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# Alteration of Human Breast Tumor Cell Membrane Functions by Chromosome-Mediated Gene Transfer

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BOT-2 cells (human breast tumor origin) have an impaired ability to utilize exogenous thymidine. Previous studies revealed this deficiency to be the permeation event rather than phosphorylation, since the cells have active thymidine kinase. Chromosome-mediated gene transfer was used to transfer genetic information in the form of metaphase chromosomes, from HeLa-65 cells to the BOT-2 cells, correcting the permease deficiency. Poly-L-ornithine or lipochromes were used for facilitation of chromosome uptake. After selection on HAT medium, transferant clones were isolated at a frequency of $4 \times 10^{-5}$ and $1 \times 10^{-5}$, respectively. Transferants MGP-1 and MGL-1 are stable after 18 months and have been characterized on the bases of purine and pyrimidine nucleoside uptake, relative thymidine kinase activities, alkaline phosphatase activities, and hydrocortisone-induced alkaline phosphatase activity. MGP-1 demonstrates positive thymidine uptake and incorporates radiolabeled thymidine into DNA. MGL-1 remains thymidine transport-deficient and survives on HAT by increasing endogenous dihydrofolate reductase activity. Alkaline phosphatase activity in MGL-1 is similar to HeLa-65, 2% of that in BOT-2, and in addition, is inducible 25–30-fold by 3 $\mu$M hydrocortisone. We have separated, genetically, a thymidine permease function from phosphorylation in cells of human origin and have transferred genetic information for the regulation of alkaline phosphatase.

Key words: **Alkaline phosphatase, chromosome-mediated gene transfer, human breast tumor cells, hydrocortisone, lipochromes, membrane bound enzymes, nucleoside uptake, thymidine kinase, thymidine transport**

BOT-2 cells are a clonal continuous cell line derived from a human mammary ductal cell carcinoma [1]. They have specific breast tumor antigens [2, 3], are not hormone dependent [4], and have high levels of endogenous alkaline phosphatase (AP) [1]. Previous studies in our laboratory have demonstrated these cells to have low uptake of pyrimidine nucleosides. There is a specific deficiency for thymidine uptake [4, 5], which is not correctable by treatment with estradiols and testosterone. These hormones have proved to be stimulatory effectors of thymidine uptake in other breast tumor cultures [6]. BOT-2 cells are unable to radiolabel DNA with exogenous $^3$H-thymidine and do not show nuclear (or cytoplasmic) labeling by autoradiography for labeling periods shorter than 15 h. Thymidine kinase (TK) is active in cell-free extracts, yet the cells fail to grow on HAT medium [7].

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The lack of thymidine uptake (presumably transport) ($TT^-$) in the presence of TK supports the concept of separation of the permeation and phosphorylation events in human cells [8–10] and provides a mechanism to separate genetically the two functions, as was done with haploid frog cells [11, 12]. Following desired treatments of the cultures, selection of $TT^+$ cells can be achieved on HAT medium.

To alter the $TT^-$ phenotype we used the established method of chromosome-mediated gene transfer (CMGT) for stable genetic transfer of genes from a donor cell type, which is $TT^+$. The donor in these studies also had very low AP activity levels. CMGT has been reported to transfer genetic information from one type of cultured mammalian cell to another with transfer frequencies from $10^{-5}$ to $10^{-8}$ [13–17]. Transfer frequencies have been increased by entrapping purified metaphase chromosomes into phospholipid vesicles (lipochromes) [16] rather than by using other facilitators such as poly-L-ornithine [13]. Treatment of recipient cells, BOT-2, with metaphase chromosomes from a donor cell line (HeLa-65, see below) followed by selection on HAT medium yielded stable and nonstable transferant cell populations expressing genetic information that corrected the original defect, $TT^-$.

HeLa-65 was selected as the donor cell system [19, 52] due to its growth on HAT medium in monolayer, its human origin, and several additional morphological and biochemical differences that distinguish it from BOT-2 [1]. In addition, HeLa-65 has extremely low alkaline phosphatase activity, which is inducible by hydrocortisone [19–24]. The increased AP activity in HeLa-65 appears to occur by a stimulation of catalytic activity [25] rather than de novo synthesis of new AP, which may occur in other AP inducible systems [24, 25–29]. HeLa cells grow in suspension culture, are subject to metaphase block by usual procedures, and have been used extensively as a donor for CMGT experiments [13, 30–32].

In this report we present evidence of successful transfer of genetic information from HeLa-65 correcting the BOT-2 thymidine transport deficiency. Both lipochromes and poly-L-ornithine were facilitators for the CMGT. The frequencies of transfer were $1 \times 10^{-5}$ and $4 \times 10^{-5}$, respectively. Several transferant clones were isolated, and two, MGP-1 and MGL-1, were used for further study. MGP-1 and MGL-1 are stable transferants. MGP-1 transports thymidine sufficiently to survive on HAT medium, and MGL-1, whose selection was due to methotrexate resistance rather than thymidine transport, has AP activities 2% of BOT-2, and the AP can be induced approximately 25-fold by hydrocortisone. Dihydrofolate reductase activity in MGL-1 was three times greater than the activities in the other cell lines.

Both transferants demonstrate alteration of membrane-associated activities by genetic supplementation. The $TT^-$, $TK^+$, BOT-2 modification to MGP-1 genetically separates thymidine transport from thymidine kinase, providing a mechanism for analyzing a specific nucleoside carrier. The MGL-1 transferant representing a change from AP constitutive to AP repressed, but inducible, enables further study of the mechanism for steroid hormone regulation in cells of human origin.

## MATERIALS AND METHODS

### Cell Cultures

BOT-2 cells were kindly provided by Dr. Robert Nordquist. HeLa-65, the chromosome donor cell line, was a gift from Dr. Martin J. Griffin and has a modal chromosome number of 65. HeLa-65 grows in suspension culture as well as monolayer. The recipient cells, BOT-2, and the donor cells, HeLa-65, differ with respect to the following characteristics: modal chromosome number (BOT-2 has 63), glucose-6-phosphate dehydro-

genase isoenzyme patterns [1], alkaline phosphatase activity, tumor antigen specificity, morphology, and growth characteristics (see Table I). Both cell lines were found to be mycoplasma-free as determined by the double-labeling technique of Schneider [33] with $^{14}$C-uracil and $^{3}$H-uridine.

Other cell cultures described are transferants derived by chromosome transfer. MGP-1 was selected, with poly-L-ornithine as the uptake facilitator. MGL-1 was obtained with lipid coated chromosomes, lipochromes.

### Conditions for Cell Growth

Monolayer cultures were grown in plastic tissue culture flasks (Corning, NY) using a Dulbecco's modified Eagle's medium, DPM (72214, GIBCO, Grand Island, NY). This was supplemented with 10% heat-inactivated fetal calf serum (FCS) (GIBCO, Grand Island, NY) and 50 μg/ml gentamicin (Schering, Kenilworth, NJ). Cultures were incubated at 36.5°C in an humidified atmosphere of 6% $CO_2$, 94% filtered air. For the preparation of metaphase chromosomes cells were grown as spinner cultures in Eagle's phosphate medium (69191 GIBCO, Grand Island, NY) supplemented with 10% FCS and gentamicin.

Growth studies, doubling times, and plating efficiencies were done in 35 mm plastic tissue culture dishes in DPM and DPM-HAT media. Doubling times were determined over a 9-day period, with feeding every third day. Cell counts were made every 24 h with a Coulter particle counter (Hialeah, FL). Plating efficiency was determined 24 h after plating of cells. Multicellular tumor spheroid (MTS) [34] formation was determined for all cell lines in 25 cm$^2$ tissue culture flasks. When necessary, 3μM hydrocortisone (Sigma Chem Co., St. Louis, MO) was added directly to the culture medium for required times.

### Donor Cell Synchronization and Chromosome Isolation

Metaphase chromosomes were prepared from HeLa-65 cells growing in suspension culture. As cells in the exponential growth phase reached a density of $2 \times 10^5$ cells/ml, they were synchronized by a double thymidine block [35]. Synchronized cultures were arrested at metaphase, with an efficiency of 95–98%, by addition of colcemid, 0.03 μg/ml, for 15 h.

Metaphase-arrested cells were harvested, under sterile conditions, for chromosome preparation. Chromosomes were isolated by using the pH 10.5 method of Wray [36, 37] and fractionated into small- and large-sized populations by sucrose density gradient centrifugation [37].

### Chromosome-Mediated Gene Transfer and Transferant Selection

Two transfer systems were used for fusion of chromosomes with the recipient BOT-2 cells.

First, recipient cell suspensions in DPM without serum, $2 \times 10^6$ cells/ml, were mixed with cellular equivalents of metaphase chromosomes and poly-L-ornithine, 12 μg/ml, in siliconized 12 ml glass centrifuge tubes. The tubes rotated slowly, 20 rpm, for 2.5 h on a roller apparatus at 37°C. This suspension was supplemented with fetal calf serum to 10% and placed in plastic tissue culture flasks for plating and subsequent selection. Medium was changed at 24 h with DPM plus 10% FCS to remove nonattached cells, debris, and nonfused chromosomes. After 48 h, cells were switched to HAT selection medium for at least 5 weeks, with biweekly medium changes.

Second, sized metaphase chromosomes were mixed with lipids to form lipochromes [18, 38]. A sterile mixture of lecithin, stearylamine, and cholesterol (4:1:3) dissolved in chloroform and methanol was evaporated to dryness on the walls of a 12 ml sterile sili-

conized glass tube with nitrogen; 4.4 mg of lipid were used for $2 \times 10^6$ cell equivalents of chromosomes. Chromosomes in DPM were added to the lipid-coated tube, and the tube was mixed on a Vortex mixer for 3 min at room temperature. Cellular equivalents of lipochromes were added to monolayer cultures at $1.33 \times 10^4$ cells/cm$^2$ in DPM + 10% FCS. The medium was replaced with DPM + 10% FCS after 8 h to remove excess lipids and nonbound lipochromes. After 24 h the medium was changed to DPM-HAT, and for the next 5 weeks cells were fed biweekly with HAT medium.

In both CMGT systems control flasks of cells were carried through similar manipulations, except for chromosomes, and with chromosomes but no facilitators. After 5 weeks no colonies were detected in control flasks. After a few days there were a few giant bizarre nondividing cells that did not detach as did the majority of the cells. Chromosome-treated cells were observed microscopically at weekly intervals. After 5 weeks the number of colonies surviving selection pressure were determined. Selected colonies were recovered and transferred to individual culture flasks and grown in HAT medium until confluence. Cultures then were split and maintained in DPM as well as HAT medium. A pilot study to measure thymidine uptake in the transferent cultures was done to aid in selection of cultures to be used for further study.

### Nucleoside Uptake Measurements

Uptake of purine and pyrimidine nucleosides was determined for donor and recipient cells, as well as for the stable transferants MGP-1 and MGL-1. MGX-100, a nonstable transferant, was also included. The following radioisotopically labeled nucleosides (Amersham, Arlington Heights, IL) were used: [methyl-$^3$H] thymidine, 50 Ci/mmole; 5-[$^3$H] uridine, 2 Ci/mmole; 5-[$^3$H] cytidine, 27 Ci/mmole; 5-[$^3$H]-deoxycytidine, 20 Ci/mmole; 2-[$^3$H]-adenosine; 20 Ci/mmole, G-[$^3$H]-deoxyadenosine, 12 Ci/mmole; 8-[$^3$H]-guanosine, 8 Ci/mmole; and 8-[$^3$H]-deoxyguanosine, 3 Ci/mmole. Each cell type was plated at $1 \times 10^4$ cells/cm$^2$ in 35 mm plastic tissue culture dishes in DPM plus 10% FCS. Following 36 h of incubation, labeled nucleosides were added to a concentration of $2 \times 10^{-5}$ M, 1 $\mu$Ci/ml, and incubated for an additional 18 h. Cultures were washed 3 times with cold Tris-Cl saline, pH 7.3 (ST), drained, and lysed with 0.5 ml 0.2M NaOH. The lysate was neutralized, mixed with Redisolv-HP (Beckman, LaJolla, CA), and counted in a liquid scintillation spectrometer (Intertechnique, Englewood, NJ). Parallel cultures were used for cell counts in a Coulter particle counter. All experiments were done in triplicate and repeated a minimum of three times.

### DNA Synthesis

To determine to what extent the intracellular labeled thymidine could be phosphorylated and incorporated into DNA, cells were plated and labeled as described above. After rinsing cells three times with ST and lysing with 0.5 ml of 0.2M NaOH, the lysate was mixed with 5 ml of cold 15% trichloracetic acid and set in an ice bath for 60 min. The precipitate was collected on nitrocellulose filter discs, washed with 5% TCA, rinsed with 95% ethanol, dried, and the radioactivity counted in a liquid scintillation spectrometer.

### Enzyme Assays

Thymidine kinase (TK) activity was measured with DEAE-52 paper disks [39, 40]. Results are expressed as pmoles phosphorylated thymidine per mg cell protein.

Alkaline phosphatase (AP) activity was measured with para-nitrophenyl phosphate

as the substrate [41]. AP activity was determined on whole lysates prepared in 0.5% sodium deoxycholate.

Protein concentrations were determined by the method of Zak and Cohen [42].

Specific breast tumor antigens were monitored by fixed-cell immunofluorescence with antitumor antibodies from human breast cancer sera [2].

## RESULTS

### Recipient and Donor Cell Characteristics

BOT-2 recipient cells were originally described to be distinct from HeLa-65, the chromosome donor, with respect to morphology, glucose-6-phosphate dehydrogenase isoenzymes, alkaline phosphatase activity, alkaline phosphatase isoenzymes, karyotype, and cell surface antigens [1, 2]. We have repeated these studies on both cell lines and on the transferant clones described, except for the glucose-6-phosphodehydrogenase analysis. These results, along with others presented here, are summarized in Table I. We reported preliminary results indicating a difference in the utilization of pyrimidine nucleosides between the BOT-2 and HeLa-65 cells [4, 5]. The differences between the two cell lines of particular interest in this study are the low levels of thymidine uptake by BOT-2, the inability of BOT-2 to survive in HAT medium, the high activity of AP in BOT-2 cells, and the breast tumor-specific antigens. These parameters, as well as characteristic morphology, modal chromosome number, and multicellular tumor spheroid formation, were used initially to differentiate BOT-2 from HeLa-65 and later to verify the ancestry of transferants selected on the basis of their altered thymidine utilization and alkaline phosphatase regulation.

### CMGT and Selection of Transferants

Treatment of BOT-2 cells with metaphase chromosomes was followed by selection on HAT medium. More than 99% of the recipient cells were killed after 5–8 days. HAT-resistant colonies, after 5 weeks of exposure, were counted, and the frequency of transference was found to be $4 \times 10^{-5}$ for the poly-L-ornithine facilitated transfer method and

**TABLE I. Characterization of Parental and Transferant Cells**

| Cell lines | Chromosome number | Growth on hat | Thymidine uptake[a] | AP[b] | AP in HC[c] | Thymidine kinase[d] | MTS[e] | BOT-2[f] Antigen |
|---|---|---|---|---|---|---|---|---|
| BOT-2[g] | 63 | – | 0.20 | ++++ | ++++ | 244 ± 17 | + | + |
| Hela-65[h] | 65 | + | 0.45 | + | ++ | 273 ± 25 | – | – |
| MGP-1 | 63 | + | 1.00 | ++++ | ++++ | 299 ± 29 | + | + |
| MGX-100 | 63 | +,– | 0.20 | +++ | ND | 242 ± 19 | + | + |
| MGL-1 | 63 | + | 0.11 | + | +++ | 169 ± 17 | + | + |

[a]Results expressed as nanomoles thymidine/$10^6$ cells.
[b]AP = relative alkaline phosphatase activity; see text for values.
[c]Relative alkaline phosphatase activity after treatment of cells with 3 $\mu$M hydrocortisone.
[d]Values expressed as picomoles TMP formed/mg protein ± SEM.
[e]MTS = multicellular tumor spheroid formation.
[f]Human breast tumor-specific antigen determined by immunofluorescence.
[g]Parental recipient.
[h]Parental chromosome donor.
ND = value not determined.

$1 \times 10^{-5}$ for the lipochrome technique. No surviving colonies were detected for untreated BOT-2 cells in HAT or for cells mixed with poly-L-ornithine or liposomes without chromosomes.

Transferant colonies were selected for further study after screening for thymidine uptake and alkaline phosphatase activity. MGP-1, MGL-1, and MGX-100 were grown in DPM and HAT media with periodic changes to the alternative medium. MGP-1 and MGL-1 were stable transferants maintaining resistance to HAT and other characteristics described later. MGX-100 was unstable, as determined by exponential increases in sensitivity to growth on HAT medium after removal of selection pressures. The instability of MGX-100 was typical of the majority of original transferants.

The population doubling times and plating efficiencies were measured in nonselective and selective media. Doubling times for MGP-1 and MGL-1 were 28 and 50 h, respectively, whereas the doubling time, using the same conditions, for BOT-2 was 32 h and that for HeLa-65 was 22 h.

Doubling time in HAT medium were extended to 43 h for MGP-1, 72 h for MGL-1, and approximately 30 h for HeLa-65. BOT-2 does not grow in HAT. The plating efficiency after 24 h for each cell type was nearly 98% in nonselective medium. This high efficiency was maintained for MGP-1 and MGL-1.

### Nucleoside Uptake Studies

Purine and pyrimidine nucleoside uptake by transferants was compared to the donor and recipient cell lines. MGP-1 demonstrated a 5-fold increase in thymidine incorporation over that of the BOT-2 cells (Fig. 1) and more than twice the level for HeLa-65 cells. MGL-1 did not show a similar increase, and its HAT resistance has been shown to be due

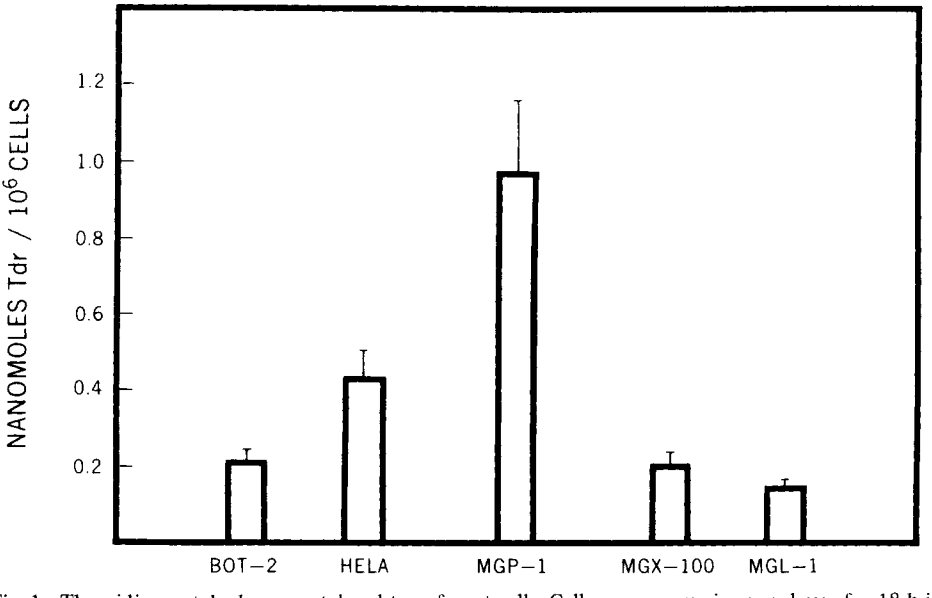


Fig. 1. Thymidine uptake by parental and transferant cells. Cells were grown in monolayer for 18 h in the presence of $1 \times 10^{-5}$ M [methyl-$^3$H]-thymidine. Whole cell lysates in 0.2 M NaOH were used to determine total uptake. Levels in BOT-2 cells are much lower than for other nucleosides (see Fig. 2). MGP-1 transports sufficient thymidine for survival on HAT medium BOT-2 cells are the parental recipient; HeLa-65 the parental chromosome donor; MGP-1 and MGL-1 are stable transferants; MGX-100 is an unstable transferant.

to other mechanisms (results not shown). The very low levels of thymidine uptake (0.2 nmoles/$10^6$ cells) in BOT-2 may actually represent plasma membrane-bound (but not incorporated) thymidine. Autoradiography did not demonstrate $^{14}$C-labeled thymidine in the cytoplasm or nucleus, whereas similar experiments with cytidine and uridine did (unpublished observation).

MGP-1 has the ability to incorporate uridine and cytidine, whereas no increase in deoxycytidine was noted. The results for nucleoside uptake, except thymidine, are summarized in Figure 2. All transferants were able to transport adenosine and deoxyadenosine equally well, except for MGL-1, with a 50% decrease in adenosine uptake. MGX-100, the unstable transferant, showed an increased ability, compared to BOT-2, to incorporate guanosine and deoxyguanosine. However, this change was unstable with increasing reversion of MGX-100. Likewise, the increases in uridine and cytidine uptake of MGX-100 are difficult to interpret, since no increase in thymidine was noted.

MGP-1, alone, showed a specific alteration in pyrimidine uptake. The 5-fold increase in thymidine uptake is critical for MGP-1 survival in HAT medium. Even though uridine and cytidine incorporation were improved, the parental BOT-2 cells incorporated both of these nucleosides into macromolecules at levels 5 to 10 times greater than thymidine, indicating a membrane specificity for thymidine.

### Incorporation of Exogenous $^3$H-Thymidine Into DNA and Thymidine Phosphorylation

Differences in thymidine incorporation were paralleled in the labeling of DNA as determined by acid precipitation of cells after long-term incubation with $^3$H-thymidine (Fig. 3). The transferant, MGP-1, had nearly a 5-fold increase over the recipient cells.

To determine whether the low levels of thymidine incorporation into DNA by BOT-2 and the higher levels by MGP-1 were due to an altered transport deficiency or to a modified ability to phosphorylate exogenous thymidine initially, the activity of thymidine kinase was determined for each cell line (Table I). Both parental cell types demonstrated equivalent TK activity. Likewise, the transferants possessed TK activity sufficient to permit cell growth in HAT medium, provided exogenous thymidine entered the cell. We previously demonstrated that BOT-2 cells made permeable to thymidine by gentle hypotonic treatment were able to phosphorylate $^3$H-thymidine and subsequently utilize this in DNA synthesis [53].

### Alkaline Phosphatase

A primary biochemical difference between the recipient and donor cells was that of alkaline phosphatase. This parameter was one of several used to distinguish BOT-2 cells from HeLa-65. It was also used to ascertain the relatedness of the transferants to the parental recipient. Normal levels of alkaline phosphatase activity in HeLa-65 are quite low (approximately 7–10 nmoles Pi/min/mg protein), and can be increased to 50–60 nmoles Pi/min/mg protein by induction with 3 $\mu$M hydrocortisone. Alternatively, BOT-2 has relatively high activity levels of alkaline phosphatase (approximately 750 nmoles Pi/min/mg protein), which are not significantly increased by hydrocortisone. Screening of the transferants revealed that MGL-1 shows alkaline phosphatase activity nearly identical to HeLa-65 (12 nmoles Pi/min/mg protein). This AP activity in MGL-1 can be increased nearly 30 times to 356 nmoles Pi/min/mg protein by growing the cells in 3 $\mu$M hydrocortisone (Fig. 4). The time course of the induction is similar to that for HeLa-65, including the lag period. Similar changes in AP activity were not seen in MGP-1.

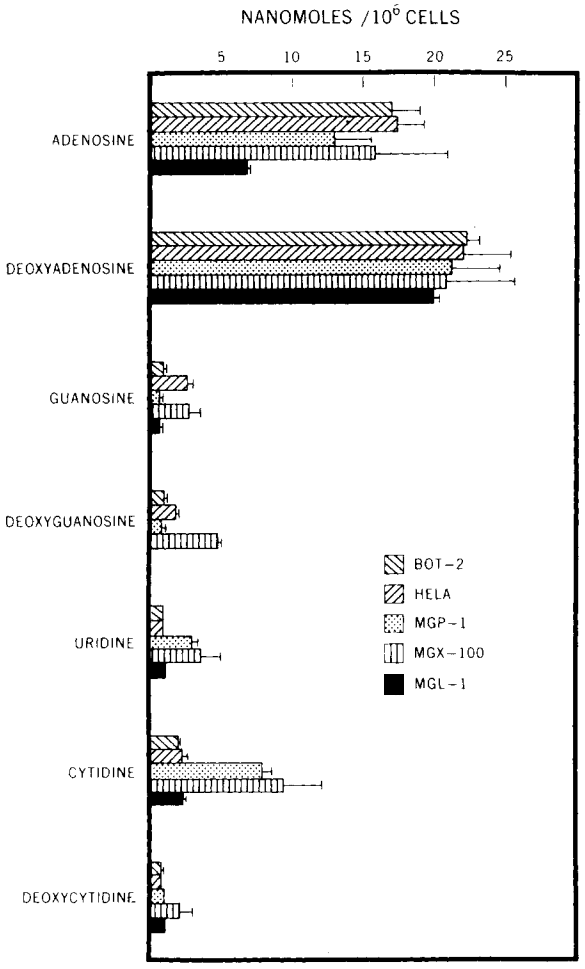


Fig. 2. Purine and pyrimidine nucleoside uptake by parental and transferant cells. All values are significantly higher than for BOT-2 ($TT^-$) (see Fig. 1). Uptake was determined after 18 h of growth in radioactive medium (see Methods), followed by whole cell lysis.

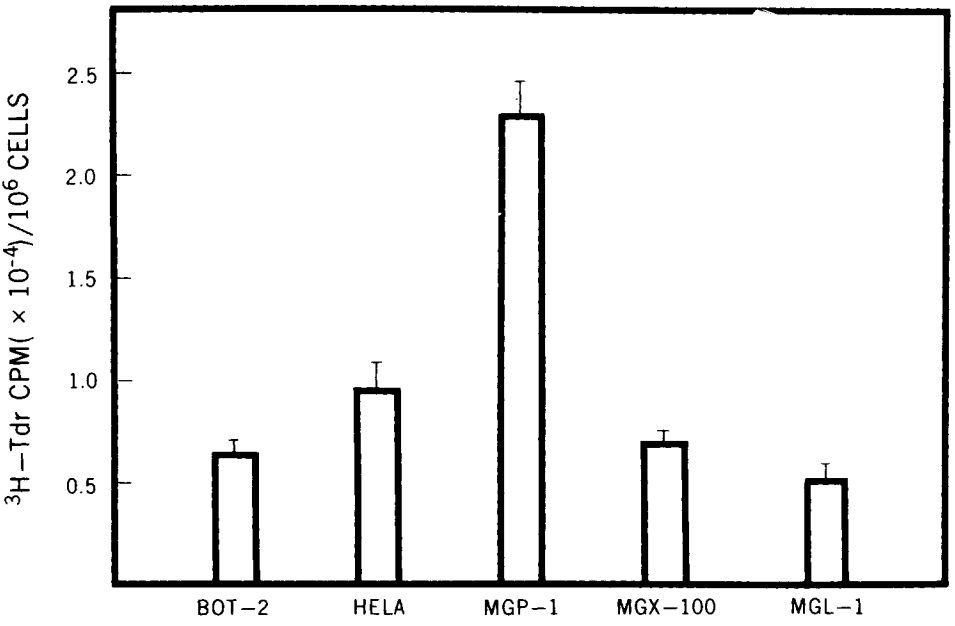


Fig. 3. Incorporation of $^3$H-thymidine into DNA. Cells were grown as in Figure 1, lysed, and DNA-precipitated with 15% trichloracetic acid (see Methods). Incorporation parallels thymidine uptake. BOT-2 nuclei do not show labeling by autoradiography after 15 h, whereas HeLa-65 nuclei do.

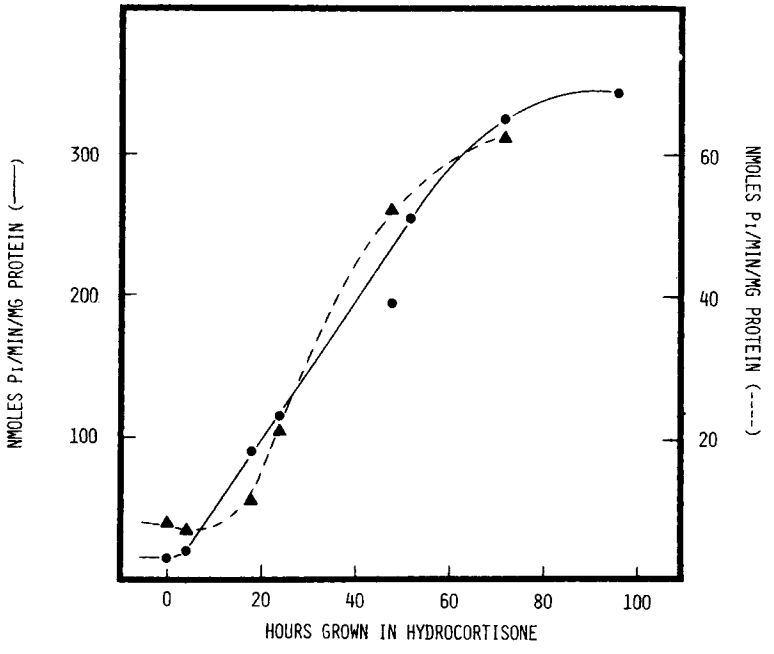


Fig. 4. Alkaline phosphatase induction by hydrocortisone in HeLa-65 and MGL-1. Cells in monolayer culture were grown in the presence of 3$\mu$M hydrocortisone for the times indicated. Alkaline phosphatase activity was determined as described in Methods. (———) MGL-1 cells. (- - - - - - -) HeLa-65 cells. Both cell types show similar patterns of induction; however, MGL-1 alkaline phosphatase activity is induced nearly 30-fold, whereas HeLa-65 activity is induced only 5–6-fold.

### Chromosome Analysis and MTS Formation

Karyotype analyses of the parental cell-lines confirmed a modal chromosome number of 65 for HeLa-65 and 63 for BOT-2, with BOT-2 having 2 minichromosomes [1]. All transferants had the same modal chromosome number as the recipient BOT-2 cells, 63. No major chromosome alterations were detected.

Multicellular tumor spheroid formation was monitored for all cell types. HeLa-65 did not form three-dimensional cellular aggregates; whereas, BOT-2 and all transferant cells presented the cellular aggregates characteristic of mammary tumor cells (Table I). In addition, the transferant cells retained the cell surface antigen specific to breast tumor cells as described for BOT-2 cells [2, 3].

## DISCUSSION

The transfer of genetic information, via metaphase chromosomes, between two human cell lines has led to the alteration in the recipient cells of two membrane-associated cellular activities. Activation of thymidine transport and regulation of alkaline phosphatase catalytic efficiency have been stabilized genetically in a human breast tumor cell line originally thymidine transport-deficient and alkaline phosphatase-constitutive. Both modifications reflect the normal phenotype of the parental donor, HeLa-65.

Recipient BOT-2 cells grow well under normal culture conditions; however, exposure to methotrexate, $4 \times 10^{-7}$ M, completely inhibits cell survival [7]. Addition of thymidine and hypoxanthine to make HAT medium does not increase survival. The reversion frequency for BOT-2 cells in HAT medium is less than $2 \times 10^{-9}$, and we have not yet been able to isolate a spontaneous revertant. Following treatment of BOT-2 cells with metaphase chromosomes in the presence of poly-L-ornithine or a mixture of lipids (see Methods), surviving cells appeared after 5 weeks of selection pressure with a frequency in the order of $1 \times 10^{-5}$. The majority of the transferants were unstable, as would be expected using these techniques [13, 14, 16]. Several clones were stable after alternate subculturing in selective and nonselective growth medium. Two of the stable transferants, MGP-1 and MGL-1, resembled the parental recipient, except for increased thymidine uptake by MGP-1 and very low levels of alkaline phosphatase activity in MGL-1. No differences in morphology, specific cell-surface tumor antigens, or multicellular tumor spheroid formation were detected. BOT-2 sensitivity to HAT medium could be accounted for by defective thymidine transport, and the resistance of MGP-1 to HAT could be accounted for by thymidine uptake and incorporation into DNA. The resistance of the MGL-1 to HAT apparently was due to another mechanism. MGL-1 cell extracts had a 3.5-fold increase in dihydrofolate reductase activity [5, 43] when measured in the presence of endogenous methotrexate. MGX-100 was unable to incorporate methotrexate (results not published).

The uptake of thymidine by eukaryotic cells in culture includes a permeation event. This event may be mediated by a specific carrier component in the cell membrane and/or by simple diffusion. Transport is followed by a phosphorylation event to a nucleoside monophosphate, with subsequent metabolism and incorporation into macromolecules [8, 44, 45]. Separation of these events in mammalian systems has been reported through the use of thymidine kinase deficient ($TK^-$) mutants [9, 11, 46, 47], rapid transport studies [8, 48–50], and ATP depletion to prevent phosphorylation [51]. Actual genetic separation of these events with thymidine transport-deficient ($TT^-$) cells having normal TK activity has been limited to a few studies, including those in Chinese hamster ovary cells [10] and haploid frog cells [11]. Whereas correction of $TK^-$ with

DNA [47], viral genome [47], and chromosome transfer [12] has become nearly routine, the reversion of $TT^-$ to $TT^+$ using genetic material, without affecting TK, has been demonstrated satisfactorily only in the haploid frog cell system [11, 12]. Our data now provide similar evidence for genetic regulation of nucleoside transport and its separation from phosphorylation in human-derived cells in culture. This is supported by results showing equivalent TK in vitro activities in all cells studied and by the in vivo TK activity and nucleotide incorporation into DNA in cells "force fed" thymidine by mild hypotonic treatment [53]. Our results support the current concept that permeation and phosphorylation are separate events in mammalian cells and that transport is not necessarily kinase mediated. Recent studies with nitrobenzylthioinosine probes also suggest transport to be the rate-limiting factor in nucleoside uptake [51, 54].

The specific carrier system for transport of purine and pyrimidine nucleosides across the plasma membrane may be either permanent or transient components of the membrane [44]. Transport capacity has been linked to both the cell cycle [44, 55] and malignant transformation [44, 56]. The nucleoside specificity of these carriers appears to be more complex and to a certain degree genus specific. There are systems that generally differentiate between purine and pyrimidine nucleosides and those that show specificities for individual nucleosides [48, 57, 59, 60]. HeLa cells are of the latter classification, having different carriers for thymidine than for uridine [58]. This has been corroborated for our donor HeLa-65 and recipient BOT-2 cells.

The lack of thymidine transport in BOT-2 may be due to a primary structural alteration of the thymidine carrier itself. It may be absent or inactivated through one or more defective functional modifications. It also could be due to a modification in a secondary membrane component that plays a role in localization and activation of the carrier. At this time we have no conclusive evidence for either hypothesis, although the specificity of the BOT-2 defect and its correction suggest a primary alteration to be more probable.

Alkaline phosphatase is found in most tissues and cells in cultures at various endogenous levels of activity and several isoenzyme forms [27, 61]. This can be readily demonstrated in the BOT-2 and HeLa-65 cells used for this study. BOT-2 cells in culture normally demonstrate relatively high activity, and HeLa-65 cells generally have quite low activity, with the activity ratio of BOT-2:HeLa-65 approaching 50. HeLa-65 alkaline phosphatase activity can be induced nearly 10-fold by addition of hydrocortisone [41, 63], 5-bromo-deoxyuridine [62], choline chloride [26], 5-iodouridine [64, 65], sodium butyrate [23], phospholipase $A_2$ [24], and Rosenthal's inhibitor [66]. Similar results have been obtained with other cells in culture such as skin fibroblasts [67], H.Ep-2 [62], mouse L-cells [28], human placental cells [29], aortic endothelial cells [68], and embryonic chick intestine [69]. In vivo, alkaline phosphatase activity has been used extensively in clinical procedures [27] and induced in rat liver by colchicine [70]. It appears initially that induction can occur with nearly any membrane perturbation. However, induction has been repeatedly linked to a requirement for protein synthesis [22, 25, 28, 70] and, possibly, DNA synthesis [63]. Induction of AP activity may occur by catalytic activation of preexisting AP polypeptides [25] or by de novo synthesis of more AP. A constitutive AP and a different inducible AP have also been suggested [71]. It is likely that both mechanisms exist.

Our experiments showing the stable transfer of AP repression in MGL-1, a decrease to nearly 2% of BOT-2 AP, and its subsequent 25-fold increase by hydrocortisone treatment suggest a specific genetic locus having positive expression that modulates AP activity. While nonspecific stimuli indirectly affect AP activity, it is now possible to ex-

amine induced cells for specific regulatory components and mechanisms for induction. In systems with large increases in stimulated AP activity such as MGL-1 and mouse L-cells [28] one may not have induction, but rather specific gene amplification [72].

Altered alkaline phosphatase expression was found in the stable transferants selected from HAT medium. MGP-1 had AP activity greater than either parental cell line and, like the parental donor, was $TT^+$. MGL-1 had AP activity similar to the parental donor but remained $TT^-$, a characteristic of the parental recipient. Since there is a HeLa variant, HeLa-75, with high AP activity, which is also $TT^+$ [22], it is difficult to postulate a linkage between the two genes. Based on the characteristics of only two transferants, it is also premature to link HAT resistance with some aspect of AP expression.

The different AP isoenzyme patterns for HeLa-65 and BOT-2 [1] enable us to determine whether MGL-1 and MGP-1 now contain, via gene transfer, either an additional HeLa-65 isoenzyme or a new or altered regulatory protein. Indeed, there may be a transition among the isoenzymes similar to that seen in human placenta during the first and third trimesters [29].

In summary, we have shown stable genetic transfer of information necessary to alter correctively a specific pyrimidine nucleoside carrier function in human cell plasma membranes. This function is genetically distinct from phosphorylation by thymidine kinase. We have also been able to transfer genetic information for regulation of alkaline phosphatase activity. The complex nature of the interaction these two functions have with cell membranes does not enable us, at this time, to specify the chemical nature of the modified behavior. At present, we do not believe there to be a necessary link between thymidine transport and alkaline phosphatase.

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