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Retroviral Infection of Syrian Hamster BHK Cells Depends

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Infection with recombinant retroviruses is a standard method to efficiently manipulate murine or human cells genetically. Due to differences in glycosylation of the hamster homologue to the murine ecotropic receptor and the absence of an amphotropic receptor, murine retroviruses are unable to infect hamster cells. Here we describe an isolated cell clone of the Baby Hamster Kidney cell line, BHK-21, that can be successfully infected with ecotropic murine retroviruses. This finding prompted us to investigate the possibility of changing the infectability of other cell strains by manipulating the receptors of these cell lines *in vitro.* We found that treatment of other BHK cell clones with sialidase from *C. Perfringens* makes these cell clones also permissive to murine ecotropic retroviruses. Long term cultivation of three tested cell clones increased this susceptibility. Although the resulting infectants show a higher rate of infectability, they still require the manipulation with sialidase for this superinfection to be efficient.

INTRODUCTION

Recombinant retroviruses can be used to genetically manipulate cells into receiving a single-copy integrated copy of the retroviral genome. Retroviral integrates exhibit a high stability of expression that depends, however, strongly on the site of integration into the genome of the host cells. On the way to define and target highly active chromosomal sites in the DNA of biotechnologically relevant cell lines, we have investigated the use of recombinant murine retroviruses.

Only a few cell lines have proven their worth for the large scale production of pharmaceutically interesting proteins. Of these, two of the most used cell lines are derived from hamster; Chinese Hamster Ovary cells (CHO) and Syrian Baby Hamster Kidney cells (BHK). The use of the classical transfection methods with these cell lines results mostly in unstable multicopy integrates. However, high expression of a transferred gene is not necessarily coupled to high copy numbers. As demonstrated before, single copy integrates are also able to direct efficient overexpression in BHK-cells (Wirth *et al.,* 1988) so proviral insertions could be used to construct high producer cell lines.

The introduction and stable integration of foreign genes via retroviral vectors into hamster cells lines is, however, hampered by the extremely low infectability of these cells by ecotropic, amphotropic, and even xenotropic retroviruses.

In this report we describe the unexpected finding of a BHK-21 cell clone that can be efficiently infected by ecotropic retroviruses and the *in vitro* manipulation with sialidase of another BHK-21 clone into permissibility with respect to ecotropic murine retroviruses.

MATERIALS AND METHODS

Retroviral constructs

pM5TKNEOFUS, a neomycin resistance conferring construct, is based on a retroviral MPSV backbone as described by Laker *et al.* (1987), driving a single gene which encodes a fusion protein of thymidine kinase and neomycin phosphotransferase. pBPLHL, a hygromycin resistance conferring construct, has the same MPSV backbone and encodes two genes, puromycin acetyltranseferase and hygromycin phosphotransferase. Both genes are driven by the retroviral promoter and are translated from a single, bicistronic mRNA via an internal ribosomal entry site derived from polio virus (Pelletier and Sonenberg, 1988).

Cell lines and media

$\psi 2$ was used as the ecotropic packaging cell line (Mann *et al.,* 1983). PA317 (Miller and Buttimore, 1986) was used as the amphotropic packaging cell line, ATCC CRL 9078. PG13 (Miller and Rosman, 1989; Miller *et al.,* 1991) was used as the xenotropic packaging cell line (ATCC CRL 10686). Tropism is seen from the murine

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point of view. PG13 cells contain the gibbon ape leukemia virus env gene. All Syrian hamster BHK (MacPherson and Stoker, 1962) cell lines used are originally obtained from ATCC (ATCC CLL10). BHK-B was obtained in 1994 and proved to be tetraploid. BHK-A was obtained in 1984. From this cell, in a number of subcloning and selection steps, a cell clone was established which exhibits significantly higher expression of recombinant genes as compared to the parental cells. Furthermore, these cells produce recombinant proteins with an altered glycosylation pattern if compared to the BHK-B cells (Grabenhorst *et al.,* 1995; H. S. Conradt, personal communication). At the beginning of these experiments described herein, this cell clone was shown to be triploid. BHK-C is a BHK-21 cell clone obtained from Paul van den Boogart (Organon) in 1988 at a low passage number. This cell clone is still diploid. All cell clones were confirmed to be of hamster origin. Karyotyping was carried out by the DSM (German collection of micro-organisms). NIH 3T3 (Jainhill *et al.,* 1969) is a murine fibroblastoid cell line, ATCC CRL 1658. RAT 2 (Topp, 1981) is a rat thymidine kinase deficient cell line, ATCC CRL 1764.

DMEM, Dulbeco's modified Eagles medium (Sigma Chemie GmbH, Deisenhofen, FGR), complemented with 10% fetal calf serum, 20 m*M* glutamine, 60 μg/ml penicillin, and 100 μg/ml streptomycin. For selection on neomycin resistance DMEM was supplemented with 1 mg/ml geneticin (Sigma Chemie GmbH, Deisenhofen, FGR) for all cells except for BHK-C cells; these require 1.5 mg/ml geneticin for selection. For hygromycin resistance the DMEM was supplemented with 400 units/ml of hygromycin B (Sigma Chemie GmbH, Deisenhofen, FGR). All cells were grown at 37°, under 5% CO_2 and 95% humidity.

Transfections and infections

The packaging cells were transfected with the retroviral vector constructs using the calcium-phosphate method originally described by Graham and van der Eb (1973).

Stably transfected packaging cell lines confluently grown were put on half of their usual amount of medium for 24 hr (5 ml/10-cm plate). After this period of time, supernatants were harvested, passed through a filter (0.45 μm Sartorius, Göttingen, FRG), and used directly to infect the indicated cells. Infections were incubated for 24 hr with virus and 8 μg/ml Polybrene (Sigma Chemie GmbH, Deisenhofen, FGR). After 24 hr the cells were trypsinized and divided in various dilutions into selective media. Simultaneously, all virus stocks were titrated on NIH 3T3 and/or RAT 2 cells.

Infection of cells with retrovirus in the presence of sialidase

Cells were plated in a 1/10 dilution in 6-well plates and allowed to grow for 24 hr. The medium was then removed and the cells were replenished with 0.5 ml medium containing the sialidase (1.7 units/ml of sialidase), Polybrene (8 μg/ml), and virus. After 24 hr the cells were trypsinized and various dilutions were incubated in the respective selective media.

Enzymes

Sialidase was from *C. Perfringens,* cloned at Oxford GlycoSystems Ltd. (Abingdon, UK).

RESULTS

In order to identify the infectious potential of retroviral constructs, different cell lines were treated with recombinant retrovirus produced by three different packaging cell lines.

The retroviral construct pM5TKNEOFUS was expressed in the packaging cells ψ2 (ecotropic), PA317 (amphotropic), and PG13 (xenotropic). Neomycin resistance conferring retroviruses from these packaging cell lines were tested on target cells as indicated in Table 1. These were selected for neomycin resistance and the number of stable cell clones was scored. As expected, mouse cells are readily infectable with the ecotropic and amphotropic retroviruses, rat cells are infectable with all three tropisms, and the human cells are infectable with both the amphotropic and xenotropic retroviruses.

To our surprise we found that one of the hamster cell clones, BHK-A, was readily infectable with ψ2-derived, ecotropic retrovirus, while all the others were not (Table 1). Ampho- and xenotropic retroviruses did not give rise to any neomycin-resistant infectants. This is in agreement with reports claiming the unsuccessful infection of hamster-derived cells with amphotropic and xenotropic retroviruses (Wilson and Eiden, 1991).

Since BHK-A is a subclone of BHK-21 (see cell lines in the Materials and Method section for the relationship of the different BHK cells), we looked for differences between the cells in order to attribute this particular behavior. One of the obvious differences between BHK-A and BHK-B is a difference in the glycosylation pattern of the same recombinant proteins produced in both cell clones (H. S. Conradt, personal communication). The difference concerns the terminal occupancy of N-glycosydic residues with α2-6 linked Neuraminic acid. In contrast to BHK-B, BHK-A cells exhibit a significant lack of this kind of terminal modification. This prompted us to check the possibility of manipulating the cell surface protein modifications on intact cells and monitoring the infectability of these cells by ecotropic retroviral vectors (Tables 1 and 2).

BHK-B and BHK-C cells were incubated with the *C. Perfringens* sialidase. This enzyme has a broad specificity and is expected to remove all types of sialic acids from glycoproteins and glycolipids. Enzyme incubations and infection conditions were optimized by varying pH, temperature, duration, and buffers with cells freshly

TABLE 1

Apparent Titer of Retroviruses of Different Tropisms on Cell Lines from Various Species

Supernatant	NIH3T3	Rat2	BHK-A	BHK-B	BHK-C	HeLa
Ψ2	5.4×10^5	3.1×10^5	3.0×10^4	0	5.4×10^2	0
PA317	6.1×10^5	2.5×10^5	0	0	0	7.2×10^4
PG13	0	1.5×10^6	0	0	0	8.0×10^6

Note. Virus stocks were assayed in three different dilutions and numbers averaged. The retroviral construct used is M5TKNEOFUS.

seeded or under rapid growth conditions. Also varied was the moment of virus addition (during or after enzyme incubations) and the length of infection (data not shown). Under most conditions the survival rate of the cells was drastically impaired. Therefore, all experiments were carried out in medium with log-phase cells (see experimental protocol).

In Table 2 (WT) a comparison of infectability of BHK clones with and without sialidase incubation is presented. BHK-C is obviously infectable with ecotropic retrovirus after sialidase treatment.

To explain the difference in susceptibility to retroviruses in variants of the same original cell line, we speculated that high age of cells influences the susceptibility to retroviral infection after sialidase treatment. Therefore, we have tested the infectiousness after sialidase treatment in dependence of the passage number of the cell lines. Indeed, the passage number of the cell clones is important for the susceptibility of infection (compare passage 3 with passage 77 of BHK-C). Even cell clone BHK-B, which is almost unresponsive to sialidase treatment, gives rise to some clones in passage 106.

From Table 2 (WT) it is obvious that only a very small portion of cells is infected without enzymatic treatment and with a maximum of 3% with treatment. Since infection was carried out in a way that the number of viruses is at least 10 times that of the number of cells, infectivity is likely to be a property of the cells. When NIH 3T3 cells are treated in the same way one cell of three becomes infected.

One possible explanation could be that only small subpopulations of cells have undergone a series of mutational events rendering these cells highly infectable. If this is correct then all descendants of such a cell would also be infectable. This means that all of the infectants described above should be fully infectable. To test this assumption, we performed a series of superinfections. Hygromycin-resistance conferring retrovirus was used to infect the various primary BHK infectants. Table 2 (SI) shows that, with the exception of the BHK-B, the percentage of infection without enzyme treatment is higher than in the first round of infection. Furthermore, the percentage of cells that can be induced into permissiveness by sialidase has also risen dramatically. We should, however, point out that this might be partly explained by the apparently gained resistance toward cell-death by sialidase treatment, since all survival rates have increased significantly. These results indicate that the increase in susceptibility is not due to mutational events but rather a consequence of a successive change in the expression of a gene which influences the infectibility of BHK cells.

The infection was repeated with recombinant hygromycin-resistance conferring retroviruses with other tropisms (see Table 3). This result demonstrates that, after enzymatic desialylation and subsequent selection, the various BHK cells are only specifically enhanced in their susceptibility toward ecotropic retroviruses but not toward amphotropic or xenotropic viruses. Note that the percentage of infectable BHK-A cells has risen twofold. Again, this is a question of passage number as these cells had 50 passages more than those described earlier. To test this, BHK-A was passed for another 20 times after which the percentage rose to 12%.

DISCUSSION

In this report we have shown that the effective range of ecotropic retroviruses can be extended by naturally occurring and enzymatically induced variants in glycosylation patterns. It is very likely that the molecules responsible for this susceptibility are cell surface molecules, most likely the hamster homologue of the murine ecotropic retrovirus receptor.

This is supported by the fact that the only barrier for murine retroviruses in BHK-21 cells is the receptor-mediated uptake through the cell membrane (Eiden *et al.,* 1994) and by the fact that amphotropic and xenotropic viruses fail to become infectable. As has been proposed (Eiden *et al.,* 1994), the unfunctionality of the hamster equivalent of the murine ecotropic receptor is possibly due to a single asparagine-linked glycosylation. Indeed, the infectability of BHK-21 was shown to be enhanced by the drug tunicamycin, an inhibitor of N-linked glycosylation. Since, in our hands, neither tunicamycin nor extensive sialidase treatment lead to more susceptible cells, we conclude that a limited number of cells can be sensitized by sialidase treatment. This argues against the normal, main glycoform of the receptor as a rate-limiting candidate for the infection process. More likely, the phe-

TABLE 2

	WT				
	BHK-A	BHK-C p3	BHK-C p77	BHK-B p56	BHK-B p106
N.O.C.	7080	29280	40000	18360	6940
"m.o.i."	57	14	10	22	58
−E					
Cells	11280	21440	33600	7760	7200
Clones	32	16	6	0	0
%	0.28	0.07	0.02	0	0
+E					
Cells	3740	4380	18880	7440	7760
Clones	110	18	310	0	2
%	2.9	0.4	1.6	0	0.03
ratio of +E/−E	3	1	51	?	∞
% +E/% −E	10	6	80	?	∞
Survivors	33%	20%	56%	100%	100%

	SI			
	BHK-A	BHK-C p3	BHK-C p77	BHK-B p106
N.O.C.	6420	21200	57840	8120
"m.o.i."	179	54	20	142
−E				
Cells	14900	43000	62200	9150
Clones	213/390	70/70	50/70	0/0
%	1.4/2.6	0.16/0.16	0.08/0.11	0/0
+E				
Cells	15200	30900	59200	10675
Clones	4450/4450	2720/2470	2190/2500	33/13
%	29/29	9/8	3.7/4.2	0.31/0.12
ratio of +E/−E	21/11	39/35	44/36	∞/∞
% +E/% −E	21/11	56/50	46/33	∞/∞
Survivors	100%	72%	95%	100%

Note. WT: Comparison of infectibility of different wild-type (WT) BHK cell clones by TKNEOFUS retrovirus with and without sialidase treatment (+E and −E indicates with and without enzyme treatment, respectively). SI: The influence of sialidase on superinfection (SI) with BPLHL retrovirus. N.O.C., number of cells infected; "m.o.i.", multitude of infectious particles, as calculated from the N.O.C. and the titer of the virus as determined on NIH 3T3 cells. −E, infections on wild-type cells without sialidase treatment where: Cells, total number of cells, measured 24 hr postinfection; Clones, number of cells that are neomycin resistant, were also measured 24 hr postinfection; %, percentage of cells that became infected cells. +E, cells treated with the enzyme sialidase (all descriptions as above). Ratio of +E/−E, fold increase for sialidase treatment absolute numbers. % + E/% −E, percentage increase. Survivors, ratio of cells that have undergone the sialidase treatment and those who have not.

notype of infectability is based on the occurrence of a certain rare glycoform of the normal receptor. We speculate that this isoform upon desialylation renders the cell susceptible to ecotropic retroviruses.

Our data also indicate that a certain percentage of those cells is infectable without sialidase treatment. Interestingly, this rate parallels the rate of cells which can be made susceptible by sialidase treatment, arguing for a rare glycoform of the receptor which, in the nonsialylated form, is active and becomes inactive by occupancy with Neuraminic acid. Indeed, the simultaneous occurrence of fully and partially glycosylated forms of glycoproteins from a cloned cell line is not unusual (Conradt *et al.,* 1990). This is thought to reflect the enzymatic capacity of a certain cell to carry out the posttranslational modification steps for all glycoproteins. For example, certain cell lines do have a low capacity to carry out the α2-6 sialylation, where all others show a high activity of this enzyme. We therefore believe that the infectable BHK cells have an altered glycosylation capacity compared to noninfectable cells. This difference should be manifested in the modification of other proteins and eventually lipids.

The results in this report imply two facts first, "natural"

TABLE 3

Comparison of Different Tropisms on Various Infected and Selected BHK Cell Lines without Prior Sialidase Treatment

	BHK-A				BHK-B				BHK-C			
Supernatant	"m.o.i."	Survivors	Infected cells	%	"m.o.i."	Survivors	Infected cells	%	"m.o.i."	Survivors	Infected cells	%
Ψ2	4.5	10800/16650	1143/988	7.8	4.1	17650/12400	0/0	0	1.3	50400/48000	542/639	1.2
PA317	3.6		0/0	0	3.3		0/0	0	1.0		0/0	0
PG13	22		11/33	0.2	20		0/1	0	6.1		1/0	0

Note. The "m.o.i." was calculated as the quotient between the number of infectious viruses, as determined by titration on RAT 2 cells and the number of cells infected. The number of cells that survived are given just once in the row of Ψ2.

barriers for retroviral contamination are less stringent then expected, and second, retroviruses can be used for gene transfer into more cell lines than hitherto have been used.

The ratio of the various glycoforms is selectable; this was shown by the superinfection experiments. Those cells that have been susceptible give rise to descendants in which this ratio is significantly increased. This observation precludes a stable mutational event but points to an epigenetic mechanism. For example, the expression of a single or a set of glycosylation modifying genes might be altered by certain chromatin conformations or DNA methylations which are basically conserved but might underlie changes during long term cultivation in cell culture. For example, instability of recombinant protein expression is often due to inactivation by DNA-methylation. In some hamster cell lines the "susceptibility"-phenotype is permanently present. In this light the fact that the Syrian hamster-derived cell line, HaK, is reported to be also infectable with ψ2-derived virions (Wilson and Eiden, 1991), becomes placed in a wider context.

The occurrence and abundance of this putative glycoform seems to be linked with the age of a cell line; the more often a cell line has been passed the better it can be infected after sialidase treatment. It could be that expression of particular glycosylation enzymes and therefore the overall change of the membrane glycoproteins gives the cell an advantage in cell culture conditions. This advantage has to be rather small to explain the subtle increases over 50 passages and more. This change in glycosylation pattern seems to reach a ceiling after long culture conditions, as the percentage of infectable cells hardly changes with passage number in the superinfection experiments with BHK-C cells.

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