

Design of vectors for efficient expression of human purine nucleoside phosphorylase in skin fibroblasts from enzyme-deficient humans

(gene therapy/retroviral vectors/gene transfer/immunodeficiency)

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ABSTRACT Purine nucleoside phosphorylase (PNP; purine-nucleoside orthophosphate ribosyltransferase, EC 2.4.2.1) deficiency is an inherited disorder associated with a severe immune defect that is fatal. Enzyme replacement therapy is an attractive approach to treatment of this disease. To this aim we constructed retroviral vectors containing a human PNP cDNA and a selectable gene encoding neomycin phosphotransferase. PNP expression was controlled by either the early promoter from simian virus 40, the immediate early promoter from human cytomegalovirus, or the retroviral promoter. Cultured skin fibroblasts from two unrelated PNP-deficient patients that were infected with these vectors expressed mean PNP activities of 0.03, 0.74, and 5.9 $\mu\text{mol/hr}$ per mg of protein, respectively. The latter infectants had PNP activities eight times the level of 0.74 $\mu\text{mol/hr}$ per mg of protein observed in normal skin fibroblasts, enabling rapid metabolism of exogenous deoxyguanosine, the cytotoxic metabolite that accumulates in the plasma of PNP-deficient patients. These experiments indicate that viral long terminal repeat was the strongest promoter for expression of PNP and suggest the potential of human skin fibroblasts as vehicles for therapeutic gene expression.

Inherited deficiency of purine nucleoside phosphorylase (PNP; purine-nucleoside orthophosphate ribosyltransferase, EC 2.4.2.1) is associated with a fatal T-cell defect (1, 2). The PNP enzyme is a trimer that catalyzes the reversible conversion of purine (deoxy)nucleosides [except (deoxy)adenosine] to purine base plus (deoxy)ribose-1-phosphate (3, 4). The major pathogenic mechanism in PNP deficiency involves accumulation of deoxyguanosine, a PNP substrate, with consequent inhibition of DNA synthesis in T cells (1). Although transplantation of normal bone marrow into these patients should be curative, none of the patients has been so treated because of clinical deterioration or lack of a suitable donor. In four patients treated with infusions of erythrocytes as a source of active PNP, two showed improved immunological function (1, 5, 6). This observation suggests that the transfer of an active PNP gene into virtually any of a patient's somatic cells should initiate normal deoxyguanosine metabolism and enable T-lymphocyte function to develop.

Current approaches to gene therapy have mainly focused on the transfer of genes into pluripotent bone marrow stem cells. Gene transfer into primitive stem cells should result in the continued expression of the gene in blood cells, including T lymphocytes. Retrovirus-mediated transfer and expression of genes in hematopoietic progenitor cells of humans (7, 8), monkeys (9), dogs (10), and mice (11, 12) have been demonstrated in culture. Gene transfer into marrow stem cells of

mice followed by continued expression of the transferred gene in progeny hematopoietic cells *in vivo* has also been reported (11-14). However, application of this technique to human gene therapy has been hindered by unreliable stem cell infection, especially in larger mammals (9, 15), and inefficient gene expression in hematopoietic cells (16, 17).

For the estimated two-thirds of patients who lack a related histocompatible marrow donor, skin fibroblasts provide an attractive alternative tissue for gene therapy. Skin fibroblasts are easily obtained, can be efficiently infected by amphotropic murine retroviruses, and can be propagated in culture to enable selection and expansion of infected cells (18). In an attempt to obtain maximum expression of PNP, we have constructed several retroviruses carrying a human PNP cDNA linked to a variety of strong viral promoter/enhancer combinations and have compared their ability to direct PNP synthesis in fibroblasts from PNP-deficient patients. These experiments provide a basis for further studies in animals to evaluate the potential of human skin fibroblasts as targets for therapeutic gene expression.

MATERIALS AND METHODS

Cell Culture. Mouse cells were grown in Dulbecco-Vogt modified Eagle's medium (DMEM) supplemented with glucose (4.5 g/liter) and 10% fetal bovine serum. Mouse cell lines included PA317 (19), PE501 (a retrovirus packaging cell line similar to PA317 but with an ecotropic host range compared with the amphotropic host range of PA317; unpublished data), ψ -2 (20), and NIH 3T3 TK⁻ (19). Human diploid fibroblast (HDF) lines were cultured in Waymouth medium and 10% fetal bovine serum and were isolated from skin biopsies of normal human donors and from two unrelated patients with PNP deficiency (HDF V and HDF D) generously provided by B. J. M. Zegers (University Children's Hospital, Utrecht, The Netherlands).

Retroviral Vector Construction. Vectors are depicted in Fig. 1. Vector nomenclature is based on the order of the different promoters and genes in the viruses, abbreviated as follows: L, long terminal repeat (LTR) promoter; S, simian virus 40 (SV40) promoter; C, cytomegalovirus (CMV) promoter; N, *neo* gene; PN, PNP cDNA. Viral sequence numbers are as described (21). All vectors have 5' LTRs from Moloney murine sarcoma virus (Mo-MSV) and 3' LTRs from Mo-MLV, but after viral replication both LTRs have Mo-MLV promoters and enhancers. Viral sequences adjacent to the 5' LTR of LPNSN-1 consist of Mo-MSV bases from 164 to 544 and those of LNPN, LNCPN, and LPNSN-2 consist

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Abbreviations: PNP, purine nucleoside phosphorylase; LTR, long terminal repeat; SV40, simian virus 40; HDF, human diploid fibroblast; CMV, cytomegalovirus; Mo-MSV, Moloney murine sarcoma virus; ADA, adenosine deaminase; cfu, colony-forming units.

of Mo-MSV bases from 164 to 544 followed by Mo-MLV bases from 569 to 1038. The *gag* start codon at Mo-MLV bases 621–623 was changed from ATG to TAG (22) in the three vectors containing this sequence. Sequences adjacent to the 3' LTR include Mo-MLV sequences from 7764 to 7815. Human PNP sequences were obtained from a cloned cDNA (23) (gift of D. W. Martin, Jr., Genentech, South San Francisco, CA). Potential poly(A) signals (AATAAA) at the 3' end of the PNP cDNA were removed before insertion into the vectors to avoid inhibition of full-length viral transcription. *neo* sequences were obtained from transposon Tn5 (24). The bacterial promoter was removed by cleavage with *Bgl* II before insertion of the gene into the vectors.

Enzyme Assays. Activity of PNP and adenosine deaminase (ADA; adenosine aminohydrolase, EC 2.5.4.4) was measured in cell extracts by spectrophotometric assays (3, 25).

Thin-Layer Starch Gel Electrophoresis. Starch gel (13%) in 10 mM sodium phosphate buffer (pH 6.7) was prepared (25) and poured into a mold constructed by gluing plastic strips 2 mm thick and 1 cm wide onto a 14-cm square glass plate. A plastic well former was placed vertically into the gel before it solidified. Sufficient gel was used to prepare a slab 2–3 mm thick. Samples (5–8 μ l) were subjected to horizontal electrophoresis at 4 V/cm overnight at 5°C using 0.1 M sodium phosphate (pH 6.7) bridge buffer. A PNP-specific stain in 0.5% agarose was poured over the gel to locate the separated isozymes (26).

RESULTS

Generation and Analysis of Vector-Producing PA317 Cells. Helper-free virus was made from the vectors depicted in Fig. 1 by using PA317 retrovirus packaging cells. Virus produced by PA317 cells has an amphotropic host range that allows infection of cells from most mammalian species, including humans (19). To generate vector-producing cell lines, the plasmid forms of the vectors were introduced into ecotropic retrovirus packaging cells (PE501 or ψ 2) by calcium-phosphate-mediated transfection, and transiently produced virus was harvested after 2 days and used to infect PA317 cells (30). G418-resistant clones that contained single unarranged proviruses by Southern analysis (31) were chosen. Maximum vector titers of 3–4 $\times 10^6$ colony-forming units

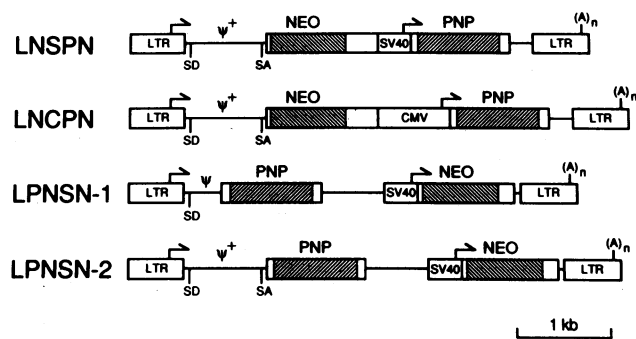


FIG. 1. Vectors for transfer and expression of PNP. Promoters are indicated as open boxes with arrows denoting the site of RNA transcription initiation; SV40 indicates a *Pvu* II to *Hind*III fragment from SV40 that contains the SV40 early region promoter and enhancers (27, 28), CMV indicates a *Bal* I to *Xma* III fragment from human CMV that contains the CMV immediate early promoter and enhancers (29), and LTR indicates the retroviral LTR that contains the retroviral promoter and enhancers (21). Boxes with hatched regions indicate fragments of the *neo* gene or a PNP cDNA, and the hatched areas represent the coding regions. Lines indicate retroviral sequences. SD, splice donor; SA, splice acceptor; ψ , retroviral packaging signal; ψ^+ , extended retroviral packaging signal; (A)_n, poly(A) site; kb, kilobase.

Table 1. Properties of vector-infected PA317 cells

Virus	Total PNP activity, μ mol/hr per mg of protein	Human PNP, % of total	Vector titer, cfu/ml
None	1.13 \pm 0.10	—	—
LNSPN	1.43 \pm 0.59	21	3 $\times 10^6$
LNCPN	1.44 \pm 0.06	22	4 $\times 10^6$
LPNSN-1	2.05 \pm 0.10	45	2 $\times 10^5$
LPNSN-2	3.12 \pm 0.57	64	3 $\times 10^6$

PNP activities are means \pm SD of two to four clones, each analyzed in duplicate. Vector titers were measured by using NIH 3T3 TK⁻ cells as recipients (19) and are those of the best virus-producing clones for each vector. No helper virus (<1 per ml) was detected in any of the cell lines by using the S⁺L⁻ assay (32).

(cfu)/ml were obtained for all vectors except LPNSN-1, which yielded 2 $\times 10^5$ cfu/ml (Table 1). The lower titer of LPNSN-1 was expected because LPNSN-1 contains only the ψ packaging signal, which results in less efficient packaging of viral RNA into virions in comparison to vectors containing the ψ^+ signal (22).

The mean percentage of human PNP in PA317 cells containing the vectors ranged from 21% for LNSPN to 64% for LPNSN-2 (Table 1). These values were calculated by comparison of control and infected PA317 cells and by PNP assays before and after immunoprecipitation of human enzyme by a specific rabbit anti-human PNP antibody (33). Both methods gave similar values. We also monitored human PNP expression in these clones by using starch gel electrophoresis (Fig. 2). The expression of human PNP in the PA317 mouse cells results in heterotrimer formation, which can yield four electrophoretically distinct isozymes. The distribution of the heterotrimers correlated with the ratio of human to mouse PNP in the PA317 infectants. For example, based on human and mouse PNP activity measurements and assuming random association of trimer subunits, we calculated the LPNSN-2 infectant in lane 9 should have an isozyme distribution of 6%, 29%, 43%, and 22% from M₃ through H₃, respectively, and this distribution was observed in the gel. MH₂ and H₃ isozymes were clearly visible in LPNSN infectants only, because of the low ratio of human to mouse PNP in the other cell lines.

The electrophoretic separation of PNP from four clones of PA317 cells infected with LPNSN-2 virus is shown in Fig. 3.

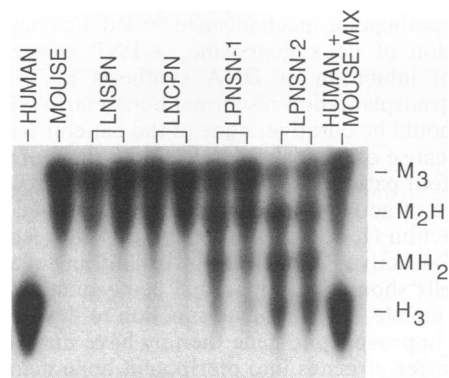


FIG. 2. Starch gel analysis of PNP from infected PA317 cells. PNP isozymes present in PA317 cells containing the indicated viruses were analyzed by using starch gel electrophoresis. Two independent clones of infected PA317 cells were analyzed for each virus. Mouse and human standards were from uninfected PA317 cells and from human T cells, respectively. The positions of the various homo- and heterotrimers formed by the mouse (M) and human (H) subunits are indicated to the right of the gel. Mixing of human and mouse cell extracts prior to analysis (human + mouse mix) does not result in heterodimer formation.

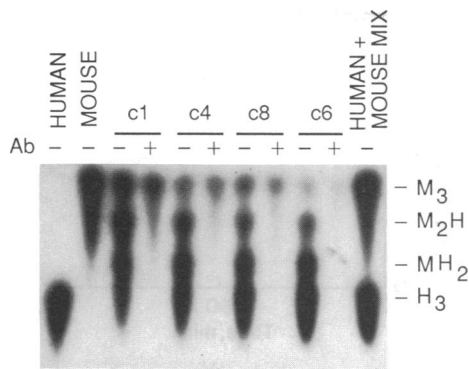


FIG. 3. Analysis of PNP in different clones of PA317 cells containing the LPNSN-2 virus. PNP isozymes were analyzed before and after treatment of cell extracts with a human PNP-specific antibody (Ab). Standards and markers are as described in the legend to Fig. 2.

The cell extracts were adjusted to have the same total PNP activity. Samples 3, 5, 7, and 9 (numbered from the left) were from different clones of specific activity 1.70, 2.40, 3.57, and 4.46 $\mu\text{mol/hr}$ per mg of protein, respectively. The greater proportion of human PNP homotrimer was clearly correlated with increasing specific activity. These samples were also subjected to immunoprecipitation using a specific rabbit anti-human PNP antibody and insolubilized protein A. Their electrophoretic patterns are shown in samples 4, 6, 8, and 10 (numbered from the left). All trimers containing human subunit are immunoprecipitated and the decreasing amounts of mouse homotrimer remaining in samples 4–10 parallel the increasing specific activity of the starting samples.

Transfer of PNP into PNP⁻ HDFs. PNP-deficient skin fibroblasts obtained from two unrelated PNP-deficient patients were infected with the vectors shown in Fig. 1 and random populations selected in G418 were assayed for PNP activity (Table 2). As a control, fibroblasts from patient D were infected with LNL6, a virus containing only the *neo* gene (22). Uninfected fibroblasts and fibroblasts infected with LNL6 had undetectable PNP activity ($<0.01 \mu\text{mol/hr}$ per mg of protein). LNSPN-infected cells expressed PNP at about 3% of normal activity and LNCPN infectants produced enzyme levels equivalent to normal cells. Fibroblasts infected with the two viruses in which PNP expression was controlled by viral LTR (LPNSN-1 and LPNSN-2) expressed PNP activities ranging from 7- to 9-fold greater than normal. These are significant increases in PNP expression compared to the viruses having PNP controlled by promoters from either SV40 or CMV. Infected fibroblasts from patient V consistently expressed slightly higher (10–22%) levels of PNP than infectants from patient D.

As an internal control we measured ADA activities in the PNP⁻ fibroblasts before and after infection. Pooling results from HDF D and HDF V cells, ADA levels (mean \pm SD) before infection were 0.89 ± 0.16 and after infection were $0.74 \pm 0.26 \mu\text{mol/hr}$ per mg of protein. Normal HDF ADA levels are $0.90 \pm 0.41 \mu\text{mol/hr}$ per mg of protein ($n = 15$). Thus, viral infection did not cause changes in a metabolically related purine enzyme.

Table 2. PNP activity in vector-infected HDFs

HDFs	Virus					
	None	LNL6	LNSPN	LNCPN	LPNSN-1	LPNSN-2
Patient D	<0.01	<0.01	0.02 ± 0.01	0.66 ± 0.01	4.81 ± 0.21	5.49 ± 0.09
Patient V	<0.01	—	0.03 ± 0.01	0.81 ± 0.08	5.29 ± 0.14	6.35 ± 0.48
Normal	0.74 ± 0.31					

PNP activities are in $\mu\text{mol/hr}$ per mg of protein \pm SD [$n \geq 3$ except for normal (control) HDFs, where $n = 12$].

Starch gel analysis of infected cells showed that the expressed PNP had normal mobility (data not shown). We measured the Michaelis constant of PNP from LPNSN-2-infected patient D HDFs and PNP from normal HDFs. With inosine as substrate, the K_m of retrovirally expressed PNP was $60.3 \pm 4.8 \mu\text{M}$, which was not different from the value of $56.0 \pm 7.3 \mu\text{M}$ obtained from normal PNP (3, 4).

We examined the structure of the proviruses present in genomic DNA from infected PNP⁻ HDFs of both patients by Southern analysis (31) using restriction enzymes that cut at both ends of the expected proviruses. After hybridization to a radiolabeled *neo* probe, single bands of the expected size were obtained from cells infected with each of the four viruses (data not shown), indicating there was no gross rearrangement of the proviruses in these infected cells. Together with electrophoretic and kinetic measurements of PNP expressed in infected cells, these results demonstrate faithful transfer of an unaltered PNP gene.

Analysis of Viral RNA in PNP⁻ HDFs. Total RNA was prepared from PNP⁻ fibroblasts from patient V or D that were infected with each of the four vectors. Following electrophoresis, the RNAs were examined by using *neo*- and PNP-specific probes (Fig. 4). The patterns of hybridization observed for a given vector were similar in fibroblasts from both patients. For each vector there was a major band that hybridized with both probes and migrated near the 28S rRNA band (4.5 kb), consistent with the size expected of full-length viral transcripts. Small differences in the sizes of these bands reflected the small differences in sizes of the viruses (Fig. 1). The major band in LNCPN-infected cells was broader than the rest because it comigrated with the very abundant 28S rRNA, tending to cause diffusion of the band.

Transcripts initiated by the internal promoters and terminating in the 3' LTRs of the different viruses should all migrate near the 18S rRNA band (1.8 kb). A small RNA species of about 1.8 kb hybridized with the *neo* probe in LPNSN-1- and LPNSN-2-infected cells, consistent with the expected *neo*-containing transcript initiated by the SV40 promoter. As anticipated, the PNP probe did not hybridize to this transcript. Small RNAs initiated by the internal promoters in LNSPN and LNCPN were not detected with either the PNP or *neo* probe. We believe the faint bands migrating just in front of the 18S rRNA bands to be artifacts caused by the presence of the abundant 18S rRNA. After long exposure a faint band that hybridized only with the PNP probe and migrated slightly more slowly than the 18S rRNA was seen in some analyses of RNA from LNCPN-infected cells but was never seen in LNSPN-infected cells (not shown). These results are consistent with the relative PNP levels in these infected fibroblasts and show that the retroviral LTR is by far the most active promoter in these cells.

By using a PNP probe we also analyzed uninfected fibroblasts from patients V and D and from a normal subject for the presence of the endogenous PNP mRNA. Although we could detect the expected RNA species of about 1.7 kb (35) in cells from the normal subject, no such species was detected in cells from patient V or D (data not shown). The amount of 1.7-kb mRNA in cells from the normal subject was similar to that of the RNA directed by the CMV promoter in LNCPN-infected PNP⁻ fibroblasts and was lower by a factor of 10–

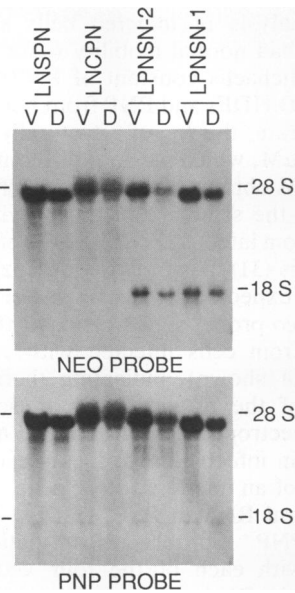


FIG. 4. Analysis of RNA from infected human fibroblasts. Enzyme-deficient skin fibroblasts from patients V and D were infected with virus produced by PA317 cells containing the indicated retroviral vectors. The cells were selected in G418 and resultant colonies were pooled. Total cellular RNA was prepared from the cells as described (34), subjected to electrophoresis in agarose gels containing formaldehyde, transferred to nylon membranes (Hybond-N, Amersham), hybridized to *neo*- or PNP-specific probe, and analyzed by autoradiography by using standard procedures. The same filter was first analyzed by using the *neo* probe, washed twice with 100°C water to remove the probe, and then reanalyzed by using the PNP probe. The positions of the 18S (1.8 kb) and 28S (4.5 kb) rRNAs are indicated.

20 than LTR-directed transcripts. Thus, if present, transcripts from the endogenous PNP gene in the PNP⁻ fibroblasts were not detectable under the conditions used in our analysis of virus-infected cells and thus did not affect analysis of viral transcripts.

Deoxyguanosine and Guanosine Metabolism. We measured the metabolism of exogenous deoxyguanosine and guanosine by skin fibroblasts from normal donors and from the PNP⁻ patients before and after gene transfer with LPNSN-2 virus. An initial nucleoside concentration of 10 μ M was chosen to approximate the level found in the plasma of PNP⁻ deficient patients (1, 33). During the first 60 min when the rates were fairly linear, the genetically modified fibroblasts metabolized deoxyguanosine at about five times the rate of the normal control cells (Fig. 5). These rates paralleled the differences in PNP activity between transduced and normal fibroblasts (Table 2). Similar results were obtained when cells were incubated with guanosine (not shown). Thus normal and genetically modified skin fibroblasts could metabolize exogenous deoxyguanosine and guanosine, the toxic substrates that accumulate in PNP-deficient children (1).

DISCUSSION

The retrovirus vector we constructed with a SV40 promoter linked to PNP (LNSPN) was modeled on one we had previously used to efficiently express human ADA (18). However, this virus, and previously constructed viruses (16) in which PNP was expressed by using the mouse metallothionein promoter, produced low levels of PNP in comparison to levels found in normal mouse or human fibroblasts. We therefore investigated other constructs that used CMV and viral LTR promoters to drive PNP and found significant improvement in PNP expression. Compared to the initial

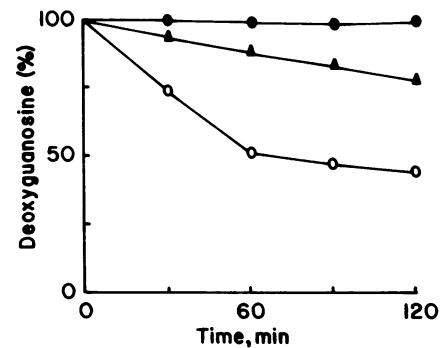


FIG. 5. Metabolism of deoxyguanosine by normal HDFs (▲) and PNP⁻ HDFs before (●) and after (○) gene transfer by LPNSN-2 infection. Nearly confluent fibroblasts were harvested and aliquots (2.5×10^5 cells) were incubated in 0.25 ml of serum-free RPMI 1640 medium containing 10 μ M deoxyguanosine with agitation at 37°C. At 30-min intervals cells were separated by centrifugation and the concentration of unmetabolized deoxyguanosine in the supernatant fluid was determined by HPLC (36). Cell viability determined by trypan blue dye exclusion was >95% after 2-hr incubations.

LNSPN virus, the vector using a CMV promoter to express PNP (LNCPN) gave a 30-fold increase, whereas a construct using the viral LTR to promote PNP (LPNSN-2) resulted in a >200-fold increase in enzyme activity in infected enzyme-deficient skin fibroblasts. In enzyme-deficient human fibroblasts the LPNSN-2 virus provided PNP expression at levels about 10-fold greater than normal. Furthermore, we demonstrated that these phenotypically corrected PNP⁻ HDFs rapidly metabolized exogenous deoxyguanosine, the cytotoxic metabolite that accumulates in the plasma of PNP-deficient children.

These data permit the consideration of autologous skin fibroblasts as a target tissue for PNP gene transfer for use in gene therapy. The number of transduced fibroblasts needed to treat a patient can be calculated on the basis of data obtained in a study using erythrocyte transfusions to improve immune function in a PNP-deficient patient (5). The urinary excretion of PNP substrates in this patient before treatment was about 6 mmol/24 hr. This level of substrate could be effectively metabolized by about 5×10^8 infected skin fibroblasts. For this calculation we reduced the *in vitro* PNP activity we had measured at saturating substrate (200 μ M) to compensate for the lower reaction velocity anticipated *in vitro*, where substrate levels are normally <5 μ M (1, 33). Transfer of this number of fibroblasts is feasible by using several techniques (18).

The initial difficulty we encountered in making vectors that efficiently express PNP contrasts with the apparent ease of making such vectors for ADA expression. A factor is the greater specific activity of ADA, which necessitates the expression of 10-fold more PNP protein to achieve comparable activities of these enzymes. This helps explain the apparent poor performance of a given vector containing PNP in comparison to the same vector containing ADA, including vectors using a mouse metallothionein promoter (16) or the SV40 early promoter (ref. 18; this report) to express the inserted gene. As might be predicted, replacement of PNP with ADA in the LPNSN-2 vector results in very high level synthesis of ADA in infected cells, 100-fold more than is normally present (R. A. Hock, W.R.A.O., and A.D.M., unpublished results), showing the general utility of this vector for high-level gene expression.

The LPNSN-2 vector provided maximal PNP expression in human fibroblasts and also had a high titer, 3×10^6 cfu/ml. Such high titer is a prerequisite for efficient infection of human fibroblasts (18). The high titer of LPNSN-2 is in part

due to the presence of the ψ^+ signal in this virus. We do not know what role potential splicing between the LTR and the start of the PNP gene (Fig. 1) has on PNP expression, although RNA analysis shows that the level of such RNAs is much lower than of the full-length viral RNA. Inclusion of the ψ (350 base pairs) or ψ^+ (800 base pairs) signals between the LTR and the PNP gene results in similar levels of PNP expression, showing that the additional sequences in ψ^+ do not affect downstream translation. The sequences in ψ^+ include part of the Mo-MLV *gag* region; however, the start codon of *gag* has been removed by alteration from ATG to TAG, thus translation initiation at the normal *gag* start codon is prevented and therefore cannot interfere with initiation at the downstream PNP start codon.

We easily detected SV40-driven *neo* transcripts in LPNSN-1- and -2-infected cells, whereas we were unable to detect SV40-driven PNP transcripts in LNSPN-infected cells (Fig. 4). We believe that this effect is most likely caused by selection for *neo* expression during isolation of these cells. Indeed, G418-resistant colonies produced by infection with LPNSN-1 or -2 were less robust and grew more slowly than colonies produced by LNSPN or LNCPN, suggesting that *neo* expression from the SV40 promoter was not optimal and that there would be selective pressure for cells that overexpressed the SV40-*neo* RNA. No such selective pressure existed when PNP was driven by the SV40 promoter.

An advantage of studying PNP gene transfer as a model for gene therapy is the existence of an animal model where increased substrate metabolism by transferred cells can be measured. Canine erythrocytes are PNP deficient and, as a result, metabolism of exogenous deoxyguanosine is much reduced when compared with rats, for example, which have erythrocyte activities similar to those of humans (refs. 37, 38; unpublished data). We calculate that it is feasible to transfer sufficient human PNP to canine skin fibroblasts, which are infectable with the amphotropic vectors developed here, to cause changes in metabolism of exogenously added PNP substrates. This model provides an *in vivo* test of the ability of modified skin cells to clear pathogenic PNP substrates implicated in the human disease and will enable studies of the persistence of reintroduced fibroblasts and the effects of engraftment on vector expression.

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