpha-chain expression and synthesis, a glycoprotein comigrating with ATPase beta chain and, with a cDNA probe (14) provided by Robert Levenson (Yale University), of the gene for the Na⁺,K⁺-ATPase alpha chain. Karyotype analysis showed that amplification of the Na⁺,K⁺-ATPase is correlated with an expanded, abnormal banded region (ABR) on chromosome 17.

MATERIALS AND METHODS

Cell culture. All cell lines used in this study were derived from the human carcinoma line HeLa S₃ (12), provided by Melvin Simon, University of California, San Diego. Except for control HeLa S₃ cultures, the direct parent of all amplified and revertant lines studied here is C⁺, a line expressing

unstable resistance to ouabain, a 10- to 12-fold increase in ouabain-binding sites, and minute chromosomes (1). The cells were grown in a medium consisting of 45% Dulbecco modified Eagle medium, 45% Ham F12 medium, 5% horse serum (Sterile Systems, Logan, Utah), and 5% calf serum (Sterile Systems). This medium, designated D/F H5C5, was supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and NaHCO₃ (2.45 g/liter). Cells were removed from the dishes for passage by incubation in a thin film of 0.05% trypsin (1:250)-0.02% EDTA for 10 min before resuspension in medium. Tissue culture reagents were obtained from Irvine Scientific, Santa Ana, Calif.

Cells were grown in plastic tissue culture dishes (Costar, Cambridge, Mass.) at 37°C in a humid 5% CO₂-95% air atmosphere. Clones were isolated by diluting freshly trypsinized cell suspensions to about 1 cell per ml and plating 0.1 ml per well in 96-well dishes. Before dilution, cell aggregates were allowed to settle and suspensions were examined by phase-contrast microscopy to ensure that they contained predominantly single cells.

Ouabain binding. Ouabain-binding measurement and calculation of the number of ouabain-binding sites were performed as previously described (1).

Reversion analysis and selection of stably resistant lines. To examine the correlation of the ouabain-resistant phenotype with the presence of minute chromosomes, C⁺ ouabain-resistant cells, selected as previously described (1), were plated in normal medium without ouabain and passaged every 4 days. At intervals, the specific ouabain binding was determined for these cells, designated C⁻², as well as for cultures of C⁺ in 225 nM ouabain and HeLa cells in normal medium. Simultaneously, parallel cultures were used for karyotype analysis, as described below, to determine minute chromosome number. A continuous record of cell counts was maintained to allow the determination of the number of population doublings as the reversion progressed.

After maintaining cultures of C⁻² continuously in the absence of ouabain for over 12 months, we isolated 15 clones as described above and determined the number of ouabain-

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binding sites for each clone. In addition, each clone was plated at 10^3 and 10^5 cells per 100-mm dish, and cloning efficiencies were determined in 0, 50, and 200 nM ouabain. Cloning efficiencies and ouabain-binding sites were also measured simultaneously for C^+ and clones of HeLa S_3 which had never been under selection with ouabain.

Membrane isolation and analysis. Membrane proteins were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis by using the discontinuous buffer system of Laemmli (10) and slab gels modeled on those of Studier (16). 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% 2-mercaptoethanol. After electrophoresis at 10 W, the gels were fixed and stained overnight in a solution of 25% 2-propanol–10% acetic acid–0.01% Coomassie brilliant blue R-250. Gels were destained with 10% methanol in 10% acetic acid. Gels of radiolabeled samples were prepared for fluorography by using En^3Hance (New England Nuclear Corp., Boston, Mass.) as specified by the manufacturer. Electrophoresis reagents were purchased from Bio-Rad Laboratories, Richmond, Calif., except for the SDS, which was obtained from BDH, Poole, England, and used without recrystallization. Molecular weight markers were obtained from Sigma Chemical Co., St. Louis, Mo., and included, in decreasing size, myosin heavy chain, β -galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase.

Depending on the information sought, samples subjected to electrophoresis were either whole-cell preparations in which the cell monolayers were solubilized in 2% SDS sample buffer (without 2-mercaptoethanol, which was added immediately before electrophoresis) or microsomal preparations. To prepare microsomes, dishes were rinsed three times in cold phosphate-buffered saline (PBS) and once in a swelling buffer consisting of 7.5 mM Tris and 1 mM EDTA (pH 8.3), followed by a 15-min incubation in the swelling buffer. Cells were removed from the dish by gentle pipetting, collected in a syringe, and lysed by being forced through a 25-gauge needle into sufficient volume of 1 M sucrose in swelling buffer to yield a final sucrose concentration of 0.25 M. Nuclei and whole cells were removed by a 10-min centrifugation at $600 \times g$, and microsomes were sedimented by centrifugation of the supernatant at $48,400 \times g$ for 30 min. Microsomes from [^{35}S]methionine-pulse-labeled cells were further purified by sedimentation through 15% (wt/wt) sucrose (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] adjusted to pH 7.5 with Tris base) layered on a 33% sucrose cushion. Membranes were collected at the 15/33% interface after centrifugation at $26,900 \times g$ for 1 h in a swinging bucket rotor. Membranes were diluted fourfold with 25 mM HEPES–Tris (pH 7.5) and collected by centrifugation at $48,400 \times g$ for 30 min. The pellets were dissolved in 2% SDS sample buffer (without 2-mercaptoethanol). Protein was determined by the Hartree modification (44) of the Lowry procedure (11) with bovine serum albumin as a standard.

The relative level of Na^+, K^+ -ATPase in whole-cell preparations was determined immunologically by using a polyclonal rabbit antibody against dog kidney holoenzyme (provided by Jack Kyte, University of California, San Diego). After electrophoresis, proteins were transferred to a nitrocellulose membrane by using a Trans-Blot cell (Bio-Rad). Proteins were transferred for 3 h at 70 V in a buffer consisting of 25 mM Tris, 192 mM glycine (pH, approximately 8.3), and 20% (vol/vol) methanol. Blots were washed

in PBS for 30 min and blocked in a solution of 3% bovine serum albumin in PBS for 2 h at room temperature to prevent nonspecific protein binding. After three rinses in PBS, blots were incubated overnight in antibody (about 2.5 $\mu\text{g}/\text{ml}$) in PBS plus 1% normal goat serum (room temperature). After three 10-min washes, the blots were incubated for 3 h with affinity-purified goat anti-rabbit serum conjugated to horseradish peroxidase (1:1,000 Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in PBS plus 1% normal goat serum. Horseradish peroxidase was detected with 4-chloro-1-naphthol as described by Hawkes (5).

Protein synthesis rates were compared in HeLa and amplified lines by pulse-labeling with [^{35}S]methionine. Before labeling, nearly confluent cultures were rinsed twice in PBS and incubated for 30 min in methionine-free medium (Hanks salts, minimal essential medium vitamins and amino acids except methionine, 0.22% NaHCO_3). This medium was removed and replaced with 4 ml of fresh methionine-free medium containing 25 μCi of [^{35}S]methionine (New England Nuclear) per ml. After 30 min, the dishes were rinsed three times in PBS, and the cultures were reincubated in D/F H5C5 for 4 h. After the chase period, membranes were prepared as described above. Samples were precipitated onto paper filter disks with 10% trichloroacetic acid, and radioactivity was determined by using Biofluor (New England Nuclear) in a Packard Tri-Carb scintillation counter (Packard Instrument Co., Inc., United Technologies, Downers Grove, Ill.).

Glycoproteins were labeled metabolically with radiolabeled sugars. The medium was removed from subconfluent dishes and replaced with fresh D/F H5C5 containing 20 μCi of [^3H]fucose (New England Nuclear) per ml. After a 20-h incubation, microsomes were prepared from labeled cultures as described above.

After electrophoresis and treatment for fluorography, gels were dried onto filter paper and exposed to Kodak XAR-5 film at -70°C . Developed autoradiographs and Coomassie-stained gels were scanned with a Multi-Media densitometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Scans were analyzed with the microprocessor and software of a Gilford 2600 spectrophotometer.

DNA preparation and blot hybridization. DNA was prepared from confluent monolayers of HeLa S_3 , C1 (a stably amplified line cloned from C^{-2}), and C4 (a revertant line cloned from C^{-2}) cells by phenol-chloroform extraction (3). After precipitation in ethanol, DNA was spooled, redissolved, treated with boiled RNase (100 $\mu\text{g}/\text{ml}$), and reextracted. The A_{260}/A_{280} ratios of resulting preparations were greater than 1.8. Dot blots (7) were prepared with 10, 5, 2, 1, and 0.5 μg of DNA from each line by using a dot blot manifold (Bethesda Research Laboratories, Gaithersburg, Md.). The blot was prehybridized and hybridized for the detection of single-copy sequences (9) with nick-translated ([^{32}P]dCTP) pRB5, a specific probe for the Na^+, K^+ -ATPase alpha-chain gene (14). pRB5, obtained from Robert Levenson (Yale University) contains a 1,200-base cDNA insert isolated from rat brain cDNA library constructed in the lambda gt11 expression system of Young and Davis (17, 18). The blot was washed, dried, and exposed to Kodak XAR-5 film.

Karyotype analysis. Karyotype analysis was performed as previously described by using Giemsa-trypsin banding techniques (N. C. Sun, E. Y. Chu, and C. C. Chang, *Mammalian Chromosome Newsl.* 14:26–28). Metaphases were analyzed for known, marker, and minute chromosomes as well as homogeneously staining regions on ABRs.

RESULTS

Correlation of loss of minute chromosomes and loss of ouabain-binding sites. In earlier experiments (1) we observed that in the absence of selection the number of ouabain-binding sites in C^+ cells reverts to the levels in parental HeLa cells by 20 to 25 population doublings. We repeated this reversion study to correlate the number of minute chromosomes with the number of ouabain-binding sites and to isolate stably amplified variants which can be identified in a background of sensitive cells.

In Fig. 1, the numbers of minute chromosomes and ouabain-binding sites are plotted against population doublings in nonselective medium. Average minute chromosome numbers dropped immediately and rapidly, and reached zero at about 50 population doublings. No minute chromosomes were observed after 60 population doublings. After the first few doublings, minute number was extremely heterogeneous, with large numbers of minute chromosomes in a small percentage of cells producing a small average number. Loss of ouabain-binding sites in C^{-2} occurred more slowly. Instead of returning to the levels in HeLa cells by 25 population doublings as was previously observed, ouabain binding plateaued at 2.5 times the levels in HeLa cells (2×10^6 binding sites per cell) by 35 doublings; it has remained at this level for over a year (nearly 400 population doublings).

Isolation of revertant and stably amplified lines. The stable intermediate (2.5 times that for HeLa cells) levels of C^{-2} ouabain binding observed in the absence of selection with ouabain could have been accounted for by a homogeneous intermediate level of binding in all cells of the population, a small number of cells expressing high levels of binding and the majority binding at normal levels, or a more complex mix of cells with heterogeneous ouabain-binding levels. To distinguish among these possibilities, ouabain binding was measured in clones of the C^{-2} population.

Figure 2 shows the distribution of the number of ouabain-binding sites in HeLa and C^+ cells and 15 clones randomly obtained from the C^{-2} population. Binding-site number in the clones clearly falls into two classes: six clones displayed elevated binding averaging 10.2-fold higher than for HeLa cells (8- to 13-fold higher), while binding of the other nine averaged within 10% of the level for HeLa cells. One C^{-2} clone (A5) bound levels of ouabain nearly twice that of HeLa cells (1.5×10^6 sites per cell). This clone appears morphologically heterogeneous and may therefore not be derived

FIG. 1. Minute chromosome number (○) and ouabain-binding site number (●) versus population doublings in C^{-2} population after withdrawal of ouabain selection.

FIG. 2. Ouabain-binding site number of HeLa S₃ and C^+ cells and 15 clones of C^{-2} isolated after 12 months in nonselective medium. Since the C^{-2} population averaged 2×10^6 ouabain-binding sites per cell, the number of high-binding clones detected is greater than expected ($P = 0.02$, assuming equal cloning efficiency). The reason for this is not known.

from a single cell. High-binding clones are morphologically distinguishable from the low-binding clones by their growth in highly adherent clonal groups.

Several clones were also reselected in two concentrations of ouabain to examine their resistance and determine the homogeneity of the populations. In all cases, the cloning efficiencies of high-binding clones were nearly unaffected by ouabain concentrations up to 200 nM (C^+ cultures were maintained at 225 nM ouabain). The average cloning efficiency of the high-binding clones examined was unchanged in 50 nM ouabain and reduced only 40% in 200 nM ouabain. Conversely, the low-binding clones, which normally had a somewhat higher cloning efficiency than the high-binding clones (0.33 versus 0.19) in nonselective medium, displayed no growth at 50 nM ouabain, even when 10^6 cells were plated. Three colonies grew when 10^6 HeLa S₃ cells were plated in 50 nM ouabain.

Amplification of expression of Na^+, K^+ -ATPase in stably resistant lines. In examining the amplification of expression of the Na^+, K^+ -ATPase, we have limited our analyses to two high-binding clones of C^{-2} , A1DP and C1, and one low-binding clone, C4, in addition to HeLa cells. The numbers of ouabain-binding sites assayed for these clones were 1.1×10^7 for A1DP (13.5 times the level for HeLa cells), 8.1×10^6 for C1 (10.0 times the level for HeLa cells), and 6.6×10^5 for C4 (0.81 times the level for HeLa cells).

Microsomal membrane preparations analyzed by SDS-polyacrylamide gel electrophoresis clearly showed amplification of a band the size of the alpha chain of the Na^+, K^+ -ATPase in A1DP and C1, but not C4 cells (Fig. 3b through d). Scanning of Coomassie-stained gels demonstrates an

FIG. 3. Coomassie-stained gel of microsomal membrane preparations of HeLa and C^{-2} clones. Lanes: a, HeLa S_3 ; b, A1DP; c, C1; and d, C4. Note the amplification of the 100-kDa band in A1DP and C1, which comigrates with the Na^+, K^+ -ATPase alpha chain. Locations of marker proteins, as described in Materials and Methods, are at the left. The same amount of protein (20 μg) was loaded on each lane.

eightfold amplification in A1DP and a sixfold amplification in C1 cells. This band (100 kilodalton [kDa]) comigrates with the amplified band in C^+ cells and with pig kidney Na^+, K^+ -ATPase alpha chain (1).

The identification of this band as the alpha chain of the Na^+, K^+ -ATPase has been confirmed by using rabbit antibody to dog kidney enzyme, provided by Jack Kyte. Equal amounts of protein (100 μg) from whole-cell preparations were separated electrophoretically, transferred to a nitrocellulose membrane, and probed with the antibody. Antibody-bound material was enzymatically visualized by using goat anti-rabbit antibody conjugated to horseradish peroxidase (Fig. 4). Although we have not accurately quantitated the amplification of the alpha chain by using immunoblotting techniques, the intensity of the 100-kDa band in Fig. 4 appears to be amplified to a comparable extent in A1DP and C1 cells, as was demonstrated by Coomassie staining of membrane proteins (Fig. 3). This antibody did not interact with any protein of a size similar to the ATPase beta chain or a possible alpha-beta heterodimer in any of the lines.

Amplification of synthesis of Na^+, K^+ -ATPase. Increased expression of an enzyme might be caused by either increased synthesis, as would be expected if the gene were amplified, or decreased degradation, a factor that could be especially significant for the ATPase, which has a reported half life of approximately 5 h in HeLa cells (8). To determine whether synthesis of the Na^+, K^+ -ATPase was amplified in cells expressing amplified protein, cultures of HeLa, C1, and C4 cells were pulse-labeled with [^{35}S]methionine. Again, a 100-kDa band was amplified in membrane preparations of C1

FIG. 4. Na^+, K^+ -ATPase alpha chain (α) visualized by rabbit antibody to dog kidney holoenzyme in a Western blot of whole-cell preparations of HeLa and C^{-2} clones. Lanes: a, HeLa S_3 ; b, A1DP; c, B4DP; d, C1; and e, C4. Approximately 10-fold amplification is evident in high ouabain-binding clones A1DP, B4DP, and C1. The same amount of protein (100 μg) was loaded on each lane.

(Fig. 5b) compared with HeLa (Fig. 5a) and C4 (Fig. 5c). Although the complexity of the band pattern in [^{35}S]methionine-labeled membrane preparations does not allow an accurate quantitation of amplification, preliminary results from immunoprecipitations with anti-ATPase anti-

FIG. 5. Autoradiogram of gel of membrane preparations pulse-labeled with [^{35}S]methionine. Lanes: a, HeLa S_3 ; b, C1; and c, C4. Note the amplification of the 100-kDa band which comigrates with the Na^+, K^+ -ATPase alpha chain (α) in C1 cells. Equal counts (200,000 cpm) were loaded in each lane. Locations of marker proteins described in Fig. 3 are at the left.

body indicate a ninefold-greater synthesis of the alpha chain in C1 than in HeLa cells (data not shown).

Amplification of glycoproteins labeled with [3 H]fucose. Because metabolic labeling with amino acids (methionine or leucine; data not shown) did not detect amplification of any peptide that could be the ATPase beta chain, we also used [3 H]fucose, which should be more sensitive to alterations in levels of that glycoprotein, for metabolic labeling. Labeling with [3 H]fucose (Fig. 6) revealed an eight- to ninefold amplification in both A1DP (Fig. 6b) and C1 (Fig. 6c) of a broad band migrating with an apparent molecular mass range of 55 to 62 kDa and a peak at 60 kDa. This band comigrated with beta chain from pig kidney. Labeling in this region was indistinguishable from that of HeLa cells (Fig. 6a) in the low-binding clone C4 (Fig. 6d). Several other amplifications of [3 H]fucose-labeled glycoproteins were also detectable in A1DP and C1: a doublet band with molecular mass of about 45 and 46.5 kDa, a band at 210 kDa, and a 17-kDa band were about three- to fourfold amplified, a 72-kDa peptide which was not detectable in HeLa was labeled, and there was a small amplification of a glycoprotein migrating at about 128 kDa. The latter glycopeptide migrates noticeably faster in C1 than in A1DP cells and is therefore not likely to be a

FIG. 6. Autoradiogram of a gel of microsomal membrane preparations from cultures metabolically labeled with [3 H]fucose. Lanes: a, HeLa S₃; b, A1DP; c, C1; and d, C4. The broad band migrating at about 60 kDa and amplified in A1DP and C1 cells comigrates with the Na⁺,K⁺-ATPase beta chain. No evidence was seen of [3 H]fucose labeling of a band comigrating with the Na⁺,K⁺-ATPase alpha chain. Amplifications seen in this autoradiogram are summarized in Table 1. Positions of protein standards are noted on the left, as in Fig. 3, and apparent sizes of labeled bands calculated from the standards are noted on the right (in kilodaltons). Equal counts (75,000 cpm) were loaded on each lane.

TABLE 1. Amplification of [3 H]fucose-labeled glycoproteins

Protein ^a (kDa)	Relative amplification ^b for		
	A1DP	C1	C4
210	3.4	2.6	3.0
128	1.7	1.7	0.3
72 ^c	+	+	—
60	8.1	8.7	1.0
46 ^d	3.6	3.0	4.4
17	3.0	2.2	0.7

^a Glycoprotein bands as identified in Fig. 6.

^b Ratio of percent total [3 H]fucose labeling in each glycoprotein band in C⁻² clones relative to HeLa cells.

^c Band not detectable in the HeLa S₃ line.

^d Doublet band at 45,000 and 46,500 Da.

homodimer of the 60-kDa band, which has identical mobility in both lines. It is also not likely to be a heterodimer of alpha and beta, since no mobility differences has been noted in the alpha chain of the ATPase in these lines (Fig. 3 and 4). Labeling in this region of the gel was somewhat (threefold) reduced in the low ouabain binder C4 compared with HeLa. The only other difference seen in [3 H]fucose labeling between HeLa and C4 cells was the persistence of the amplification of the doublet band at about 46 kDa and the 210-kDa band in the low-binding clone of C⁻². Differences in [3 H]fucose labeling among the four lines are summarized in Table 1, in which they are expressed as -fold amplifications of labeling levels in HeLa cells.

Amplification of gene for Na⁺,K⁺-ATPase alpha chain. DNA from HeLa, C1, and C4 cells was probed on a dot blot (Fig. 7) for amplification of the alpha-chain gene for Na⁺,K⁺-ATPase by using a cDNA clone obtained from Robert Levenson. C1 was clearly amplified over HeLa for sequences binding this probe. All dots were scanned and integrated, and binding was plotted against DNA loaded in the dots for each cell line. From the curves fitted to these points, concentrations of DNA giving identical probe binding were calculated, and -fold differences were calculated for C1 and C4 compared with HeLa cells. These calculations revealed a sixfold amplification in C1, whereas C4 DNA did not bind significantly more probe than did HeLa DNA (<1.2-fold difference).

FIG. 7. Dot blot of HeLa S₃ (H), C1, and C4 genomic DNA hybridized to cDNA clone pRB5 which is specific for sequences in the gene for Na⁺,K⁺-ATPase alpha chain. The amount of genomic DNA in each blot is noted at the bottom. Hybridization to C1 cells is about sixfold higher than to HeLa cells. C4 hybridization is not significantly different from that of HeLa cells.

FIG. 8. Giemsa-banded C1 chromosomes. N-R refers to Nelson-Rees marker chromosomes, and M refers to Morgan marker chromosomes (see reference 1 for comparison). An ABR was noted on the q arm of one chromosome 17 in all six karyotypes prepared.

Karyotype analysis. Figure 8 shows a Giemsa-trypsin-banded karyotype of the C1 cell line. Despite the previously known disposition of HeLa cells toward cytogenetic heterogeneity, the quantitative integrity of the C^+ line has been preserved in C1 metaphases (for comparison, see reference 1). Curious qualitative cytogenetic alterations, however, were discovered in C1 metaphases, including six from which complete karyotypes were prepared. No double or single minute chromosomes were found in any C1 cells. An ABR on one number 17 chromosome was observed in all six metaphases. The overall size of this chromosome has been dramatically increased to almost twice its original length. The Morgan marker number 3 also acquired additional material. The previously existing p arm has disappeared; translocated into its place is what appears to be the q arm of a number 12 chromosome. Also, the Nelson-Rees marker number 10 has acquired additional material on the q arm. Karyotypes of A1DP, the other amplified line studied here, revealed a similar or identical ABR on the q arm of one chromosome 17, while neither the ABR on chromosome 17

nor minute chromosomes were seen in metaphases of C4 cells examined (data not shown).

DISCUSSION

In this study we isolated and characterized cell lines stably amplified in the expression of the $\text{Na}^+, \text{K}^+ \text{-ATPase}$ from C^+ , an unstably amplified derivative of the HeLa S_3 line (1). Stable amplification of enzyme expression is correlated with an abnormal banded region on chromosome 17. Such ABRs, including the frequently described homogeneously staining regions, are often observed to be the sites of amplified genes (13, 15). Analysis of our lines by *in situ* hybridization is currently in progress to verify the chromosomal location of the amplified gene, since other alterations have been noted in marker chromosomes. These stable lines were derived during an analysis of reversion of excess ouabain binding of C^+ cells, correlated with loss of minute chromosomes. Because the parent population had apparently become a mixture of stably and unstably amplified cells, the loss of ouabain

binding did not completely parallel the loss of minute chromosomes.

From the mixed C^{-2} revertant population we have isolated six stably amplified clonal lines represented in this report by A1DP and C1 and nine revertant clones represented by C4. Although A1DP and C1 are morphologically distinguishable and express differences in the electrophoretic mobility of an amplified glycopeptide (128 kDa; Fig. 6), it is not clear that they arose independently. Possibly arguing against independent origins is the observation that amplification in both stable lines is correlated with a large ABR on the q arm of chromosome 17. Analysis of more, independently isolated, stable lines may help determine whether the q arm of chromosome 17 is a preferred site of insertion of amplified Na^{+},K^{+} -ATPase genes, perhaps as the normal chromosomal location of either the alpha or beta chain gene.

In addition, this study confirms that the gene for the alpha chain of the Na^{+},K^{+} -ATPase is amplified in cells stably overexpressing the enzyme. Using a probe specific for the alpha chain gene, we demonstrated a sixfold amplification in C1 (Fig. 7), comparable to the increase seen in synthesis and expression of this peptide (Fig. 3 through 5). Schneider et al. (14) have documented a significantly greater amplification in C^{+} (40-fold over that in HeLa), which may be due to the continuous selection (in 1 μ M ouabain) under which these cells were maintained. We have observed ouabain binding as high as 25-fold higher than for HeLa in lines unstably resistant to and maintained in 0.8 μ M ouabain (T. Kalka and J. F. Ash, unpublished observations). It is reasonable to suggest there is a good correlation in the levels of gene amplification, enzyme synthesis, and steady-state expression in this system.

It is possible that the expression of the Na^{+},K^{+} -ATPase beta subunit is also amplified in the stably resistant lines. A glycoprotein (labeled with [3 H]fucose) which comigrates with the beta chain is eight- to ninefold amplified in both A1DP and C1 cells (Fig. 6b and c) compared with the levels in HeLa and C4 cells. However, this protein is not detected by polyclonal antibody to the dog kidney holoenzyme in any of our HeLa-derived lines (Fig. 4). We are currently attempting to isolate and further characterize this protein to determine whether it is the Na^{+},K^{+} -ATPase beta subunit or an unrelated glycoprotein whose gene has been coamplified with that for the Na^{+},K^{+} -ATPase alpha chain. Labeling with [3 H]fucose also revealed the amplified expression of several other peptides which may or may not be related to the ATPase. Some of these, including the 128, 72, and 17-kDa peptides, may represent products of genes that were nonselectively coamplified with those of the ATPase. This would be consistent with the absence of amplification of these bands in the revertant C4 line. We have not excluded the possibility that the 17-kDa peptide is a cleavage product from one of the other amplified bands, although protease inhibitors were routinely included during microsome isolation.

Amplification of two other bands labeled with [3 H]fucose was not lost in C4 cells. Although C4 appears to represent a complete reversion of the amplified Na^{+},K^{+} -ATPase phenotype seen in C^{+} , it exhibits significant physiological differences from the HeLa S_3 line (6). The presence of 10^7 sodium pumps in the plasma membrane of amplified cells could represent a powerful selection for secondary membrane alterations not linked to amplification of the ATPase. Such changes would not necessarily be expected to revert coordinately with the loss of amplification of the ATPase gene and could persist in lines such as C4. The physiological and

biochemical differences between C4 and HeLa cells are currently under investigation.

The stably amplified cell lines described in this study should be extremely useful in further investigations of synthesis, assembly, and regulation of the Na^{+},K^{+} -ATPase. Because these lines do not require continuous selection with ouabain to maintain amplification, the effects of ATPase-binding ligands such as the cardiac glycosides and physiologically significant factors thought to exist in serum (2) on ATPase expression, turnover, and distribution can be examined less ambiguously. Also, analysis of the physiology of amplified and revertant cells may help to further elucidate cellular mechanisms which modulate electrochemical gradients and transmembrane fluxes in concert with the Na^{+},K^{+} -ATPase.

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