Simultaneous Up-regulation of Viral Receptor Expression and DNA Synthesis Is Required for Increasing Efficiency of Retroviral Hepatic Gene Transfer*

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To understand the relative contribution of viral receptor expression and cell proliferation in retroviral gene transfer, we created human hepatocyte-derived HuH-7.MCAT-1 cell lines. These cells constitutively express the murine ecotropic retroviral receptor MCAT-1 without changes in morphology or proliferation states. The MCAT-1 receptor is also a cationic amino acid transporter, and the HuH-7.MCAT-1.7 cells showed increased $V_{\text{max}}$ of uptake and steady-state accumulation of the cationic amino acids L-arginine and L-lysine. In HuH-7.MCAT-1 cells, L-arginine uptake was significantly up-regulated by norepinephrine and dexamethasone, and hepatocyte growth factor also increased L-arginine uptake along with cellular DNA synthesis. Gene transfer was also markedly increased in HuH-7.MCAT-1.7 cells incubated with an ecotropic LacZ retrovirus, and this further increased with hormones and hepatocyte growth factor. To define whether viral receptor up-regulation by itself increased gene transfer, cell cycling was inhibited by a recombinant adenovirus expressing the Mad transcription factor (AdMad), which is a dominant-negative c-Myc regulator. This restricted cells in $G_0/G_1$, without attenuating MCAT-1 activity, as shown by flow cytometry and L-arginine uptake analysis, respectively. When asynchronously cycling HuH-7.MCAT-1.7 cells were first infected with the AdMad virus and then exposed to the ecotropic LacZ virus, gene transfer was virtually abolished. The data indicate that while up-regulation of viral receptors can greatly enhance retrovirally mediated gene transfer, DNA synthesis remains an absolute requirement for hepatic gene therapy with this approach.

Retroviral gene transfer vectors are desirable because of their safety and integration into the host genome, which permits permanent expression of introduced genes. However, viral integrations in the host genome require cell proliferation and even ongoing mitosis (1). On the other hand, the frequency of cell proliferation-related events differs among cell types and may govern why cycling cells incorporate retroviruses far more avidly than quiescent cells (2). Additional factors determining retroviral gene transfer include the presence or absence of specific receptors that determine viral entry into cells (3, 4).

The specificity of retroviral infection is exhibited at two levels: species-specific and cell type-specific. The former is determined by differences in the viral envelope, and the latter by the presence of specific cell membrane receptors, which make cell types susceptible to infection with a given retrovirus. Alternative vectors, particularly adenovirus, which in contrast with retroviruses can be produced in extremely high titers, suffer from episomal deposition of introduced genes, leading eventually to gene losses, as well as from deleterious host immune responses preventing repeated virus administration (5).

Ecotropic retroviruses utilize the murine cationic amino acid transporter (MCAT-1)1 as their cellular receptor, which has recently been characterized (6–11). MCAT-1 possesses 622 residues and 14 transmembrane-spanning domains; transports the cationic amino acids arginine, lysine, and ornithine; and belongs to the so-called $y^+$ transporter system (see Refs. 11–13 for review). Transfection experiments showed that MCAT-1 expression makes nonpermissive cells susceptible to ecotropic retroviruses (4). Although the MCAT-1 mRNA is expressed under basal conditions in many adult organs, including the brain, intestine, stomach, bone marrow, and spleen, this is not so in the normal adult liver (4, 11). However, proliferating liver cells do express MCAT-1, as shown by studies in the newborn rat, partial hepatectomy-induced liver regeneration, and cultured primary hepatocytes undergoing DNA synthesis (4, 11). Indeed, a number of studies showed that retrovirally mediated gene transfer is increased under similar conditions (2, 10, 14, 15). Therefore, it has been unclear as to the individual contribution of viral receptor expression and DNA synthesis in retroviral gene transfer.

To develop a suitable system for dissociating retroviral receptor expression and cellular DNA synthesis, we created novel hepatic cell lines capable of constitutively expressing the MCAT-1 retroviral receptor. The task was facilitated by using the established HuH-7 cell line, which was originally derived from a human hepatocellular carcinoma (16). HuH-7 cells are resistant to ecotropic retroviruses because appropriate receptors are lacking. A number of cell clones were stably transfected with an MCAT-1 cDNA, and the model was verified for MCAT-1 expression by using L-arginine uptake as a reporter, including testing hormonal and growth factor regulation of MCAT-1 activity. To suppress cell cycling without interfering with MCAT-1 expression, we overexpressed the Mad transcription factor, which preferentially binds to Max and serves to antagonize c-Myc activity in cells (17). Use of these systems allowed us to investigate whether overexpression of viral receptors could lead to greater retroviral gene transfer, whether...
DNA synthesis was an absolute requirement for proviral integrations in the setting of receptor overexpression, and whether a combination of receptor overexpression and cellular DNA synthesis would be most effective in retroviral gene transfer.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Cell Culture, and Selection**—The pJET plasmid (18) was digested with BamHI and EcoRI to isolate MCAT-1 cDNA (2.279-kilobase pair fragment), which was subcloned into the pGem3zf (+) plasmid (Promega, Madison, WI) between the BamHI and EcoRI sites (pGEM3ZMCAT-1 plasmid). The MCAT-1 cDNA was then cut with XbaI and EcoRI and subcloned into the multilconing sites between the HindIII and EcoRI positions of the eukaryotic expression vector pcDNA3 containing the cytomegalovirus (CMV) promoter/enhancer and the 231-base-pair-long polyadenylation sequence from the bovine growth hormone gene (Invitrogen, San Diego, CA). In the final pcDNA3MCAT-1 plasmid, the MCAT-1 cDNA retained the original BamHI site of the pJET plasmid. This cloning strategy allowed us to use the CMV promoter and polyadenylation site of the pcDNA3 plasmid with no further manipulations. The pcDNA3MCAT-1 plasmid coexpressed the neo<sup>+</sup> gene under the control of the SV40 promoter/enhancer and allowed for cell selections. The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (Gemini Biochemicals Inc., Calabasas, CA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). The NIH 3T3 mouse fibroblasts were originally from the American Type Culture Collection (Rockville, MD). For hormonal induction experiments, 10<sup>−5</sup> to 10<sup>−2</sup> M dexamethasone, 5–20 μg/ml insulin, 10<sup>−4</sup> to 10<sup>−2</sup> M norepinephrine, or 20 ng/ml recombinant human hepatocyte growth factor (HGF) (Promega) were cultured both with and without arginine in the culture medium for 24 h before exposure for 2 h to arginine-free medium containing l-[3H]arginine for analyzing steady state intracellular accumulation (see below). In some experiments, primary rat hepatocytes isolated by previously described collagenase perfusion methods were also included (19). The viability of primary rat hepatocytes, tested by trypan blue dye exclusion, was 85–90%, and 2.5 × 10<sup>6</sup> cells/cm<sup>2</sup> were cultured in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics for 24 h on dishes coated with rat tail collagen. The NIH 3T3 mouse fibroblasts were cultured both with and without arginine in the culture medium for 24 h. For demonstrating steady-state accumulation of the reporters, one such clone (designated HuH-7.MCAT-1.7) was used for all subsequent experiments, and this was reconstituted by limiting dilutions to ensure cellular homogeneity.

**DNA Synthesis and Cell Proliferation**—[3H]Thymidine incorporation into trichloroacetic acid-precipitable DNA was measured as described previously (19). Cells were incubated with 3 μCi of [3H]thymidine/ml of medium containing 0.8 mM unlabeled arginine plus [3H]lysine in individual cultures was standardized to the protein content and fit by least triplicate and repeated several times.

**Transgene Analysis**—Total cellular RNA and genomic DNA from the parental HuH-7 and HuH-7.MCAT-1 cells were isolated by a single-step procedure as described (20). Before electrophoresis, DNA samples (10 μg each) were digested with BamHI endonucleases (Promega), electrophoresed on a 1% agarose gel, transblotted, and UV-cross-linked to Hybond-N<sup>+</sup> membranes (Amersham Pharmacia Biotech). Fifteen μg of RNA/sample was electrophoresed on 1.2% agarose gels containing 0.6 M formaldehyde, transblotted, and UV-cross-linked to Hybond-N<sup>+</sup> membranes. Equivalent RNA loading was analyzed by ethidium bromide staining. The blots were prehybridized for 8 h and hybridized for 16 h with a full-length MCAT-1 cDNA at 42 °C as per the manufacturer. The purified MCAT-1 cDNA insert was labeled with [α<sup>32</sup>P]dCTP by random primer extension to 2–5 × 10<sup>6</sup> cpm/μg of DNA using a commercial kit (Amersham Pharmacia Biotech) (21). The transblots were washed under stringent conditions with a final wash using 0.1× SSPE at 65 °C (1 × SSPE = 0.18 M sodium chloride, 0.01 M sodium phosphate (pH 7.7), and 0.001 M Na<sub>2</sub>EDTA). Autoradiography was performed at −70 °C with X-Omat AR film (Eastman Kodak Co.). Ribonuclease protection assays were performed with the HybSpeed<sup>™</sup> ribonuclease protection assay kit (Ambion Inc., Austin, TX). The [32P]UTP-labeled riboprobes were produced using a MAXiScript<sup>™</sup> in vitro transcription kit (Ambion, Inc.). A 720-base pair MCAT-1 riboprobe was synthesized by T7 RNA polymerase with linearized plasmid pGEM3ZMCAT-1 as the template. A 250-base pair internal control riboprobe was prepared with the SP6 RNA polymerase using a linearized pTRI mouse β-actin cDNA as the template. Fifteen μg of RNA/sample was mixed with 8 × 10<sup>6</sup> cpm of [32P]UTP-labeled riboprobe and hybridized at 68 °C for 1 h, the single-stranded unhybridized riboprobe molecules were then degraded with RNase A/T1 at 37 °C for 30 min. After precipitation, the samples were loaded on either denaturing 4% polyacrylamide gels or nylon membranes by slot blotting.

**Amino Acid Transport Assays**—The assay using Earle’s balanced salt solution was based on the method initially described by Gazzola et al. (22). l-[2,3,4,5-3H]Arginine (64 Ci/mmol), l-[1,2-14C]Proline (250 μCi/mmol) (Amersham Pharmacia Biotech), and l-[1,2-14C]lysine (4.7 Ci/mmol; Sigma) were obtained commercially. After culturing 1 × 10<sup>5</sup> cells in RPMI 1640 medium for 24 h in 24-well dishes, the medium was switched, and cells were incubated for 1 h in amino acid-free Dulbecco’s modified essential medium (Life Technologies, Inc.) containing 10% dialyzed fetal bovine serum. Cells were then washed with amino acid-free Earle’s balanced salt solution and incubated with Earle’s balanced salt solution containing various amounts of unlabeled arginine or proline and a constant amount of [3H]arginine, [3H]lysine, or [14C]proline (−1 × 10<sup>6</sup> cpm). Amino acid uptake was terminated by adding ice-cold PBS containing 0.1% bovine serum albumin, and cells were transferred immediately to ice. Cells were washed three times with ice-cold PBS/bovine serum albumin and extracted with 200 μl of 5% trichloroacetic acid, and activity in the soluble phase was counted in a liquid scintillation counter. The extent of nonspecifically associated [3H]arginine, [3H]lysine, or [14C]proline was estimated by incubating cells at 4 °C, and this value was subtracted as an uptake blank from each sample. The kinetic constants K<sub>m</sub> and V<sub>max</sub> were determined for the initial uptake of l-[3H]arginine, [3H]lysine, and [14C]proline in the presence of Na<sup>+</sup>. After depletion of amino acids for 1 h, the initial reaction velocity was estimated from a 60-s incubation over concentrations ranging between 0.02 and 1.00 mM. The protein concentration of each sample was measured by the Bio-Rad assay after dissolving 5% trichloroacetic acid-insoluble phase in 200 μl of 0.1 N sodium hydroxide (Bio-Rad). For demonstrating steady-state accumulation of the reporters, 1 × 10<sup>5</sup> cells were incubated in amino acid-free Dulbecco’s modified essential medium containing 10% dialyzed fetal bovine serum and medium of 0.8 mM unlabeled arginine plus [3H]lysine and incorporation was analyzed as described above. The amino acid uptake in individual cultures was standardized to the protein content and fit by least squares to the Michaelis-Menten equation. The least-square fitting analysis provided computer-derived K<sub>m</sub> and V<sub>max</sub> estimates ± S.E. of the estimate.

**Viruses**—Recombinant adenoviruses were grown in E1a-transformed 293 embryonic kidney cells; purified with two rounds of cesium chloride gradient ultracentrifugation; and dialyzed in 10 mM Tris-HCl (pH 7.4), 1 mM MgCl<sub>2</sub>, and 10% glycerol (23). The adenoviral titer was determined by measuring optical density at 260 nm. For infection, cells were incubated with medium containing adenoviruses at a multiplicity of infection (m.o.i.) of 20 for 2 h. AdMad virus, which expresses the Mad protein, was used to suppress cell cycling. The AdJagal virus, which expresses *Escherichia coli* LacZ, was used as a control to exclude non-specific changes due to adenoviral proteins, and this virus was provided by the Cell Culture and Genetic Engineering Core of the Liver Research Center at the Albert Einstein College of Medicine. An additional adenovirus control was generated by inserting antisense hepatitis B virus sequences, using the EcoRI/XbaI fragment from the pCP10 hepatitis B virus transferrin receptor plasmid, into the BamHI site of the pJET plasmid using the pMJ17 system, obtained with the permission of Dr. M. Imperiale (University of Michigan) and Dr. F. L. Graham (McMaster University), respectively, from Dr. M. Horwitz at the Albert Einstein College of Medicine. After digestion of the AdBglII plasmid with BglII, the antisense hepatitis B virus sequences previously ligated into an expression cassette containing the immediate-early CMV promoter and...
Although additional faint hybridization bands could have represented cross-hybridization with partially homologous endogenous sequences, these were not further characterized. RNA transblots showed that HuH-7.MCAT-1.7 cells contained mRNA transcripts that hybridized with a full-length MCAT-1 cDNA probe and corresponded to the 2.4-kilobase pair size of transfected MCAT-1 cDNA (Fig. 1B). In addition, $10^{-5}$ M dexamethasone up-regulated the steady-state abundance of this transgene transcript by severalfold. The RNase protection assay showed that HuH-7.MCAT-1.7 cells expressed unique mRNA transcripts hybridizing with the MCAT-1 riboprobe that were absent in parental HuH-7 cells (Fig. 1, C and D). In addition, the steady-state abundance of the MCAT-1 transgene transcript was up-regulated by several fold after exposure to $10^{-5}$ M dexamethasone. Interestingly, RNase protection showed that $\beta$-actin mRNAs were also up-regulated by dexamethasone in HuH-7.MCAT-1.7 cells, although the significance of this observation was unclear. Direct demonstration of the MCAT-1 gene product in the HuH-7.MCAT-1.7 cells was impossible due to the nonavailability of a reliable antibody for transblotting or immunoprecipitation, despite the use of an antibody for immunostaining (26).

**Effect of MCAT-1 Activity on Amino Acid Transport and Uptake**—The transport and intracellular retention of arginine were markedly increased in HuH-7.MCAT-1.7 cells. The initial velocity of $\L$-arginine uptake appeared to be greater in HuH-7.MCAT-1.7 and NIH 3T3 cells compared with HuH-7 cells (Fig. 2A). The relevant parameters of the transport kinetics obtained from three independent experiments are summarized in Table II. The $\L$-arginine uptake showed saturable kinetics in parental HuH-7 cells, with $V_{\text{max}} = 3.0 \pm 0.5$ nmol/mg of protein/min and $K_m = 257 \pm 16$ $\mu$M. In contrast, the $V_{\text{max}}$ of $\L$-arginine transport was significantly greater in HuH-7.MCAT-1.7 cells (mean, 160% greater), indicating higher capacity as well as higher affinity for arginine transport. In contrast, in the NIH 3T3 cells, which exhibit remarkable avidity for ecotropic retroviruses, the $\L$-arginine uptake was characterized by even greater affinity, but with a lower capacity (mean, 77% less), in comparison with the parental HuH-7 cells. Interestingly, when compared with NIH 3T3 cells, the $V_{\text{max}}$ and $K_m$ of arginine transport in the HuH-7.MCAT-1.7 cells were significantly different ($p < 0.03$ and $p < 0.002$, respectively), with the $V_{\text{max}}$ of $\L$-arginine transport was showing lower affinity and higher apparent capacity for $\L$-arginine transport. The $\L$-lysine transport analysis demonstrated a greater initial velocity of $\L$-lysine uptake in HuH-7.MCAT-1.7 cells (Fig. 2B). The mean $V_{\text{max}}$ (7.03 $\pm$ 0.52 nmol/mg of protein/min) for $\L$-lysine in these cells was significantly greater (161%) than that in parental HuH-7 cells ($V_{\text{max}} = 4.36 \pm 0.21$ nmol/mg of protein/min; $p < 0.01$), indicating again a higher affinity and capacity for another cationic amino acid. We found that arginine uptake could not be saturated in primary rat hepatocytes, confirming previous studies (12) and further contrasting with the arginine transport observed in HuH-7.MCAT-1.7 cells. On the other hand, the proline transport was similar in HuH-7 and HuH-7.MCAT-1.7 cells, with $V_{\text{max}} = 2.35 \pm 0.11$ versus $2.38 \pm 0.14$ nmol/mg of protein/min and $K_m = 0.138 \pm 0.10$ versus 0.124 $\pm$ 0.09 $\mu$M, respectively ($p = \text{not significant}$) (Fig. 2C).

MCAT-1 overexpression is known to increase the steady-state intracellular accumulation of $\L$-arginine as shown in *Xenopus* oocytes and mammalian cells (27), although the previous systems were dissimilar to ours. Utilizing retention of $\L$-arginine allowed us to conveniently examine the hormonal regulation of MCAT-1 activity. Our studies of steady-state arginine accumulation showed that $\L$-arginine retention in HuH-7.MCAT-1.7 cells was markedly increased compared with

### Table I

| HuH-7.MCAT-1 cell clones | $L^{\text{3H}}$Arginine uptake | p values (against HuH-7) |
|--------------------------|-------------------------------|--------------------------|
| HuH-7                    |                               |                          |
| HuH-7.MCAT-1.1           | 300 ± 31                     | 0.05                     |
| HuH-7.MCAT-1.2           | 1970 ± 332                   | <0.05                    |
| HuH-7.MCAT-1.3           | 2550 ± 529                   | <0.05                    |
| HuH-7.MCAT-1.4           | 2330 ± 499                   | <0.05                    |
| HuH-7.MCAT-1.5           | 2130 ± 501                   | <0.05                    |
| HuH-7.MCAT-1.6           | 1850 ± 321                   | <0.05                    |
| HuH-7.MCAT-1.7           | 1797 ± 299                   | <0.05                    |
| HuH-7.MCAT-1.8           | 2001 ± 211                   | <0.05                    |
| HuH-7.MCAT-1.9           | 2123 ± 438                   | <0.05                    |
| HuH-7.MCAT-1.10          | 1973 ± 328                   | <0.05                    |
| HuH-7.MCAT-1.11          | 2375 ± 483                   | <0.05                    |
| HuH-7.MCAT-1.12          | 2483 ± 634                   | <0.05                    |
| HuH-7.MCAT-1.13          | 893 ± 432                    | NS<sup>a</sup>           |
| HuH-7.MCAT-1.14          | 1475 ± 239                   | <0.05                    |

<sup>a</sup> NS, not significant.

* Development of System to Overexpress MCAT-1 Receptors in Human Cells

**MCAT-1 Transgene Expression**—A total of 14 HuH-7.MCAT-1.7 cell clones were analyzed 4 weeks after transfection with the plasmid pcDNA3MCAT-1. In all positive clones, steady-state $\L$-arginine accumulation was markedly increased compared with the untransfected parental cells (Table I). From among these cell clones, the HuH-7.MCAT-1.7 clone was randomly chosen for detailed studies because these cells expressed the introduced MCAT-1 cDNA with increased susceptibility to ecotropic retroviral infection, although not uniquely so. DNA transblot analysis showed that the HuH-7.MCAT-1.7 cells contained integrated MCAT-1 cDNA sequences that were absent in the parental HuH-7 cells (Fig. 1A).

**RESULTS**

**Development of System to Overexpress MCAT-1 Receptors in Human Cells**

Enhancer and the SV40 small T-antigen splice and SV40 polyadenylation sites were cloned. The antisense hepatitis B virus adenovirus was produced by Lipofectam™-mediated transfection of the modified pAdBGIII and pM plasmids into E.coli-transformed 293 embryonic kidney cells. The transfected 293 cells were cultured for 10–12 days until cytopathic effect became apparent, and cells were lysed by three freeze-thaw cycles. The recombinant adenovirus was grown and purified as described above.

Ecotropic and amphotropic retrovirus vectors expressing *E. coli* LacZ were used for demonstrating gene transfer. The “-CRE-nls-LacZ” vector was originally developed by Somatix Corp. (Los Angeles, CA) and provided by the Cell Culture and Genetic Engineering Core of the Marion Bessin Liver Research Center. The producer cells were maintained in $\alpha$-minimal essential medium (Life Technologies, Inc.) supplemented with 10% calf serum and antibiotics. The culture supernatant was harvested after overnight incubation of cells with fresh medium and passed through a 0.45-µm filter to remove debris. Target cells were infected with retroviruses in serum-free Dulbecco’s modified essential medium containing 8 µg/ml Polybrene for 2 h and cultured for an additional 72 h before fixation in 0.5% glutaraldehyde in PBS for 10 min. After washing with PBS, fixed cells were incubated overnight with 1 mg/ml 5-bromo-4-chloro-3-indolyl-$\beta$-galactopyranoside in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride. For the viral m.o.i., a constant number of transduced cells was counted in random areas. For some experiments, retroviruses were concentrated by precipitation with 5 M calcium chloride as described (25).

**Statistical Methods**—Data are expressed as means ± S.E. Data were analyzed by SigmaStat software (Jandel Scientific, San Rafael, CA). The significance of differences was tested where appropriate by Student’s t test. All experiments were carried out in triplicate at least; p values were calculated with Student’s t test.
MCAT-1 Expression Does Not Alter Cell Proliferation

When HuH-7.MCAT-1.7 cells were cultured for 24 h in arginine-deficient RPMI 1640 medium containing $10^{-6}$ or $10^{-5}$ M dexamethasone, steady-state accumulation of L-arginine significantly increased by up to 2-fold (Table III). Interestingly, $10^{-3}$ M norepinephrine also increased L-arginine accumulation by ~1.4-fold in HuH-7.MCAT-1.7 cells, whereas insulin alone did not change steady-state arginine accumulation. Finally, in response to dexamethasone and HGF, the steady-state accumulation of L-arginine was maximally increased by up to ~3-fold. These results indicated that the MCAT-1 activity in HuH-7.MCAT-1.7 cells could be modulated by hormonal stimulation because the CMV promoter used for driving transgene expression is up-regulated by dexamethasone (28).

States—The doubling times of HuH-7.MCAT-1.7 cells were similar to those of HuH-7 cells, 28 ± 2 versus 30 ± 3 h, respectively ($p < 0.001$). In contrast, the doubling times of both these cell lines significantly differed from those of NIH 3T3 fibroblasts, which doubled in 18 ± 3 h ($p < 0.001$). When [3H]thymidine incorporation was measured as a marker
of DNA synthesis at 24 and 72 h after plating 2 × 10^4 HuH-7 or HuH-7-MCAT-1.7 cells, again no significant differences were observed (Fig. 4). Finally, flow cytometry of propidium iodide-stained HuH-7 and HuH-7-MCAT-1.7 cells showed similar cell cycle profiles, confirming that MCAT-1 overexpression did not alter the proliferative status of the cell clone (data not shown).

Overexpression of the Heterologous Retroviral Receptor and Hepatic Gene Transfer

The transduction rates were dependent upon virus concentration. The ecotropic LacZ retrovirus transduced 21 ± 3, 93 ± 2, and 98 ± 1% NIH 3T3 cells at m.o.i. values of 0.2, 2, and 25, respectively. In contrast, HuH-7-MCAT-1.7 cells showed gene expression in 5 ± 1, 18 ± 2, and 65 ± 2% cells at corresponding m.o.i. values of 0.2, 2, and 25, respectively (p < 0.02; analysis of variance). The number of blue-stained cells in parental HuH-7 cells was essentially unaffected, with at most 1.0 ± 0.7% cells at the highest m.o.i. of 25. Further testing with single HuH-7-MCAT-1.7 cell clones (n = 25) derived by dilutional cloning showed susceptibility to the ecotropic retrovirus vector in all cell clones, with 7–35% cells staining blue at a m.o.i. of 2. In contrast, the parental HuH-7 cells showed LacZ expression with a m.o.i. of 2 in only 0.7 ± 0.8% cells and, despite the maximal m.o.i. of the virus used, in <1.5% cells. In contrast with these results, infection with the amphotropic LacZ virus was similar in both HuH-7 and HuH-7-MCAT-1.7 cells (55 ± 8 versus 57 ± 7% blue-stained cells, respectively; p = not significant).

Up-regulation of Viral Receptor Expression and Gene Transfer—When HuH-7-MCAT-1.7 cells were infected along with hormone treatments, gene transfer improved significantly (Fig. 5). Exposure of cells to either 10^{-6} M dexamethasone alone or with 20 ng/ml HGF increased ecotropic retroviral transfer in HuH-7-MCAT-1.7 cells by up to 2- and 4-fold, respectively (p < 0.001). In contrast, exposure of HuH-7-MCAT-1.7 cells to the ecotropic virus in the presence of insulin alone, norepinephrine alone, or dexamethasone at concentrations <10^{-6} M did not have any effect upon gene transfer efficiency. Exposure of either HuH-7 cells or NIH 3T3 cells to the hormones had no effect upon retroviral gene transfer. On the other hand, NIH 3T3 cells became unhealthy and started to detach from tissue culture dishes in the presence of dexamethasone. The differential response of fibroblast- and hepatocyte-derived cells to hormonal stimulation was not surprising because unlike hepatocytes, dexamethasone might have had a negative effect upon gene expression or altered differentiation states in fibroblasts as suggested by previous studies (29, 30). Assays of [3H]thymidine incorporation into DNA indicated that after exposure to 20 ng/ml HGF for 24 h, DNA synthesis in HuH-7 and HuH-7-MCAT-1.7 cells increased by −2-fold (p < 0.05).

Abolition of Cell Cycling Interferes with Retroviral Gene Transfer—The AdMad virus allowed us to dissect the roles of receptor overexpression and cell proliferation in retroviral gene transfer. When HuH-7 cells were incubated with AdMad, there was increased expression of the Mad protein as demonstrated by immunoblotting (data not shown). Studies with the Adgal virus showed that virtually 100% of the HuH-7 or HuH-7-MCAT-1.7 cells could be infected by the adenovirus. Upon exposure of log phase growth HuH-7 cells to the AdMad virus, the cell number increased only by 28 ± 6% compared with 242 ± 47% in untreated control HuH-7 cells (p < 0.001). Flow
cytometry corroborated these findings and demonstrated accumulation of AdMad-treated cells primarily in G0/G1 (Fig. 6). However, despite exposure of the HuH-7.MCAT-1.7 cells to AdMad, the MCAT-1 activity was unchanged, as shown by accumulation of [3H]arginine in response to stimulation by 10^{-5} M dexamethasone (6312 ± 430 versus 6188 ± 168 dpm/μg of protein/2 h; p = not significant). Finally, when asynchronously cycling HuH-7.MCAT-1.7 cells were first infected with the AdMad virus and 72 h later infected with the ecotropic LacZ virus, gene transfer was virtually abolished (32 ± 3 versus 1 ± 0.6% blue-stained cells in control and AdMad-treated cells, respectively; p < 0.001). In contrast, infection of the HuH-7.MCAT-1.7 cells with the adenovirus containing antisense hepatitis B virus sequences had no effect upon ecotropic retroviral infection. Similarly, infection with an amphotropic LacZ retrovirus of HuH-7.MCAT-1.7 cells previously exposed to AdMad resulted in no gene transfer.

**DISCUSSION**

Our findings are in agreement with retroviral receptor overexpression alone without DNA synthesis being insufficient for improving retroviral gene transfer. The studies showed that retroviral gene transfer was virtually abolished when either of these processes was abrogated. We demonstrated that resistance of human cells to ecotropic retroviruses was overcome by MCAT-1 overexpression, which is in agreement with results obtained in mink fibroblast and Chinese hamster ovary cells that are also resistant to ecotropic retroviruses in their native states (4).

Although our studies were not directed at detailed analysis of the cationic amino acid transport mechanism, we nonetheless found that MCAT-1 overexpression increased L-arginine and L-lysine transport in a high affinity fashion in HuH-7.MCAT-1.7 cells. In contrast, the parental HuH-7 cells exhibited a lower affinity for L-arginine and L-lysine transport, which probably indicates the presence of alternative transporters. Additional MCAT transporters, designated MCAT-2 and MCAT-2A, have been identified (31, 32), but no viruses utilizing these transporters as their cellular receptors have yet been recognized. The MCAT-2A gene product, which shares substrate specificity with MCAT-1, but exhibits much higher capacity for cationic amino acids in hepatocytes (~10-fold greater) (32), could be one such candidate. In fact, the total L-arginine transport in our HuH-7.MCAT-1.7 cells most likely represents the sum effect of MCAT-1 plus other transporters. Therefore, by using the L-arginine transport alone, MCAT-1 activity cannot possibly be directly compared between the HuH-7.MCAT-1.7 and NIH 3T3 cells. However, increased L-arginine transport did serve as a useful surrogate reporter for demonstrating MCAT-1 activity in our cells, although the efficiency of ecotropic retroviral infection was the best measure of receptor activity. Hormonal treatment increased retroviral infection in HuH-7.MCAT-1.7 cells, which was most likely due to up-regulated transgene expression since the CMV promoter is regulated by dexamethasone, although hormones may also up-
regulate endogenous receptor activity (11, 29). Judging from l-arginine transport, however, it would appear that the endogenous MCAT-1 receptor in NIH 3T3 cells was far more efficient in its retroviral receptor function than the MCAT-1 in HuH-7.MCAT-1.7 cells. Whether this was directly related to quantitative differences in MCAT-1 expression or qualitative differences, e.g. the presence or absence of unidentified regulatory subunits that improve receptor binding to specific viral domains, is unknown. Overexpression of other retroviral receptors showing low base-line organ expression, e.g. the amphotropic gibbon ape leukemia virus receptor, which shares a unique membrane-spanning domain determining retroviral infection with MCAT-1, can render murine cells susceptible to the appropriate virus (34–36). Analysis of the Moloney murine leukemia virus indicates that 246 amino-terminal gp70 residues contain the MCAT-1-binding domain and that the variable region designated VRA regulates direct interactions with the receptor and thus the host range (37–39), whereas resistance of Chinese hamster ovary cells to ecotropic Moloney murine leukemia virus is due to glycosylation of the hamster chloramphenicol acetyltransferase, which may transiently be overcome by the N-glycosylation inhibitor tunicamycin (40). On the other hand, in cells infected by retroviruses, the turnover of MCAT-1 itself is unchanged (41), and our results indicate that ecotropic retroviruses require specific receptor domains that may not be substituted by alternative cellular cationic amino acid transporters, which is consistent with the resistance of both HepG2 and HuH-7 cells to infection with ecotropic retroviruses.

It is remarkable, from a teleological point of view, that for entry into cells, retroviruses exploit an ubiquitous transporter, which is up-regulated during cell proliferation. This two-headed approach is most efficient because of the obvious advantages for viral entry and proviral integrations. Although rapidly cycling cells, such as progenitor or stem cells, could potentially be infected with retroviral vectors (42), this is not the case with adult hepatocytes, which are proliferatively quiescent and restricted mostly to G0/G1, thus limiting retroviral receptor expression as well as proviral integrations. In view of the attractiveness of liver-directed gene therapy for metabolic disorders, strategies to improve hepatic gene transfer include ways to increase retroviral titer, which is currently limited to $1 \times 10^{10}$ to $10^{12}$ virion particles/ml. However, the dose-dependent increases in retroviral gene transfer in cells overexpressing viral receptors in our studies support such a strategy and need for further work in this area. We believe that additional strategies based upon overexpression of viral receptors alone will also be successful, as shown by increased retroviral gene transfer upon hormonal up-regulation of MCAT-1 activity in our studies. Clearly, however, retroviral gene transfer will be most efficient when viral receptors are overexpressed in the setting of ongoing cell proliferation. While overexpression of retroviral receptors in the presence of increasing viral titer but constant proliferative activity was effective, abolition of cell cycling by exposure of cells to the Mad transcription factor prevented proviral integrations and gene transfer.

The translational implications of these findings are that if retroviral receptors were iatrogenically overexpressed in cells capable of high grade proliferative activity, such as stem cells, gene transfer would be very efficient, with permanent gene expression in daughter cells. In addition, overexpression of heterologous viral receptors in tissues, such as the liver, could offer one way to target retroviral vectors in a tissue-specific manner. In view of their broad activities, amphotropic or xenotropic retroviral receptors will be less suited for such a task in humans, whereas the MCAT-1 receptor might well be a candidate (43). This could potentially be accomplished with a dual vector strategy, e.g. by first transiently expressing the MCAT-1 retroviral receptor with efficient adenoviral, herpes simplex virus-1, or other vectors, followed by exposure to the retrovirus containing a therapeutic gene. The potential of dual vector approaches for hepatic gene transfer has begun to be addressed (15). However, the strategy to overexpress retroviral receptors by itself will be ineffective in proliferatively quiescent hepatocytes, which will also require a mitogenic stimulus for inducing cell proliferation and proviral integrations. Nonetheless, the first arm of the strategy should be quite successful because hepatocytes contain abundant receptors for transduction with adenoviral or herpes simplex virus-1 vectors, and the second arm would be facilitated by recent insights into liver growth control by exogenously administered growth factors, such as HGF, transforming growth factor-α, epidermal growth factor, and others (5, 44, 45). Use of an ecotropic retrovirus for gene transfer via such a dual vector strategy should be specially safe for laboratory personnel and care givers. However, the recipient could potentially be susceptible to murine retroviruses, although such an exposure would be temporary if the introduced MCAT-1 cDNA were to be localized episomally and survive transiently, as would be expected with adenoviral and physical gene vectors.

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