A transmissible retrovirus expressing human hypoxanthine phosphoribosyltransferase (HPRT): Gene transfer into cells obtained from humans deficient in HPRT

(Lesch–Nyhan syndrome/hypoxanthine phosphoribosyltransferase cDNA/infectious virus/gene therapy)

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ABSTRACT A cDNA corresponding to the human gene for hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) has been ligated into murine retroviral vectors such that it is under the transcriptional control of viral long terminal repeats. Transfection of HPRT+ cells followed by superinfection with various helper viruses has led to the rescue of chimeric virus capable of transmitting the HPRT+ phenotype to HPRT− rodent or human cells. These genetically transformed cells contain authentic human HPRT at levels similar to normal HPRT+ cells.

The ability to introduce eukaryotic genes into mammalian cells has contributed much to our current knowledge of the mechanism and regulation of gene expression. A variety of methods have been used to transfer foreign genetic material into cells. The widely used calcium phosphate-mediated gene-transfer technique (1–3) is relatively inefficient and is limited to use in cultured cells. Introduction of foreign sequences by microinjection into target cells is efficient but requires sophisticated instrumentation (4). An alternative approach for transferring genes into cells involves the use of viral vectors (5). The commonly used papovavirus vectors such as simian virus 40 (SV40), however, have major limitations: (i) the length of the foreign DNA segment cannot exceed 2.5 kilobase pairs (kb) and (ii) papovaviruses have a limited host range (6). Because of their unique structure and mode of propagation, retroviruses appear to be ideally suited as gene-transfer vehicles. Some of their unique properties include (i) the viral genome (RNA) is efficiently transmitted to the recipient cells and integrated into the chromosome(s) as DNA (7), (ii) integration is specific with respect to the viral genome (7), (iii) the plasticity of the viral genome allows packaging of DNA inserts of up to at least 7 kb, (iv) retroviruses have a wide host range and can infect a variety of cell types (8), and (v) viral long terminal repeats (LTRs) provide efficient signals for initiation and termination of transcription (9, 10). Several groups have recently used retroviral DNA vectors to generate infectious virus containing either the herpes simplex virus thymidine kinase (TK) gene (11–13) or the Escherichia coli guanine phosphoribosyltransferase gene (14). We have been interested in the development of retroviral vector systems for gene replacement in human genetic disease and have recently reported the isolation and characterization of a functional and expressible full-length cDNA clone of the human hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) gene (15). Lesions in the HPRT gene are associated with the devastating Lesch–Nyhan syndrome and forms of gouty arthritis (16). Here, we report the construction of a retroviral DNA vector containing human HPRT cDNA. Virions containing HPRT as an integral part of the viral genome can be rescued from cells transfected with this vector and are capable of transferring expressible HPRT sequences into a variety of rodent and human cells.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. The 208F HPRT− clonal cell line was derived from Fischer rat cells by selection in 6-thioguanine (17). The BALB 3T3 HPRT− cell line 2TG0R (18) was a gift of H. L. Ozer (Hunter College, New York). The HPRT− rat cell line WA, derived from Wag-Rij rats, was a gift from M. Maszynsky (Case Western University) and G. Rosenfeld (University of California at San Diego). All cell lines, including NIH 3T3 TK− cells (12), SV40-transformed HPRT− Lesch–Nyhan (LNSV) cells (19), GM637 SV40-transformed normal (HPRT+) human fibroblasts (20), and D98 HPRT− HeLa cells (21) were maintained in Dulbecco–Vogt modified Eagle medium/10% fetal bovine serum (Sterile Systems, Logan, UT). HPRT− cells were selected in the same medium containing 30 μM hypoxanthine/1.0 μM amethopterin/20 μM thymidine (HAT medium).

Transfection. HPRT− cells were plated at 5 × 10^5 cells per 5-cm dish on day 1. On day 2, 0.5 μg of uncut recombinant plasmid DNA and 8 μg carrier DNA (from NIH 3T3 TK− cells) were transfected onto the cells by using the calcium phosphate precipitation procedure (1, 3). On day 3, the cells were split 1:5 into HAT medium and, every 3 days thereafter, the medium was replaced with fresh HAT medium. For quantitation, colonies were stained on day 9 with Coomassie blue (1 g/liter in 50% methanol/10% glacial acetic acid/40% water).

Virus Rescue. HPRT-expressing virus was rescued from cells using either Moloney murine leukemia virus (Mo-MuLV) (22), FBJ murine leukemia virus (FBJ-MuLV) (23), or 1504A amphotropic virus (24). Medium from helper virus-producing cells was filtered through a 0.45-μm filter and applied to cells at a multiplicity of about 1 in the presence of Polybrevine (Sigma) at 4 μg/ml. The cells were passaged 3 days after infection, medium was replaced with fresh medium on day 5, and supernatant was harvested on day 6 from nearly confluent cells. The supernatant was centrifuged at 5,000 × g for 7 min to remove

Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; SV40, simian virus 40; LNSV, simian virus 40-transformed Lesch–Nyhan cells; kb and kbp, kilobase(s) and kilobase pair(s); LTR, long terminal repeat; TK, thymidine kinase, HAT, 30 μM hypoxanthine/1 μM amethopterin/20 μM thymidine, Mo-MuLV and FBJ-MuLV, Moloney and FBJ murine leukemia virus, respectively.
cells, and aliquots of the supernatant were applied to 208F HPRT+ and NIH 3T3 TK− cells (plated the day before at 5 × 10^5 per 5-cm dish) in medium containing Polybrene at 4 μg/ml. HPRT+ 208F cells were scored as in the transfection assay, and Mo-MuLV and FBJ-MuLV were quantitated by using the XC plaque assay (25) on NIH 3T3 TK− cells. 1504A amphotropic virus is XC-plaque negative and was not quantitated.

RESULTS

Construction of Viral Vectors. HPRT-containing viral constructs were made as outlined in Fig. 1. The cDNA clone of the gene for human HPRT was obtained from the SV-40-based expression vector (p4aA8) in which it was initially isolated (15). Viral LTRs were linked to the HPRT cDNA such that the coding regions for viral proteins were either completely removed (gag and pol) or truncated (env); thus, no viral proteins would be expressed from the constructs. The 5′ LTR and adjacent sequences were derived from a cloned Mo-MSV provirus, pMSV-

![Diagram of viral vectors](image)

FIG. 1. Construction of retrovirus-HPRT hybrid plasmids. Recombinant plasmids were made using standard techniques (26, 27). Restriction enzyme abbreviations are P, Pst I; B, BamHI; H, HindIII; E, EcoRI; R, Rsa I; Hp, Hpa I; S, Sal I. To reduce unwanted ligation products, the indicated fragments were treated with bovine alkaline phosphatase (BAP). Cross-hatched segments represent HPRT cDNA, boxes with interior arrows represent viral LTRs with orientation indicated by the arrow (5′ → 3′); thin lines indicate viral sequences, thick lines indicate pBR322, and wavy lines indicate mouse cell sequences surrounding proviral DNA.

1L (28, 29), and the 3′ LTR and adjacent sequences were obtained from cloned unintegrated Mo-MuLV DNA, pMLV-1 (30, 31). Retained in the constructs are 5′ sequences presumably involved in packaging of viral RNA (32). The HPRT gene is situated so that the ATG start codon is in approximately the same position as the start codon for the gag polyprotein in the parental virus. Two potential polyadenylation signals exist near the 3′ end of the Pst I to BamHI HPRT cDNA fragment. Thus, in addition to the construct containing these sites (pLPLA), another construct was made in which these sites were removed and replaced with Mo-MuLV sequences (pLPL). In pLPL, the HPRT cDNA was joined to the viral env gene downstream of the env splice acceptor junction and start codon, so that pLPL should be transcribed as a single RNA species of about 4.0 kb, which would encode HPRT.

Rescue of Virus Expressing HPRT. In a transfection assay, recombinant plasmids pLPA, pLPAL, and pLPL conferred HAT resistance (HAT+) to HAT-sensitive (HAT-) HPRT− 208F rat cells. The efficiency of HAT+ colony production was the same
for these constructs as for the original SV40–HPRT hybrid plasmid p44a8, in which HPRT is expressed from a SV40 promoter (400–700 colonies per μg 10^6 cells). No HAT+ colonies were observed in control 208F cells.

Cells transfected with the HPRT-containing viral constructs are unable to produce virus because the coding regions for viral proteins necessary for viral replication have been deleted. However, it should be possible to rescue these defective viruses with replication-competent helper virus. In initial experiments, HPRT+ rat cells transfected with pL PAL or pLPL were infected with helper virus and the virus produced by these cells was able to confer HAT resistance to HPRT+ cells. Furthermore, transmissible HPRT-virus was also obtained when molecularly cloned helper virus DNA was cotransfected with pL PAL or pLPL (data not shown). Under both these conditions, the relative titer of HPRT-expressing virus was uniformly low (<1% of helper virus titer). Analysis of the transfected cells revealed a number of aberrant forms of viral RNA (see below), which may not be properly packaged or may produce noninfectious virions.

In an attempt to obtain high HPRT-expressing virus titers, we used the following strategy. HPRT+ rat cells were infected with HPRT-expressing virus obtained from helper virus-infected pL PAL- or pLPL-transfected rat cells. HAT+ clones that did not produce helper virus (nonproducer cells) were isolated. Nonproducer cell lines were superinfected with helper virus and the culture medium was assayed for HPRT-expressing virus and helper virus. As shown in Table 1, Mo-MuLV, FBJ-MuLV, and an amphotropic virus were able to rescue HPRT-expressing virus. Although HPRT-expressing virus could be rescued from cells infected with either L PL or LPL, the titer from cells infected with the construct (L PL) containing the HPRT polyadenylation signals was no more than 10% of that from which these signals had been deleted (L PL). Averaged over many experiments, the HPRT-expressing virus/helper virus ratio using the LPL-infected nonproducer line was about 10%.

The relatively low titers of HPRT-expressing virus rescued from 208F rat cells reflects the inefficient replication of murine retroviruses in rat cells. In another experiment, a HPRT- murine cell line (2T GOR) was infected with virus from rat 208F cells producing HPRT-expressing virus (LPL) and Mo-MuLV. HAT+ cells were selected and passaged once to allow for helper virus spread. Supernatant from a nearly confluent dish of these HPRT+ mouse cells had a titer of 2 × 10^5 HPRT-expressing virus and 2 × 10^5 of the Mo-MuLV helper virus (Table 1). Thus high titers of HPRT-expressing virus can be generated in an appropriate cell line.

Infection of Human Cells. The amphotropic helper virus used in the rescue experiments is able to infect human cells. We investigated its usefulness for transmitting the human HPRT-expressing virus into HPRT–human cells. All HPRT–human cells tested, including D98 HeLa cells, SV-40-transformed Lesch–Nyhan (LNSV) fibroblasts, and Epstein–Barr virus-transformed Lesch–Nyhan lymphoblasts, could be converted from HAT+ to HAT- by infection with culture medium from LPL-infected nonproducer rat cells superinfected with amphotropic virus. The efficiency of formation of HAT+ colonies was similar to that obtained for infection of 208F rat cells.

We analyzed the HPRT activity of the HPRT-expressing virus-infected LNSV cells to ascertain whether authentic human HPRT protein was present and at what levels. Several rat cell lines that had been transfected or infected with the HPRT-expressing viral constructs were also analyzed. Both the LNSV cells and rat 208F cells infected with HPRT-expressing virus contain HPRT activity that comigrates with authentic human HPRT from HeLa or GM367 cells and migrates differently from rat HPRT from WA cells (Fig. 2). Control LNSV cells and 208F HPRT+ rat cells have undetectable activity in this assay. The levels of enzyme activity in LNSV and rat cells infected with HPRT-expressing virus obtained from the LPL construct are similar to those of control HPRT+ human cells. 208F rat cells infected with HPRT-expressing virus from the LPAL construct display human HPRT activity but at significantly lower levels than the LPL-infected cells.

Analysis of RNA Transcripts. We analyzed RNA from cells either transfected or infected with the HPRT vectors (Fig. 3) to determine whether the RNA patterns corresponded to those predicted from the DNA organization of the vectors. We used two probes, one to HPRT cDNA and the other to the Mo-MSV LTR. The LTR probe does not hybridize to RNA transcripts from control 208F cells while the HPRT probe hybridizes slightly to control cell RNA. A complex pattern of RNA transcripts hybridizing to either the LTR or HPRT probe is observed in cells transfected with the vectors. RNA blot analysis of RNA from cells infected with HPRT virus, on the other hand, produced a readily interpretable pattern, because viral integration is precise with respect to the viral genome. In cells infected with HPRT-expressing virus from pL PAL-LPAL-transfected cells, two species of RNA hybridizing to both probes were identified (Fig. 3 A and B). The size of the larger RNA species corresponds to the full-length RNA transcript expected from L PAL (3.9 kb). The smaller species of RNA (1.6–1.9 kb) probably represents transcripts that initiated at the 5’ LTR but terminated at the putative polyadenylation signals in the HPRT cDNA.

![FIG. 2. HPRT enzymatic activity in cells transfected or infected with HPRT-containing constructs. Enzymatic activity of HPRT was detected in polyacrylamide gels as described (33) after electrophoresis of cell extracts on 7.5% polyacrylamide gels using 0.1 M Tris (pH 8.6) buffer. TX indicates transfection with the indicated plasmid, INF indicates infection with the indicated virus. All cells were helper virus-free except L PAL/FBJ-INF, which expresses FBJ-MuLV as well as L PAL.](image-url)
In cells, electrophoresed or the fragment. In HPRT\(\text{LPL}\)-expressing virus-infected cells only a single species of RNA hybridized to either the HPRT probe or the LTR probe (Fig. 3C and D). The size of this RNA corresponds to the full-length RNA expected from LPL (4.0 kb) if the normal retroviral transcriptional signals are used. The fact that all major species of RNA in HPRT-expressing virus-infected cells hybridize to both viral LTR and HPRT probes is consistent with the idea that transcripts initiate in the LTR and transcribe through the HPRT cDNA sequences.

Analysis of Integrated Chimeric DNAs. Retroviral DNA integrates into host chromosomal DNA in a fashion that maintains the colinearity of the viral genome. To determine the organization of chimeric proviral DNA in HPRT-expressing virus-infected cells, we analyzed the DNA in recipient LNSV cells by using restriction endonucleases (Fig. 4). The restriction endonuclease Sst I cleaves only once in each LTR (9) of LPL and does not cut in the SV40-based expression vector p4aA8. When high molecular weight DNA from LNSV cells was cleaved with Sst I and then hybridized to a HPRT probe, two fragments of about 14.5 and 7.2 kb were identified. These fragments presumably represent the endogenous HPRT gene of LNSV cells. In cells transfected with the HPRT expression vector p4aA8, an additional fragment of >25 kb was detected, which is consistent with integration of the p4aA8 plasmid in a tandemly repeated unit (36), which would not have internal Sst I sites. Sst I-cleaved DNA from LNSV cells either transfected or infected with LPL shows the control cell DNA fragments plus a single new fragment of about 3.9 kb, as expected. Thus, like other retroviruses, the HPRT-proviral DNA appears to have an uninterrupted 5' LTR-HPRT-LTR 3' organization.

DISCUSSION

Retrovirus Vectors. We have been interested in developing retroviruses as vectors for efficient gene transfer into eukaryotic cells. As a model system, we have constructed recombinant plasmids containing retroviral sequences linked to a human HPRT cDNA fragment. Transfection of such recombinant plasmids into HPRT\(^{-}\) rodent or human cells can convert them to HPRT\(^{+}\). A replication-defective retrovirus containing HPRT cDNA as an integral part of its genome can be rescued from transfected cells by infection with a variety of helper retroviruses. The HPRT-expressing virus can infect rodent and human cells and is present at about 10% of the helper virus titer. Titers as high as 2 \(\times\) 10\(^{6}\) HPRT-expressing virus per ml were obtained.

The presence of internal polyadenylation signals in the LPL retroviral genome can generate truncated RNA transcripts that may be efficiently packaged but will be unable to initiate synthesis of plus-strands of DNA (7). This would explain the lower efficiency of rescue in cells transfected with pLPAL. Similar observations have previously been made in constructs that contain the TK gene in spleen necrosis virus (11).

The size of the HPRT-expressing virus reported here is small, less than 4.5 kb, including both LTRs. At the 5' end of the virus, 1.8 kb includes the 5' LTR, tRNA binding site, presumed...
Fig. 4. Analysis of integrated retroviral packaging signal, and the HPRT cDNA. About 0.7 kb at the 3′ end includes the 3′ LTR and sequences involved in the synthesis of plus-strand DNA during viral infection. Thus, only 2.5 kb of the viral sequences are required for HPRT expression and virus rescue. Therefore, 6–7 kb of additional sequences could be added to the HPRT-encoding virus without exceeding the limit for retroviral genome size. Hence, this vector could be used as a vehicle for other genes and would carry a convenient and powerful selectable marker.

Gene Introduction in Viro. Transmissible viral vectors offer several potential advantages over other methods of gene transfer, including the possibility of studying the expression and fate of genes newly introduced into whole animals. It should also be possible to select and design vectors that would allow insertion of sequences into specific cells or organs in the body by taking advantage of specificity of infection of the helper virus. The ability of retroviruses to infect stem cells, such as the pluripotent bone marrow stem cells (37, 38), promises to allow stable gene insertion into a regenerating and permanent cell type.

The use of transmissible viral vectors raises questions about the effect of infectious helper virus on the recipient animal. The presence of helper virus may help in the body-wide dissemination of the specific gene carrying vector but also may lead to tumor development or other unanticipated illness. One way to overcome some of the potential problems is to design high-titer HPRT-encoding virus particles free of any helper particles. We have recently developed cell lines in which helper virus functions are provided but the production of infectious helper virus is prevented. Preliminary results indicate that infectious HPRT-encoding virus can be produced by such a cell line (unpublished data).

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