ELSEVIER

Biophysical Chemistry 97 (2002) 159–172

Biophysical Chemistry

www.elsevier.com/locate/bpc

Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes

Howard E. Davis[a,b], Jeffrey R. Morgan[a], Martin L. Yarmush[a,*]

[a]*Center for Engineering in Medicine/Surgical Services, Massachusetts General Hospital, Harvard Medical School and the Shriners Hospital for Children, Boston, MA 02114, USA*
[b]*Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA 02139, USA*

Received 26 December 2001; received in revised form 11 March 2002; accepted 11 March 2002

Abstract

Cationic polymers, such as polybrene and protamine sulfate, are typically used to increase the efficiency of retrovirus-mediated gene transfer, however, the mechanism of their enhancement of transduction has remained unclear. As retrovirus transduction is fundamentally limited by the slow diffusion of virus to the target cell surface, we investigated the ability of polybrene to modulate this initial transport step. We compared the ability of both envelope (gp70) and capsid (p30) protein based assays to quantitate virus adsorption and found that p30 based assays were more reliable due to their ability to distinguish virus binding from free gp70 binding. Using the p30 based assay, we established that polybrene concentrations, which yielded 10-fold increases in transduction also, yielded a significant increase in virus adsorption rates on murine fibroblasts. Surprisingly, this enhancement, and adsorption in general, were receptor and envelope independent, as adsorption occurred equivalently on receptor positive and negative Chinese hamster ovary cells, as well as with envelope positive and negative virus particles. These findings suggest that the currently accepted physical model for early steps in retrovirus transduction may need to be reformulated to accommodate an initial adsorption step whose driving force does not include the retrovirus concentration, and the reclassification of currently designated 'receptor' molecules as fusion triggers. The implication of these findings with respect to the development of targeted retrovirus-mediated gene therapy protocols is discussed.

Keywords: Recombinant retroviruses; Gene therapy; Retrovirus adsorption; Polybrene

1. Introduction

Recombinant retroviruses are a widely used vector in human gene therapy clinical trials, yet despite advances in both the productivity of viral packaging cell lines, transduction efficiencies have remained unacceptably low for many applications [1–4]. While the presence of endogenous inhibitors of transduction in virus stocks explains some of the difficulties encountered [5,6], there exist

*Corresponding author. Tel.: +1-617-371-4882; fax: +1-617-371-4950.
E-mail address: ireis@sbi.org (M.L. Yarmush).

0301-4622/02/$ - see front matter
PII: S0301-4622(02)00057-1

basic biophysical constraints which limit the efficiency of retroviral gene transfer. Several groups have demonstrated that the slow diffusion and rapid inactivation of retroviruses are major contributors to the low observed efficiencies [7–10]. With virus particles able to diffuse only a few microns before losing bioactivity, a large proportion of the initially active virus particles are inactive before they can interact with a target cell, fundamentally limiting achievable levels of transduction efficiency.

To circumvent these constraints, mechanical approaches, including centrifugation [8,11,12] and flow-through transduction [13], have been developed to increase the likelihood and frequency of active virus-cell interactions by adding a convective component to the mass transport of virus. While these strategies do yield enhanced transduction efficiencies, their expense and difficult scale-up limit their usefulness for large-scale experiments or in vivo clinical work. The classical approach to enhancing retrovirus transduction efficiency has been to add cationic polymers during the transduction process [14–16], however, the precise mechanism of their enhancement has remained unclear. As the cell and virus lipid membrane both possess a net-negative charge, it has been suggested that cationic polymers act by counteracting repulsive electrostatic effects, thereby enhancing adsorption [14,17]. To date, however, there have been no reports demonstrating cationic polymer effects on virus binding with either the FACS-based immunofluorescence [18,19] or any other virus binding assay [20–22].

In this report, we describe a new retrovirus-binding assay based upon quantitation of the amount of adsorbed capsid (p30) protein in cell lysates. Using this assay, we have been able to quantify retrovirus adsorption to target cells under conditions identical to those used in traditional transduction experiments. In initial experiments, we showed that p30 is the more reliable marker of virus adsorption as its detection is not confounded by the high levels of free gp70 we found to be present in retrovirus vector stocks. We used the p30 assay to study the mechanism of polybrene's enhancement and found that polybrene increased the relative rate of virus adsorption several-fold, and, surprisingly, that this enhanced adsorption could be demonstrated in the absence of both the virus envelope and cellular receptor for the virus. Based upon our findings, we propose a new model of retrovirus adsorption, which is diffusion-limited, receptor and tropism independent, and modulable by positively charged compounds. The implications of our findings for the development of targeted retrovirus-mediated gene therapy protocols is discussed.

2. Experimental

2.1. Chemicals

Nonidet P-40 and 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (Polybrene) were purchased from Sigma Chemical Co., St. Louis, MO. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), Pefabloc SC, aprotinin and o-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from Roche Biochemicals (West Germany).

2.2. Cell culture

NIH 3T3 cells and virus-producing cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD) with 10% bovine calf serum (HyClone Labs Inc., Logan, UT) containing 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco BRL). Phoenix-gp cells, which package envelope (-) 'bald' virus particles, were cultured in DMEM with 10% heat inactivated fetal bovine serum containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (Gibco BRL). Virus containing medium from the ecotropic packaging cell line CRE-BAG (ATCC CRL 1858) and the Phoenix-gp cell line (ATCC SD 3514) were harvested from confluent cultures, filtered through 0.45 μm syringe filters (Gelman Sciences, Ann Arbor, MI), frozen on pulverized dry ice and stored at −85 °C. CHO-K1 cells (ATCC CCL-61) were cultured in Ham's F12 medium (ATCC, Rockville, MD) supplemented with 10% fetal bovine serum containing 100 units/ml penicillin and 100 μg/ml streptomycin. Stable CHO-based cell lines express-

ing the ecotropic (MCAT-1) or the amphotropic receptor (rPIT-2) virus receptor were established by transfection of expression vectors encoding the appropriate cDNAs (gift from R. Cunningham). These cell lines were maintained in complete F12 supplemented with 500 μg/ml G418 (Gibco BRL) for selection. Expression of the receptors was verified via binding of free ecotropic or amphotropic gp70 to the relevant cell line.

2.3. Transduction assay

A microplate assay was used to measure virus infectivity [23]. The day before infection, a 10-cm dish of confluent 3T3 cells was treated with trypsin, and the cells were counted with a Coulter counter model ZM (Coulter Electronics, Hialeah, FL). Five thousand cells in 100 μl of medium were plated per well in a 96-well flat-bottomed tissue culture dish with a low-evaporation lid (Costar Corp., Cambridge, MA). The next day (19–25 h later), the medium was removed, and dilutions of virus in culture medium with Polybrene (8 μg/ml) were added to each well (final volume, 100 μl per well). Two days after the infection, the culture medium was removed, and the cells were washed once with 100 μl of phosphate-buffered saline (PBS) with 1 mM $MgCl_2$. After removal of the wash solution, 50 μl of lysis buffer was added (PBS with 1 mM $MgCl_2$ and 0.5% Nonidet P-40) to each well, and the plate was incubated at 37 °C. After 30 min, 50 μl of lysis buffer with 6 mM ONPG warmed to 37 °C was added to each well, and the plate was incubated at 37 °C for another 15 min. The reactions were halted by the addition of 20 μl of stop buffer (1 M Na_2CO_3). The plate was brought to room temperature, and the optical density at 420 nm (OD_{420}) was measured using an absorbance plate reader (Molecular Devices, Menlo Park, CA); non-specific background at 650 nm was subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the averages of at least triplicate wells.

2.4. Virus adsorption assay

Adsorption of amphotropic or ecotropic virus to cells was analyzed by ELISA. NIH-3T3 or CHO cells were plated in 6-well dishes (1.5×10^5 per well). The cells were incubated at 37 °C for 72 h, yielding a confluent monolayer. The medium was removed and replaced with 1 ml of virus stock with or without 8 μg/ml of polybrene. For dose response experiments, the virus was serially diluted in fresh medium and incubation of the cells with virus was carried out for 2 h. For time course experiments, undiluted virus was used. Following incubation, the virus supernatant was diluted 1:1 in cell lysis buffer and frozen at −80 °C for analysis of unbound p30 and gp70 via ELISA. The cells were washed once with fresh medium and incubated in 0.5 ml of cell lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 2 mM Pefabloc SC, 1 μg/ml aprotinin, 0.02% sodium azide) for 20 min on ice. Cell debris was removed by centrifugation at $12\,000\times g$ for 2 min at 4 °C, and the resulting supernatant was stored at −80 °C until analysis for levels of p30 and gp70 by ELISA. Relative virus adsorption rates were extracted from the time courses by linear regression analysis performed on early time points ($t<1.5$ h), before p30 or gp70 degradation is significant.

2.5. ELISAs for p30 and gp70

Mouse anti-p30 and anti-gp70 antibodies were purified from supernatants harvested from the CRL-1912 (ATCC, Rockville, MD) and 83A25 [24] hybridoma cell lines, respectively, following standard protocols [25]. ELISAs for p30 and gp70 were performed as described by Forestell et al. [26] The wells of a 96-well ELISA plate (Fisher Scientific, Agawan, MA) were coated with the anti-p30 or anti-gp70 capture antibody by overnight incubation at 4 °C with 100 μl of a 10 μg/ml solution of the antibody in PBS. The next day, non-specific binding sites were blocked by incubating the plate for 30 min at 37 °C with 200 μl of BLOTTO® Blocker in TBS (Pierce, Rockford, IL). Samples of clarified cell lysates or supernatant were added to the ELISA plate (100 μl per well) and incubated for 1 h at 37 °C. Bound p30 and gp70 were sandwiched by addition of secondary goat polyclonal antibodies (78S221 and 79S834, respectively: Quality Biotech, Camden, NJ) diluted

1:300 and 1:1000, respectively, in BLOTTO® and incubated for 1 h at 37 °C. A horseradish peroxidase conjugated rabbit anti-goat immunoglobulin G polyclonal antibody (Zymed Laboratories, South San Francisco, CA) diluted 1:5000 in BLOTTO® was added to the ELISA plate (100 μl per well) and incubated for 1 h at 37 °C to enable detection and quantitation of the sandwiched antigens. A stock of retrovirus diluted 1:1 into cell lysis buffer was used as a reference standard to normalize for plate to plate variations in the ELISA results. Serial dilutions of this reference stock produced a linear dose response curve with an $r^2 = 0.99$ up to an O.D. of 1.5 and 1.2 for p30 and gp70, respectively.

3. Results

3.1. Polybrene enhances virus binding as measured by p30 but not gp70

To measure the adsorption of virus on target cells, we used ELISAs to detect bound gp70 or p30 and compared the results for both assays. NIH 3T3 cells were exposed to serially diluted ecotropic virus for 2 h with or without 8 μg/ml polybrene. The medium was removed, the cells were lysed, and the level of cell-associated gp70 and p30 was measured by ELISA. In the presence of polybrene, levels of both gp70 and p30 increased linearly with the concentration of retrovirus (Fig. 1a,c, respectively). A time course of virus adsorption using the same samples showed that bound gp70 and p30 levels increased initially, but reached a maximum within 2 h (Fig. 1b,d, respectively). When binding of virus samples with and without polybrene was compared, levels of gp70 again increased linearly with the concentration of retrovirus, but there was no statistically significant difference between samples with or without polybrene (Fig. 1e). When we measured virus adsorption by monitoring levels of p30 protein, however, samples containing polybrene exhibited approximately 3 times the level of adsorption as compared to those without (Fig. 1f). Polybrene's enhancement of p30 binding is in line with its enhancement of transduction, however, the lack of an effect on gp70 was somewhat surprising.

3.2. Retrovirus binding is independent of the viral envelope–cellular receptor interaction

One explanation for polybrene's differential effect on gp70 vs. p30 adsorption could be the presence in virus stocks of free gp70 not associated with virus particles. To test this, we exposed serially diluted ecotropic virus with and without polybrene to CHO cells, which had been transfected with vectors expressing the ecotropic receptor, the amphotropic receptor, or no receptor. After 2 h, the virus was removed, the cells lysed, and the lysates were analyzed for the presence of p30 and gp70 by ELISA. When the ecotropic receptor was present on the cell surface (Fig. 2a), levels of adsorbed gp70 were 10-fold higher than adsorption on cells with the amphotropic (Fig. 2b) or no receptor (Fig. 2c). The difference between gp70 adsorption levels with and without polybrene, however, was at most 10%. When virus binding to these same cell lines was assayed by p30, however, the presence of polybrene resulted in at least a 10-fold increase in the level of adsorbed p30, with adsorption occurring equivalently whether or not an appropriate virus receptor was present (Fig. 2d–f). As it was clear that the presence of free gp70 made it impossible to characterize virus-binding behavior with a gp70-based assay, the p30 assay was used to characterize virus adsorption in the remainder of our experiments.

The receptor–independence of the virus adsorption process observed in Fig. 2d–f suggests that a receptor-envelope interaction is not necessary for initial virus attachment. To investigate whether polybrene's effect requires a specific interaction between polybrene and the virus envelope, CHO cells were exposed to serially diluted, envelope negative, 'bald' virus particles with and without polybrene. After 2 h, the medium was removed, the cells were lysed and the lysates were analyzed for the presence of p30 by ELISA. We found that virus bound to the cells even in the absence of the envelope protein, and that polybrene enhanced that binding (Fig. 3).

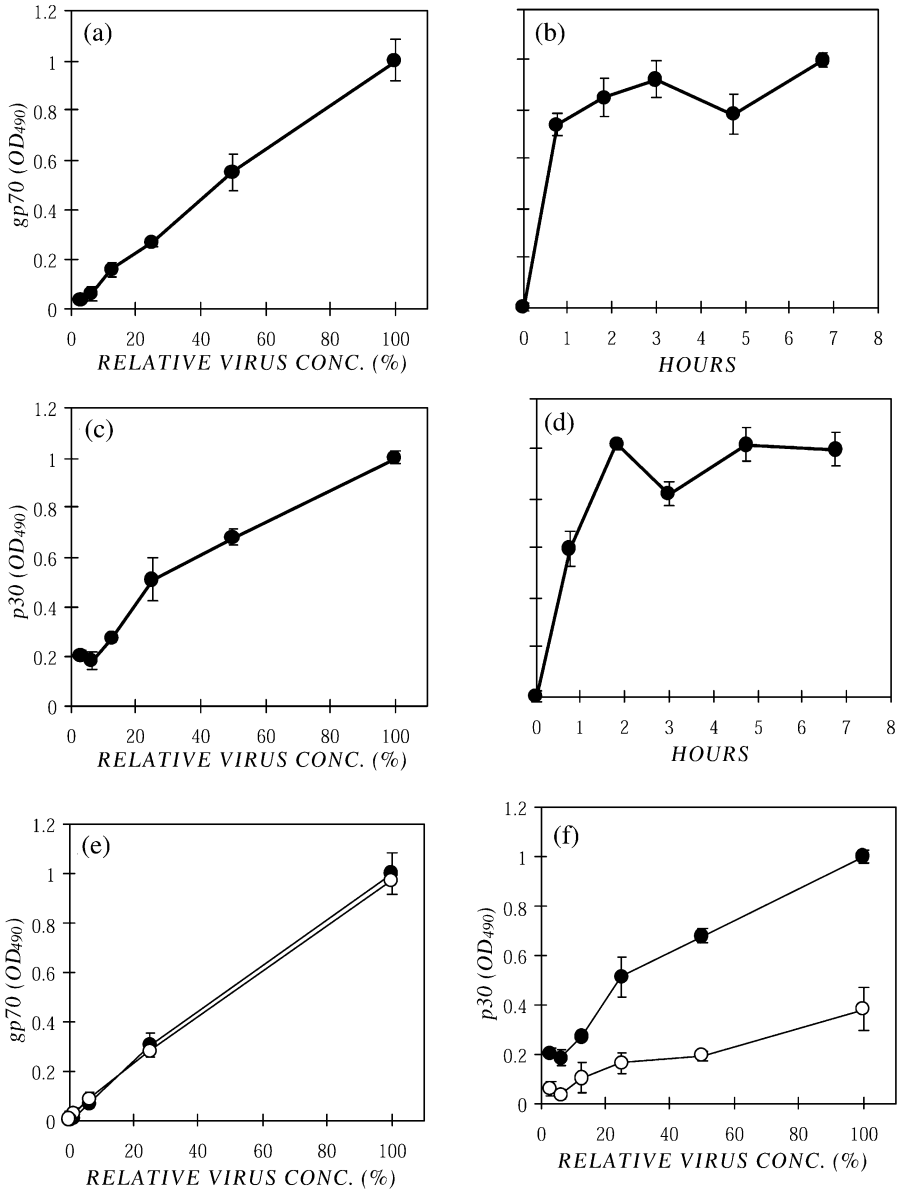

Fig. 1. p30 and gp70 binding is dose-dependent and plateaus after 2 h of adsorption, however, polybrene differentially affects their adsorption. (a–d) NIH 3T3 cells (1.5×10^5 per well) were plated in a 6-well dish. After 72 h, the medium was replaced with varying dilutions (a,c) or undiluted (b,d) ecotropic virus, containing 8 μg/ml polybrene. After 2 h (a,c), or at regular intervals (b,d), virus was removed, the cells lysed, and the lysates were analyzed for the presence of gp70 (a,b) or p30 (c,d) by ELISA. (e–f) NIH 3T3 cells (1.5×10^5 per well) were plated in a 6-well dish. After 72 h, the medium was replaced with varying dilutions of ecotropic virus containing 8 μg/ml (closed circles) or 0 μg/ml (open circles) polybrene. After 2 h, virus was removed, the cells lysed, and the lysates were analyzed for the presence of gp70 (e) and p30 (f) by ELISA.

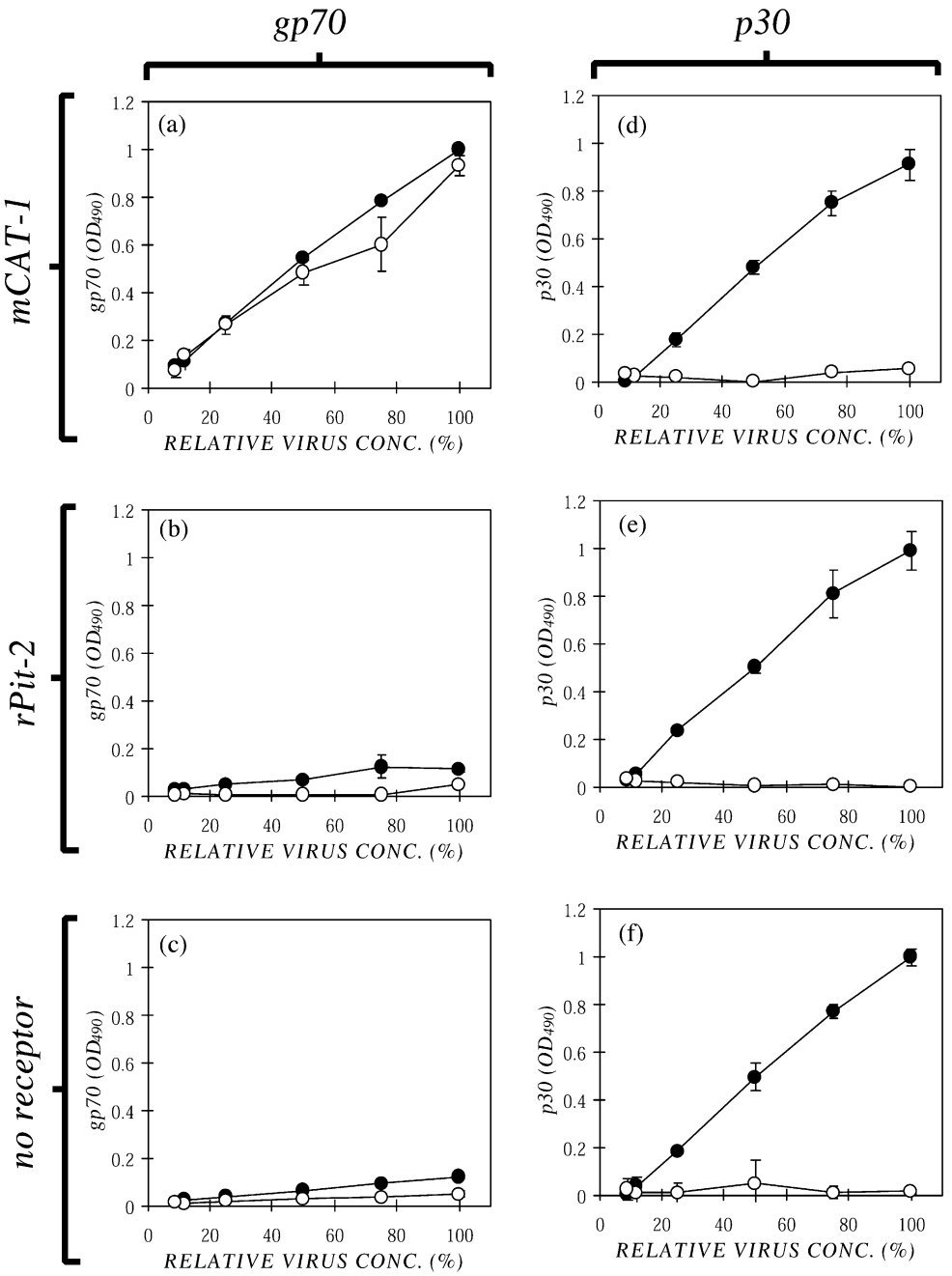

Fig. 2. gp70-based detection of virus binding is complicated by the presence of free gp70, whereas p30-based detection is not and demonstrates receptor independent virus binding. CHO cells which had been stably transfected with an expression vector encoding for the ecotropic receptor (a,d), the amphotropic receptor (b,e) or no receptor (c,f) were plated (1.5×10^5 per well) in a 6-well dish. After 72 h, the medium was replaced with varying dilutions of ecotropic virus containing 8 μg/ml (closed circles) or 0 μg/ml (open circles) polybrene. After 2 h, virus was removed, the cells lysed, and the lysates were analyzed for the presence of gp70 (a–c) or p30 (d–f) by ELISA.

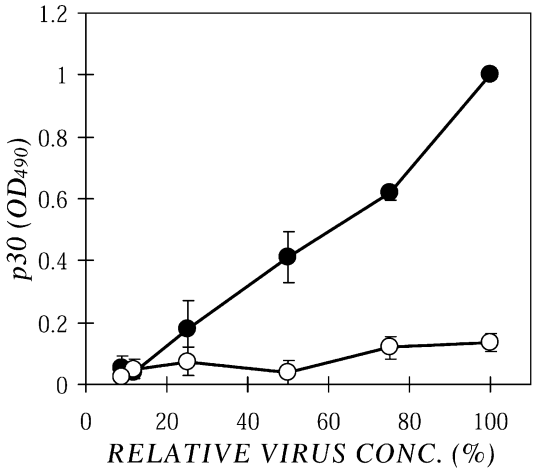

Fig. 3. Polybrene's enhancement of virus adsorption is not envelope dependent. CHO cells were plated in a 6-well dish (1.5×10^5 per well). After 72 h, the medium was replaced with varying dilutions of envelope (-) 'bald' virus containing 8 μg/ml (closed circles) or 0 μg/ml (open circles) polybrene. After 2 h, the virus was removed, the cells lysed, and the lysates were analyzed for the presence of p30 by ELISA.

3.3. Retrovirus binding is irreversible, diffusion limited, and non-saturable

The current physical model of retrovirus binding holds that virus adsorption is a reversible process, which is limited by the cellular receptor concentration, the putative driving force. However, these conclusions were drawn based upon results obtained using suspect gp70-based assays. This uncertainty coupled with the receptor-envelope independent nature of the virus binding which was observed in Figs. 2 and 3 prompted us to re-evaluate these basic conclusions regarding the nature of the virus adsorption process. Our primary focus was on the mechanism behind the plateau in virus adsorption after 2 h of binding.

As virus particles lose bioactivity relatively rapidly, we tested whether the plateau in virus adsorption was due to limiting concentrations of active virus by measuring the binding of decayed virus. We thawed a stock of virus and divided it into two aliquots. The control aliquot was re-frozen immediately, while the other was re-frozen after incubation at 37 °C for 24 h. When supplemented with 8 μg/ml polybrene and tested in a transduction assay, the decayed aliquot showed essentially no ability to transduce, whereas the control aliquot retained its activity (Fig. 4a). We then tested these same aliquots in a 2-h adsorption experiments on NIH-3T3 cells. The control and

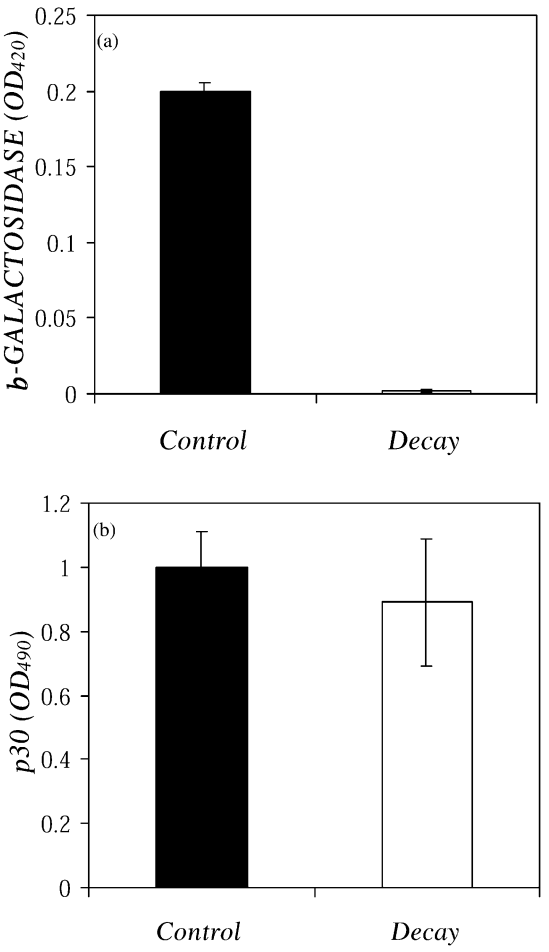

Fig. 4. Virus adsorption plateau is not due to virus decay. Ecotropic virus was divided into two aliquots; one was decayed by incubation at 37 °C for 24 h then frozen, whereas the control aliquot was frozen immediately. (a) NIH-3T3 cells (5000 per well) were plated in a 96-well dish. After 24 h, the medium was replaced with control (dark bar) or decayed (light bar) virus containing 8 μg/ml polybrene. Forty-eight hours later, transduction was measured using an assay for β-galactosidase. (b) NIH 3T3 cells (1.5×10^5 per well) were plated in a 6-well dish. After 72 h, the medium was replaced with control (dark bar) or decayed (light bar) virus containing 8 μg/ml polybrene. After 2 h, the virus was removed, cells were lysed, and the lysates were analyzed for the presence p30 by ELISA.

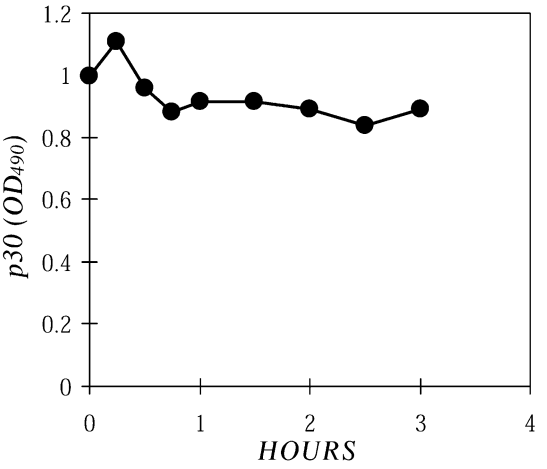

Fig. 5. Virus adsorption plateau is not due to depletion of virus from the supernatant. NIH 3T3 cells (1.5×10^5 per well) were plated in a 6-well dish. After 72 h, the medium was replaced with undiluted ecotropic virus containing 8 μg/ml polybrene. At regular intervals, the virus was removed, diluted 1:1 into Triton X-100 lysis buffer, and analyzed for the presence of p30 by ELISA.

decayed aliquots produced identical levels of adsorption (Fig. 4b), indicating that adsorption is independent of virus bioactivity.

While the concentration of virus is not limited by decay, it was still possible that the total amount of virus in the medium was limiting. To investigate this possibility, we exposed NIH-3T3 cells to ecotropic virus and measured the time course of virus depletion from the supernatant by p30 ELISA (Fig. 5). After 3 h of adsorption, 85% of the initial p30 was still present in the supernatant. Since the maximum in the time course occurs within the first 2 h, this result suggests that depletion from the supernatant does not explain the maximum in the adsorption time course.

We next considered whether the maximum level in the time course might be due to an equilibrium between virus adsorption and desorption, as suggested in previous studies using gp70-based assays. To determine whether such an equilibrium existed, we performed a pulse-chase experiment. A stock of ecotropic virus was adsorbed for 5 h onto NIH-3T3 cells in the presence of polybrene (pulse), followed by removal of the virus-containing medium and replacement with fresh medium (chase). Following the virus pulse, the medium chase was monitored for levels of desorbed p30 (Fig. 6). Through 11 h of the fresh medium chase, no significant desorption of p30 was detected. During this same pulse-chase experiment, however, we tested whether the maximum in the adsorption time course was due to degradation of the cell-associated p30 proteins. Levels of cell-associated p30 declined significantly on the time-scale of the adsorption time-course experiments, with a half-life of 7.2 h, when modeled using first order reaction kinetics. The plateau in virus adsorption can therefore be explained as a balance between the diffusion and adsorption of new p30-containing virus particles and the degradation of previously adsorbed particles.

3.4. Polybrene increases virus adsorption rates 10-fold on receptor-negative cells

To determine whether a simple model of irreversible virus adsorption, coupled with p30 deg-

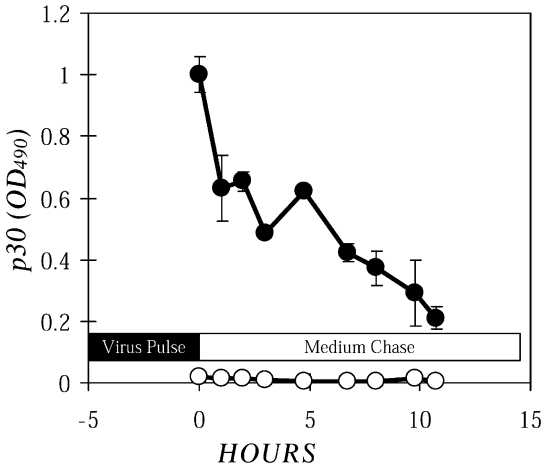

Fig. 6. Virus adsorption plateau is due to degradation of p30 and gp70, not desorption. NIH 3T3 cells (1.5×10^5 per well) were plated in a 6-well dish. After 72 h, the medium was replaced with ecotropic virus containing 8 μg/ml polybrene (virus pulse). After 5 h, the virus was replaced with fresh medium (chase). At regular intervals after the virus pulse, the medium was removed, diluted 1:1 into Triton X-100 lysis buffer, and analyzed for the presence of p30 by ELISA (open circles). Following removal of the medium at each time point, the cells were also lysed and analyzed for the presence of p30 (closed circles).

Table 1
Relative virus adsorption rates on CHO cells

	p30 adsorption rate Constant[a] (OD_{490}/cell-h$\times 10^{-8}$)
+ Polybrene	16.38
− Polybrene	1.69
Fold increase	9.68

[a] Rate constant was determined from short-time course (<1 h) adsorption experiments assuming irreversible virus binding and negligible p30 degradation.

radation, could be used to describe polybrene's enhancement of virus adsorption, we compared our experimental data to a series of model predictions. Our physical model assumed that the accumulation of cell-associated retrovirus proteins, V^{CA}, was an irreversible process governed by the effective rate of adsorption, k_a, the number of target cells, C, and the concentration of virus in the stock, V^S. The rate of intracellular degradation of cell-associated virus proteins was assumed to be directly proportional to V^{CA} (i.e. first order), with a degradation rate constant, k_d. With this physical model of virus protein adsorption, the level of cell associated virus as a function of time, t, is governed by the following equation:

$$\frac{dV^{CA}}{dt} = k_a V^S - k_d V^{CA} \quad (1)$$

We modeled virus accumulation on CHO cells by using Eq. (1) and independently determined values of k_d and k_a. The k_d values were derived from the virus pulse-chase experiments (Fig. 6), whereas the k_a values (Table 1) were determined from a short time course ecotropic virus adsorption experiment on CHO cells (Fig. 7a). The presence of polybrene yielded an approximately ten-fold increase in the rate of virus adsorption. When the adsorption profiles predicted from Eq. (1) were compared to experimental time courses, the model curves generated provided a reasonable fit to the p30 adsorption data with an r^2 value of 0.94 and 0.87 for the plus and minus polybrene curves, respectively (Fig. 7b). It is interesting to note that while polybrene enhances transduction roughly 10-fold, only a 3-fold increase in the level of adsorbed p30 was observed in Fig. 1e, as p30 degradation is not accounted for. However, when rates of p30 adsorption are compared, such as those determined

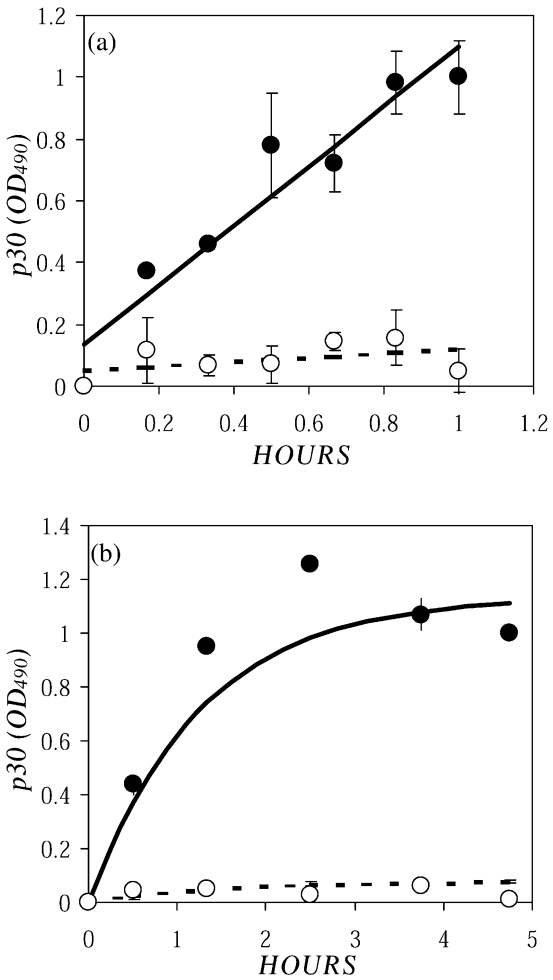

Fig. 7. Polybrene's enhancement of virus adsorption can be modeled using a simple model of irreversible adsorption and degradation. (a) CHO cells were plated in a 6-well dish (1.5×10^5 per well). After 72 h, the medium was replaced with ecotropic virus containing 8 μg/ml (closed circles) or 0 μg/ml (open circles) polybrene. At regular intervals, the virus was removed, the cells lysed, and the lysates were analyzed for the presence of p30 by ELISA. (b) Independently determined adsorption and degradation rate constants were used to model the adsorption time course of ecotropic virus p30 on CHO cells containing 8 μg/ml (solid line) or 0 μg/ml (dashed line) polybrene. The simulation was compared to experimental adsorption measurements of ecotropic virus containing 8 μg/ml (closed circles) or 0 μg/ml (open circles) polybrene.

Table 2
Physicochemical properties of virus particles and free envelope protein

	Mol. Wt. (kD)	Effective radius (nm)	Diffusivity (cm^2/s)
Virus particle	$\sim 10^5$	50	4.3×10^{-8}
Envelope protein	210[a]	5.8	3.7×10^{-7}

[a] Assumes a trimer of three gp70 molecules.

from initial adsorption data (Table 1), polybrene shows the expected ten-fold enhancement consistent with its effects on transduction. Thus, with knowledge of k_d for a given target cell type, Eq. (1) can be used to determine effective adsorption rates.

4. Discussion

Polybrene and other cationic polymers are well known enhancers of transduction [14–16], however, the mechanism by which these charged molecules modulate transduction is not well understood. As retrovirus transduction is fundamentally limited by the slow diffusion and rapid decay of virus [7,9,27] we hypothesized that cationic polymers enhance transduction efficiency by increasing the flux of virus on the cell surface. The initial step in the currently accepted model for retrovirus transduction is the diffusion of virus to the target cell, followed by gp70 mediated binding to a specific receptor on the cell surface [28]. For amphotropic viruses, the cellular receptor is a phosphate symporter (rPit-2) [29], while for ecotropic viruses, the receptor is a cationic amino acid transporter (mCAT-1) [30]. Since the amino acid sequence of gp70 determines virus tropism, the detection of gp70 adsorption to the cell has been the basis for a variety of virus binding assays [18–22]. While these studies have shown that successful binding of a specific gp70 is a prerequisite for successful infection, it is unclear whether virus binding parameters can be inferred from isolated gp70 binding studies, as intact virus particles and gp70 proteins have vastly different molecular weights and diffusivities (Table 2). Moreover, it has been suggested that the presence of free gp70 in retrovirus stocks may complicate the interpretation of the results of gp70-based assays [31,32]. Although many have hypothesized that polybrene enhances virus adsorption, few studies have characterized the effect of polybrene on this initial step of transduction [33], and direct, quantitative measurements have not been reported. In this report, we have demonstrated that polybrene increases retrovirus transduction efficiency by enhancing adsorption, and that this enhancement is both receptor and envelope independent.

To measure virus adsorption, we developed a novel assay based on the detection of cell-associated virus capsid (p30) protein. p30 is physically located inside the lipid membrane of the virus, whereas gp70 is attached to the outside of the membrane via a labile disulfide bond to the p15E transmembrane protein. The labile nature of the gp70 bond increases the likelihood of its release from the particle as free gp70, while the intraparticle nature of p30 ensures that it will remain virus-associated. To determine whether p30 and gp70 yielded equivalent results when used to characterize virus binding, we measured polybrene's effect on virus adsorption using assays based on both proteins. When time courses and dose responses were measured, polybrene enhanced p30 adsorption, but had no effect on gp70 adsorption. To determine whether the lack of correlation between the adsorption behavior of these two virus proteins was due to interference by free gp70 present in retrovirus, we repeated the virus adsorption experiments on CHO cells which expressed the appropriate virus receptor (ecotropic, MCAT-1), an alternate receptor (amphotropic, rPIT-2), or no receptor at all. The adsorption dose response curves for an ecotropic virus demonstrated high levels of gp70 binding on cells expressing MCAT-1, as compared to those without, which supports the concept of tropism dependent virus binding. When levels of p30 were measured on the same cells, however, identical levels of virus binding were found on all cell types. As the ratio of gp70 to p30 in a virus particle is constant, the increased binding of ecotropic gp70 on cells expressing the ecotropic receptor must be due to the presence of free gp70. As free gp70 is more abundant and capable of more rapid diffusion and binding than a virus particle (Table 2), its presence in virus

stocks interferes with the ability of gp70 based assays to reliably measure virus particle binding. To circumvent this limitation, we used the p30 based assay for all further characterization of virus binding.

When virus adsorption was measured using a p30-based assay, polybrene caused a 9.7-fold increase in the rate of adsorption of ecotropic virus. To address the mechanism of polybrene's enhancement of virus adsorption, we considered the role of the gp70-receptor (mCAT-1 or rPIT-2) interaction in the adsorption process. As this interaction is a prerequisite for infection, we hypothesized that polybrene enhanced adsorption by interacting directly with one of these two molecules. However, the equivalency of virus binding on CHO cells, with and without an appropriate receptor, was indicative of the receptor independence of virus adsorption, as well as polybrene's enhancement of it. Similarly, the identical binding behavior of envelope negative, 'bald' virus particles suggested that adsorption and polybrene's enhancement are envelope independent, as well. The receptor-envelope independence of binding, coupled with polybrene's enhancement of it, may have interesting implications for the currently accepted physical model of retrovirus adsorption, which designates the receptor-gp70 interaction as the driving force for initial virus attachment [28]. The actual driving force may involve an as of yet uncharacterized co-receptor, however, the charged nature of polybrene and the virus and cell membranes suggests the central role that electrostatic interactions may play in mediating early virus–cell interactions.

While virus binding in the absence of a receptor is at odds with the currently accepted model of retroviral entry, this may not represent such a surprising finding when one considers the molecular dimensions of the receptor protein on the surface of the cell. The amphotropic and ecotropic receptors are both transmembrane proteins [29,30], whose extracellular domains consist of loops less than 30 amino acids in length. If these domains were maximally extended, there would be a virus-binding site that extends a mere 10–15 nanometers from the surface of the plasma membrane. This is in stark contrast to the glycocalyx, a negatively charged, carbohydrate-rich layer of proteoglycans and glycoproteins which coats the cell membrane and extends up to 400 nanometers from the surface of the plasma membrane in certain cell types [34]. The difference in size between the glycocalyx and the receptor domain suggests that before a virus can access the receptor, an initial adsorption event at the surface of the glycocalyx may be necessary. Polybrene likely enhances transduction by facilitating this initial adsorption, however, further work will be necessary to establish this.

In light of the receptor and envelope independence of virus adsorption and cationic polymer enhancement of it that we observed, it was somewhat surprising that the time course of adsorption reached a maximum. In previous studies using gp70-based assays, a maximum in retrovirus binding has been interpreted as either saturation of receptors or attainment of a binding equilibrium [19]. In light of the inaccuracies associated with gp70-based assays, we revisited these conclusions about retrovirus binding using the p30-based assay. Our findings suggest that rPit-2 and mCAT-1 receptor saturation does not occur because adsorption is equivalent in the presence or absence of receptor. Nonetheless, the actual driving force for adsorption may be an unidentified receptor or simply the electrostatically amenable surface area of the cell membrane, either of which could become saturated during adsorption. To investigate this possibility, we looked at the dose-response of virus adsorption and found that it was uniformly linear, suggesting that saturation of the cell surface or of an unidentified receptor is not achieved. We also considered whether virus decay or depletion of virus in the supernatant were potentially responsible for the maximum by limiting the concentration of adsorbable virus, however, neither explained the virus-binding maximum. Moreover, when we performed virus pulse-chase experiments to consider the possibility of an adsorption–desorption equilibrium process, we were unable to detect any level of virus desorption. This finding is consistent with the theoretical predictions of Chuck et al. [7], who concluded that virus adsorption is diffusion limited—a condition inconsistent with an adsorption–desorption equilibrium. In contrast, Yu et al. [19] showed evidence of an adsorp-

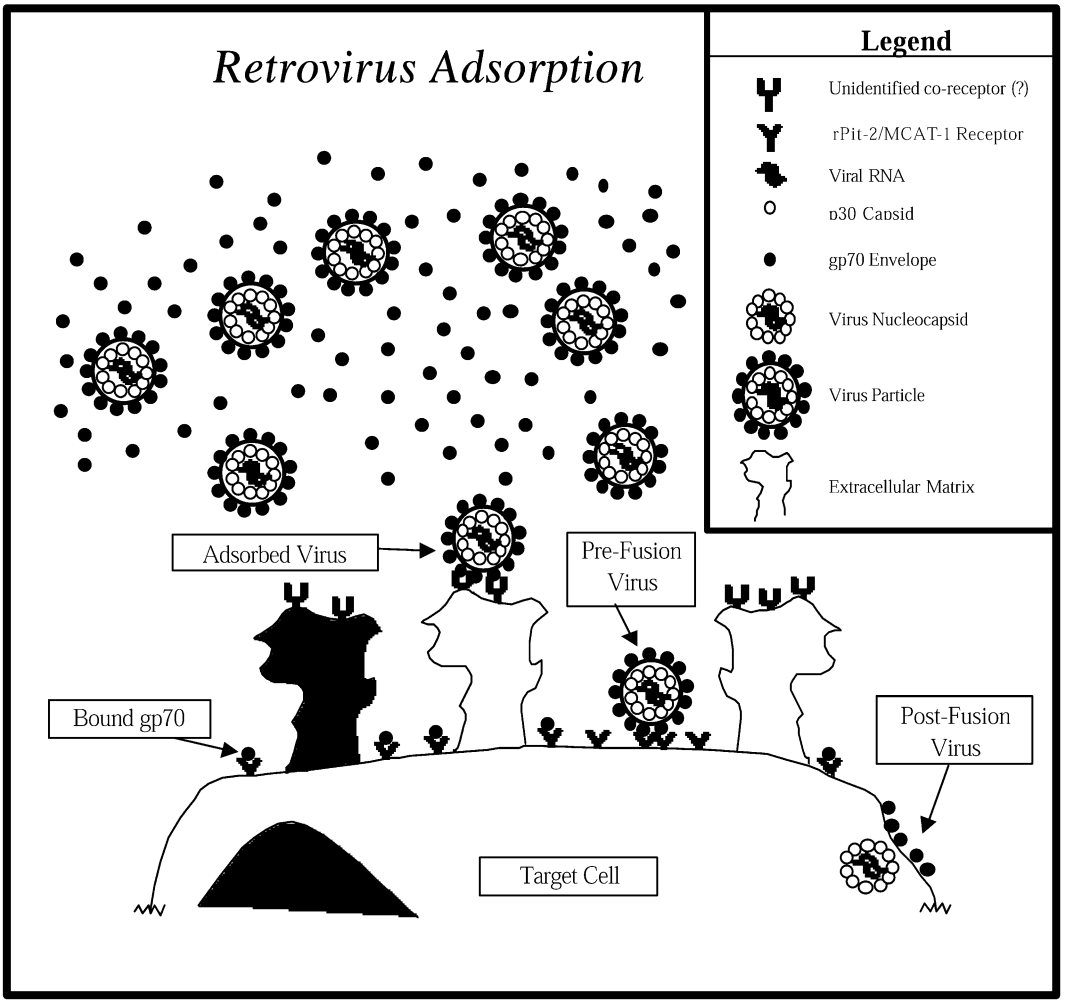

Fig. 8. A revised physical model of early steps of retroviral transduction. Initial virus adsorption occurs via an unidentified co-receptor or non-specific charge interaction with the extracellular matrix. Adsorbed virus interacts with mCAT-1/GLVR-2 receptor and virus-cell membrane fusion is triggered.

tion/desorption equilibrium process, however, the use of a gp70-based assay may have enabled free gp70 to mask the actual virus adsorption behavior. Our adsorption pulse-chase experiments did indicate, however, that the maximum in the adsorption time course could be explained by degradation of adsorbed p30. By combining this degradation half-life with our measured adsorption rate constant, we were able to show a good correspondence between the predicted and experimental binding data.

Based on our results, we propose a revised physical model of the early steps of transduction (Fig. 8), in which a retrovirus diffuses to the cell surface, where it becomes adsorbed. This initial adsorption is mediated by either a currently unidentified co-receptor, or via non-specific electrostatic interactions. Next, the adsorbed virus encounters a secondary 'receptor' (e.g. rPIT-2) which triggers virus fusion. In this model, initial adsorption occurs independently of the mCAT-1 or rPIT-2 receptors, enabling virus adsorption to reach

comparable levels on cells, which are permissive or non-permissive, with respect to transduction. This revised model is supported by the findings of Pizzato et al. [32], who detected comparable levels of virus adsorption on permissive and non-permissive cell lines using a fluorescence microscopy based assay. Furthermore, the binding behavior we observed brings murine leukemia virus adsorption in line with that of other enveloped viruses, such as vesicular stomatitis virus [35] and Rous sarcoma virus [33,36], which also exhibit receptor-independent binding.

This physical model of adsorption has significant implications for the targeting of retroviruses for in vivo gene therapy. Most efforts have focused on altering gp70 structure in order to redirect virus binding to a new receptor [37–41]. However, if initial virus adsorption is independent of gp70-receptor interactions, and desorption is negligible, only a fraction of the introduced virus may encounter the intended target cell because most of it will be non-specifically, and therefore inappropriately, adsorbed on the first population of cells encountered. Thus, it will be important to define the molecular determinants of the virus surface that are important for this initial adsorption step and to alter these properties, as well as gp70 tropism, when devising strategies for targeted retrovirus gene therapy.

5. Conclusions

We investigated the mechanism of polybrene's enhancement of retrovirus gene transfer by measuring its effect on virus flux onto target cell surfaces. Polybrene enhanced virus adsorption rates approximately ten-fold when measured via a p30-based assay, however, adsorption measurements based on a gp70 assay could not detect this enhancement due to interference by significant levels of free gp70 in virus stocks. Surprisingly, the enhancement of adsorption by polybrene was observed in the presence and absence of a competent receptor-envelope interaction, indicating that the initial adsorption event is independent of gp70-receptor interactions. Taken together, these findings provide support for a revised model of the early steps of retrovirus transduction in which the initial adsorption step is independent of the mCAT-1/rPit-2 receptor, diffusion limited, and enhanceable by cationic polymers, such as polybrene.

Acknowledgments

This work was supported by grants from the National Institutes of Health (PO1HD 28528-07) (J.R.M.) and the National Science Foundation (BES-9800617) (M.L.Y.). H.E.D. was supported by a Biomedical Engineering Graduate Fellowship from the Whitaker Foundation.

References

[1] W.F. Anderson, Human gene therapy, Nature 392 (1998) 25–30.
[2] C.M. Roth, M.L. Yarmush, Nucleic acid biothenology, Annu. Rev. Biomed. Eng. 1 (1999) 265–297.
[3] T. Friedmann, The maturation of human gene therapy, Acta Paediatr. 85 (1996) 1261–1265.
[4] I.M. Verma, N. Somia, Gene therapy—promises, problems and prospects, Nature 389 (1997) 239–242.
[5] J.M. Le Doux, J.R. Morgan, R.G. Snow, M.L. Yarmush, Proteoglycans secreted by packaging cell lines inhibit retrovirus infection, J. Virol. 70 (1996) 6468–6473.
[6] J.M. Le Doux, J.R. Morgan, M.L. Yarmush, Removal of proteoglycans increases efficiency of retroviral gene transfer, Biotechnol. Bioeng. 58 (1998) 23–34.
[7] A.S. Chuck, M.F. Clarke, B.O. Palsson, Retroviral infection is limited by Brownian motion, Hum. Gene Ther. 7 (1996) 1527–1534.
[8] H. Kotani, P.B. Newton, S. Zhang, et al., Improved methods of retroviral vector transduction and production for gene therapy, Hum. Gene Ther. 5 (1994) 19–28.
[9] J.M. Le Doux, H.E. Davis, J.R. Morgan, M.L. Yarmush, Kinetics of retrovirus production and decay, Biotechnol. Bioeng. 63 (1999) 654–662.
[10] B. Palsson, S. Andreadis, The physico-chemical factors that govern retrovirus-mediated gene transfer, Exp. Hematol. 25 (1997) 94–102.
[11] B.A. Bunnell, L.M. Muul, R.E. Donahue, R.M. Blaese, R.A. Morgan, High-efficiency retroviral-mediated gene transfer into human and non-human primate peripheral blood lymphocytes, Proc. Natl. Acad. Sci. USA 92 (1995) 7739–7743.
[12] A.B. Bahnson, J.T. Dunigan, B.E. Baysal, et al., Centrifugal enhancement of retroviral mediated gene transfer, J. Virol. Methods 54 (1995) 131–143.
[13] A.S. Chuck, B.O. Palsson, Consistent and high rates of gene transfer can be obtained using flow-through transduction over a wide range of retroviral titers, Hum. Gene Ther. 7 (1996) 743–750.

[14] R.J. Coelen, D.G. Jose, J.T. May, The effect of hexadimethrine bromide (polybrene) on the infection of the primate retroviruses SSV 1/SSAV 1 and BaEV, Arch. Virol. 75 (1983) 307–311.
[15] J.S. Manning, A.J. Hackett, N.B. Darby, Effect of polycations on sensitivity of BALB-3T3 cells to murine leukemia and sarcoma virus infectivity, Appl. Microbiol. 22 (1971) 1162–1163.
[16] K. Toyoshima, P.K. Vogt, Enhancement and inhibition of avian sarcoma viruses by polycations and polyanions, Virology 38 (1969) 414–426.
[17] B.S. Coller, Polybrene-induced platelet agglutination and reduction in electrophoretic mobility: enhancement by von Willebrand factor and inhibition by vancomycin, Blood 55 (1980) 276–281.
[18] M.J. Kadan, S. Sturm, W.F. Anderson, M.A. Eglitis, Detection of receptor-specific murine leukemia virus binding to cells by immunofluorescence analysis, J. Virol. 66 (1992) 2281–2287.
[19] H. Yu, N. Soong, W.F. Anderson, Binding kinetics of ecotropic (Moloney) murine leukemia retrovirus with NIH 3T3 cells, J. Virol. 69 (1995) 6557–6562.
[20] M.S. McGrath, A. Decleve, M. Lieberman, H.S. Kaplan, I.L. Weissman, Specificity of cell surface virus receptors on radiation leukemia virus and radiation-induced thymic lymphomas, J. Virol. 28 (1978) 819–827.
[21] M.S. McGrath, I.L. Weissman, AKR leukemogenesis: identification and biological significance of thymic lymphoma receptors for AKR retroviruses, Cell 17 (1979) 65–75.
[22] K.B. Andersen, B.A. Nexo, Entry of murine retrovirus into mouse fibroblasts, Virology 125 (1983) 85–98.
[23] J.R. Morgan, J.M. LeDoux, R.G. Snow, R.G. Tompkins, M.L. Yarmush, Retrovirus infection: effect of time and target cell number, J. Virol. 69 (1995) 6994–7000.
[24] L.H. Evans, R.P. Morrison, F.G. Malik, J. Portis, W.J. Britt, A neutralizable epitope common to the envelope glycoproteins of ecotropic, polytropic, xenotropic, and amphotropic murine leukemia viruses, J. Virol. 64 (1990) 6176–6183.
[25] D. Lane, E. Harlow, Antibodies: a laboratory manual. xiii, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988, p. 726.
[26] S.P. Forestell, E. Bohnlein, R.J. Rigg, Retroviral endpoint titer is not predictive of gene transfer efficiency: implications for vector production, Gene Ther. 2 (1995) 723–730.
[27] S. Andreadis, T. Lavery, H.E. Davis, J.M. Le Doux, M.L. Yarmush, J.R. Morgan, Toward a more accurate quantitation of the activity of recombinant retroviruses: alternatives to titer and multiplicity of infection, J. Virol. 74 (2000) 3431–3439.
[28] J.M. Coffin, S.H. Hughes, H.E. Varmus, Retroviruses, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1997, pp. 71–119.
[29] D.G. Miller, R.H. Edwards, A.D. Miller, Cloning of the cellular receptor for amphotropic murine retroviruses reveals homology to that for gibbon ape leukemia virus, Proc. Natl. Acad. Sci. USA 91 (1994) 78–82.
[30] L.M. Albritton, L. Tseng, D. Scadden, J.M. Cunningham, A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection, Cell 57 (1989) 659–666.
[31] P. Kurre, H.P. Kiem, J. Morris, S. Heyward, J.L. Battini, A.D. Miller, Efficient transduction by an amphotropic retrovirus vector is dependent on high-level expression of the cell surface virus receptor, J. Virol. 73 (1999) 495–500.
[32] M. Pizzato, S.A. Marlow, E.D. Blair, Y. Takeuchi, Initial binding of murine leukemia virus particles to cells does not require specific Env-receptor interaction, J. Virol. 73 (1999) 8599–8611.
[33] M.F. Notter, J.F. Leary, P.C. Balduzzi, Adsorption of Rous sarcoma virus to genetically susceptible and resistant chicken cells studied by laser flow cytometry, J. Virol. 49 (1982) 345–348.
[34] H. Vink, B.R. Duling, Identification of distinct luminal domains for macromolecules, erythrocytes, and leukocytes within mammalian capillaries, Circ. Res. 79 (1996) 581–589.
[35] R. Schlegel, M.C. Willingham, I.H. Pastan, Saturable binding sites for vesicular stomatitis virus on the surface of Vero cells, J. Virol. 43 (1982) 871–875.
[36] F. Piraino, The mechanism of genetic resistance of chick embryo cells to infection by Rous sarcoma virus-BRYAN strain (BS-RSV), Virology 32 (1967) 700–704.
[37] M. Marin, D. Noel, S. Valsesia-Wittman, et al., Targeted infection of human cells via major histocompatibility complex class I molecules by Moloney murine leukemia virus-derived viruses displaying single-chain antibody fragment-envelope fusion proteins, J. Virol. 70 (1996) 2957–2962.
[38] F. Martin, J. Kupsch, Y. Takeuchi, S. Russell, F.L. Cosset, M. Collins, Retroviral vector targeting to melanoma cells by single-chain antibody incorporation in envelope, Hum. Gene Ther. 9 (1998) 737–746.
[39] W.H. Gunzburg, A. Fleuchaus, R. Saller, B. Salmons, Retroviral vector targeting for gene therapy, Cytokines Mol. Ther. 2 (1996) 177–184.
[40] J.D. Harris, N.R. Lemoine, Strategies for targeted gene therapy, Trends Genet 12 (1996) 400–405.
[41] F.L. Cosset, S.J. Russell, Targeting retrovirus entry, Gene Ther. 3 (1996) 946–956.