Expression of phosphoenolpyruvate carboxykinase (PEPCK) chimeras in renal epithelial cells

Retention of appropriate physiological responsiveness using enhancerless retroviral vectors

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We used an enhancerless U3 mutant retroviral vector to deliver chimeras of the phosphoenolpyruvate carboxykinase (PEPCK) promoter region to a renal epithelial cell line capable of expressing PEPCK mRNA. Chimeras consisting of the PEPCK promoter and chloramphenicol acetyltransferase, neomycin phosphotransferase or human growth hormone genes were expressed after viral infection of the NRK52E renal epithelial cell line. Virus-delivered sequences in which the direction of PEPCK promoter transcription was antegrade to the normal direction of the long terminal repeat (LTR)-initiated transcription correctly upon stimulation with dexamethasone or 8-bromo cyclic AMP and upon lowering of the extracellular pH. Fluorescent primer extension *in situ* using primers specific for virus-delivered sequences of antegrade constructs indicated that a large fraction of NRK52E cells could be infected by co-cultivation with virus-producing ψ -2 cells without G418 selection. Virus-delivered constructs whose orientation was opposite to that of the LTRs were expressed at very low levels, with transcripts detectable by PCR only in RNA from cyclic AMP-treated cells. Using reverse transcription/PCR, we demonstrated that the chimeric transcripts were from the internal PEPCK promoter rather than a functional or reconstituted Moloney LTR. PEPCK-reporter chimeras delivered by retroviral vectors demonstrated a level of expression more consistent with the level of expression of the native PEPCK gene than did transfected chimeras. This expression system should prove useful for studies of the physiological modulation of gene expression in renal tissues.

INTRODUCTION

The expression of the gene for phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32), which is expressed in liver and kidney, has been extensively investigated *in vivo*, in PEPCK-expressing cultured cell systems and in *in vitro* transcription systems [1–5]. Correlations with the physiologically regulated expression of this gene in liver have led to the identification of cyclic AMP (cAMP)- and glucocorticoid-responsive regions within the 5' and 3' flanking regions of the gene. Most studies have centred on the regulation of PEPCK expression in liver. For example, the immediate ~ 500 bp of the 5' flanking PEPCK region is sufficient to confer much of the hormonal specificity to chimeras expressed in hepatocytes as well as to correctly target expression of PEPCK chimeras in transgenic animals [6,7].

The renal cortex of several species, including the rat, expresses the PEPCK mRNA at high levels. Regulation of PEPCK gene expression in kidney shares cAMP- and glucocorticoid-sensitivity with hepatic expression, but lacks insulin-sensitivity [8]. The unique and perhaps most physiologically significant property of renal PEPCK gene expression is the rapid increase in transcription following induction of systemic metabolic acidosis. The kidney is also unique in that elevated rates of PEPCK transcription persist in acidotic states [1,8].

The identification of acidosis-responsive promoter regions which might mediate the pH-sensitivity of PEPCK transcription in renal cells has been hindered by the lack of a cultured renal epithelial cell model system which expresses PEPCK at levels comparable with those in the kidney or, by comparison, in the hepatoma cell lines which are generally used in PEPCK expression studies. The rat NRK52E renal epithelial cell clone is

gluconeogenic and basally expressed PEPCK at very low levels [9–11]. We have shown that PEPCK enhancer/promoter chimeras transiently expressed in NRK52E cells do retain cAMP- and glucocorticoid-responsiveness, as well as a degree of pH-responsiveness [12]. The level of transcription from such PEPCK chimeras was typically much higher than the level of native PEPCK transcription, thereby making extrapolation to the physiological regulation of PEPCK mRNA transcription ambiguous.

Retroviral vectors offer the advantage of delivery of a relatively uniform copy number per infected cell, as well as stable genomic integration. In the case of cDNAs delivered by these vectors, the proviral DNA flanking the insert is invariant, decreasing the likelihood of adventitious alteration of transcriptional properties by the site of genomic integration. Enhancerless or self-inactivating retroviral vectors have been described by several groups, and rely on a peculiarity of the Moloney retroviral life cycle. After retroviral RNA is reverse-transcribed within the infected cell, the enhancer-bearing U3 region of the viral 3' long terminal repeat (LTR) is transposed to the 5' LTR. A deletion of the enhancer in the 3' LTR U3 region can be propagated, appearing in the 5' LTR of the target cell. Thus the integrated proviral cDNA in the target cell will not contain Moloney transcriptional enhancers, yet an engineered internal promoter may still be active. This allows evaluation of enhancer/promoter activity in the absence of interference from the strong transcriptional activity of the retroviral LTR. Using this type of retroviral system, we demonstrate that PEPCK enhancer/promoter chimeras can be transferred into renal cells and expressed at levels more comparable with that of the native PEPCK gene, but retain pH-sensitivity in a context suitable for physiological study.

Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase; cAMP, cyclic AMP; SV40, simian virus 40; CAT, chloramphenicol acetyltransferase; HGH, human growth hormone; DTT, dithiothreitol; PBS, Hanks' phosphate-buffered saline; NEO, neomycin phosphotransferase; AMV, avian myeloblastosis virus; CRE, cyclic AMP-responsive element; GRE, glucocorticoid-responsive element; LTR, long terminal repeat.

Fig. 1. Schematic of the retroviral constructs described in these studies

The PEPCK promoter fragment extends from the first *EcoRI* site 5' to the transcription start site through the first *BglII* site 3' to the transcription start. The *HindIII*–*EcoRI* fragment of the pUC19 polylinker, which contains a *BamHI* site, was ligated to the *EcoRI* site of the promoter fragment. The map of pZH was redrawn from the map of pZIPNEOSVenH[−] provided by Dr. R. C. Mulligan, and is not to scale. The *BamHI* cloning site is located at the *PstI* site of MoMLV (base 739 [13,29] from the left end of the 5' LTR). The arrows denote the direction of transcription from the PEPCK promoter. The approximate locations of the 5' splice donor (5'SD), the 3' splice acceptor (3'SA) and the packaging signal (ψ) are schematically indicated. ori, origin of replication; Aⁿ, SV40 polyadenylation signal; B/B, *BamHI*/*BglII* ligation site.

MATERIALS AND METHODS

Materials

Rat renal NRK52E cells [9] were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The H4IIE cell line [2] was a gift from Dr. Carl Grunfeld (University of California, San Francisco, CA, U.S.A.). The retroviral vector pZIPNEOSVenH[−] was a gift from Dr. Richard Mulligan, Massachusetts Institute of Technology, Cambridge, MA, U.S.A. [13]. Cell culture medium was from GIBCO; DNA-modifying enzymes were from BRL and US Biochemical. Avian myeloblastosis virus (AMV) reverse transcriptase was from Life Sciences. Immunological reagents were from Zymed. All other reagents were from Sigma Chemical Co.

Plasmid construction and retroviral preparation

A genomic clone, λ PC1, bearing an ~15 kb insert consisting of three *EcoRI* fragments and containing the entire coding sequence of the rat PEPCK gene, was isolated from a rat genomic library as described [12]. A single *EcoRI* fragment containing almost the entire PEPCK gene was subcloned into pGEM7Zf (p λ PC1.1) and was the source of the promoter fragments described. A 529 bp fragment (*EcoRI*–*BglII*) containing the transcription start site as well as upstream cyclic AMP-responsive element (CRE) and glucocorticoid-responsive element (GRE) elements was isolated. Four retroviral constructs were created using this PEPCK 5' region to drive transcription of the neomycin phosphotransferase (NEO), the chloramphenicol acetyltransferase (CAT) and the human growth hormone (hGH) genes. These are shown in Fig. 1. The starting material was the plasmid pZH, which was derived from the plasmid pZIPNEOSVenH[−] [13] by removal of the *HindIII* site located outside of the retroviral

cassette. pZIPNEOSVenH[−] is a self-inactivating retroviral vector which contains, from 5' to 3': (1) an intact 5' LTR from the Moloney murine leukaemia virus, (2) a ψ packaging site and the Moloney 5' splice donor, (3) the 3' splice acceptor site surrounding a *BamHI* cloning site, (4) the TN5 NEO gene without eukaryotic promoters, (5) the simian virus 40 (SV40) and pBR322 origins of replication, and (6) the Moloney 3' LTR containing an *XbaI*–*PvuII* deletion in the U3 region [13,14].

Plasmid pZH-f-PCP-NEO was constructed by removing the *BamHI*–*BglII* fragment of the NEO gene and replacing it with a *BamHI*–*EcoRI* polylinker fragment (from pUC19) ligated to the *EcoRI*–*BglII* PEPCK 5' region described. This placed the Moloney 5' splice donor region just upstream from the PEPCK enhancer/promoter region.

Plasmid pZH-r-PCP-CAT was constructed by ligating the *HindIII*–*EcoRI* pUC19 polylinker, the *EcoRI*–*BglII* PEPCK 5' region and the *HindIII*–*BamHI* fragment of pTKCAT from which the TK promoter had been removed. Digestion with *BamHI* gave a cassette containing the 5' PEPCK region, the CAT gene and an SV40 polyadenylation signal. This fragment was ligated into the *BamHI* site of pZH. Recombinants were selected with the transcriptional orientation of the PEPCK–CAT chimera retrograde to the 5'→3' LTR direction. A similar plasmid, pZH-PCP-CAT/a, containing the PEPCK 5' region and a CAT cassette (from pCM4) without a polyadenylation signal, was created in which the PEPCK transcription direction was antegrade to the LTRs.

Plasmid pZH-r-PCP-GH was constructed by first ligating the *HindIII*–*EcoRI* pUC19 polylinker and the *EcoRI*–*BglII* PEPCK 5' region together in a holding vector, then deleting the *EcoRI* site by digestion and Klenow fill-in, excising the *HindIII*–*BglII* cassette and ligating this with the *HindIII*–*BamHI* fragment of

plasmid p0GH [15]. This plasmid contains a genomic HGH clone with a terminal *EcoRI* site at the 3' end of the HGH gene. The resultant plasmid, pPCP-2.0-GH[3' Δ E], was linearized with *EcoRI* and adapted with *BclI* linkers, and the PEPCK-HGH cassette was isolated from an agarose gel. This fragment was ligated into the *BamHI* site of pZH and recombinants were selected with the PEPCK-HGH transcriptional direction retrograde with respect to the LTRs.

The identity of each of these plasmids was verified both by restriction digestion and by limited double-stranded sequencing using primers 3' to the *BglII* site of the NEO gene or to the 3' Moloney splice acceptor region surrounding the *BamHI* cloning site.

Plasmids were introduced into the ecotropic ψ -2 packaging cell line [16] by electroporation [17], and stable transfectants were selected in 800 μ g of G418/ml (Gibco). The packaging cells were grown to confluence in 150 cm² flasks and the supernatant medium was filtered through a 0.22 μ m filter after addition of 8 μ g of polybrene/ml. The target NRK52E cells were infected with this virus by applying the virus-bearing medium to subconfluent cells for 24 h. After 48 h, the cells were selected in 800 μ g of G418/ml until resistant colonies appeared. The pooled colonies were maintained in 800 μ g of G418/ml.

For the CAT and HGH constructs, no dominant selectable marker which could be transduced by viral infection was expressed (see below), and a different strategy for viral infection was pursued. The appropriate ψ -2 producer cells, which had been selected in G418, were grown to ~90% confluence in 150 cm² flasks and then exposed to 12.5 μ g of mitomycin-C/ml in Hanks phosphate-buffered saline (PBS) for 2 h [18]. The monolayers were rinsed several times and NRK52E cells were added to achieve 5–10% confluence. After 24 h of co-cultivation, the cells were passaged. Passaging was repeated at 5 days, at which time only the NRK52E cells remained. The surviving cells were pooled and expanded. A portion of the pooled cultures was grown in the presence of G418. By this time, control cultures of mitomycin-C-treated ψ -2 cells alone had no surviving cells.

Analysis of transcription products

After experimental treatment, cell monolayers were rinsed with PBS at 4 °C and poly(A)⁺ RNA was prepared. For poly(A)⁺ RNA preparation, the medium was aspirated and a solution containing NaCl (500 mM), Tris/HCl, pH 7.5 (10 mM), EDTA (1 mM), SDS (1%) and 200 μ g of proteinase K/ml was added. The viscous homogenate was sheared by passage through a 25 gauge needle and incubated at 37 °C for 1 h. Oligo(dT)-cellulose (10 mg; Pharmacia, type 77F) was added and incubated for an additional 1 h at room temperature. The oligo(dT)-cellulose was washed three times with 0.1 M-NaCl/10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA/1% SDS, and poly(A)⁺ RNA was eluted with two aliquots of water at 60 °C.

Primer extension analysis of PEPCK-NEO chimeric transcripts was performed using a ³²P-end-labelled 25-mer primer whose 3' terminus was at the *BglII* cleavage site of the NEO cDNA. Approx. 5×10^4 c.p.m. of end-labelled primer was annealed to 10–15 μ g of poly(A)⁺ RNA at 60 °C in 30 μ l of 250 mM-KCl/1 mM-EDTA/10 mM-Tris/HCl, pH 8.3, for 4 h. The mixture was brought to 37 °C, and 70 μ l of a mixture containing MgCl₂ (10 mM), Tris/HCl, pH 8 (10 mM), dithiothreitol (DTT; 3 mM), actinomycin D (100 μ g/ml), dNTPs (300 μ M), and AMV reverse transcriptase (100 units/ml) was added. After incubation at 37 °C for 2 h, the mixture was precipitated with 3 vol. of ethanol/0.5 M-ammonium acetate at –70 °C. The pellet was rinsed with ethanol and resuspended in 6 μ l of 100 mM-NaOH/1 mM-EDTA for 1 h. Samples were analysed on an 8%

acrylamide/7 M-urea sequencing gel, along with an M13 ladder labelled with [³⁵S]dATP. Dried gels were exposed directly to Kodak XAR film using one intensifying screen at –70 °C.

Rare RNAs were analysed by PCR of first-strand reverse-transcribed products. The PCR primers and oligonucleotide probes used for these studies were as follows. All PCR probes to the 5' end of PEPCK fusion products used the sequence 5'-CGGTTGGCCTTCCCTCTGGGAACAC (**pep-1*), corresponding to the sequence beginning at +1 of the normal PEPCK transcript. To detect the authentic PEPCK transcript, the 3' primer was 5'-GCATGATGACCTTGCCCTTATGCTC, i.e. the inverse complement of the PEPCK cDNA beginning 25 bases 3' from the second internal *PstI* site of PEPCK cDNA. The sequence 5'-GCTTTCTCGAAGTCCTCTTCTGACA, corresponding to the inverse complement of the PEPCK cDNA at position +517, was end-labelled with [γ -³²P]ATP and used as a probe of Southern blots of PCR products. For the PEPCK-HGH fusion product *pep-1* was used as the 5' primer, and 5'-AGAGCGCACTGCACGATGCGCAG, corresponding to bases 690–668 of the predicted HGH cDNA (bases 1505–1481 of the genomic HGH clone relative to the transcription start), was used as a 3' primer. The HGH fusion product was probed with 5'-GAAGAAGCCTATACCCAAGGAAC, corresponding to bases 232–254 of the HGH cDNA. For the PEPCK-CAT fusion product, *pep-1* was used as the 5' primer, 5'-TTGTAATTCATTAAGCATTCTGCCG was used as the 3' primer, and 5'-CCGGCAGTTTCTACACATATATTCG was used as a probe. For the PEPCK-NEO fusion product, *pep-1* was used as the 5' primer, 5'-AACTCGTCAAGAAGGCGATAGAAGG was used as the 3' primer, and 5'-ATCGAGCGAGCACGTA CTGGATGG was used as a probe. In order to probe reverse-transcribed RNA for a full-length genomic MoMLV transcript originating from regions 5' to the inserted sequences, the primer 5'-CGGTTTGGGACCGAAGC [corresponding to bases beginning at +527 (near the 5' splice donor) of intact MoMLV virus] was used as the 5' primer. 5'-TCAGGGTCAGGGAAGTT, corresponding to the inverse complement of bases 5456–5440 (near the 3' splice acceptor) of MoMLV [29], was used as 3' primer. This site had been mobilized to the *BamHI* cloning site in the parent vector.

Poly(A)⁺ RNA (0.1–1 μ g) was reverse-transcribed using random hexamers (5 μ g/50 μ l reaction) in a 50 μ l volume containing Tris/HCl, pH 8.3 (50 mM), KCl (50 mM), MgCl₂ (3 mM), dNTPs (500 μ M each), DTT (10 mM), BSA (50 μ g/ml), AMV reverse transcriptase (200 units/ml), Triton X-100 (0.01%, v/v) and actinomycin D (100 μ g/ml) at 40 °C for 2 h. Aliquots of the reverse-transcription reaction were amplified with Taq polymerase for 35 cycles using 18- or 25-mer primers in a thermal cycler (Ericomp). The buffer was as described above, except that the magnesium concentration was 1.5 mM and the dNTP concentration was 200 μ M (each); actinomycin and DTT were omitted. The PCR parameters included a 10 s denaturation at 94 °C, a 10 s annealing time at 56 °C (50 °C for the 18-mer retroviral splice primers), and a 1 min extension time. Amplification products were chloroform-extracted, precipitated with 1 vol. of propan-2-ol, electrophoresed in agarose gels and transferred to nylon membranes. The Southern blots were probed with ³²P-end-labelled 25-mer oligonucleotides which were distinct from the PCR primers and designed to hybridize with an internal portion of the predicted amplified sequence.

In situ primer extension

Cells expressing the CAT message after retroviral infection with pZH-PCP-CAT/a were grown on etched 12 mm-diam. round glass coverslips. Cultures were processed prior to con-

fluence when discrete colonies were identifiable. Cells were fixed for 20 min at 22 °C in 4% buffered paraformaldehyde, rinsed, dehydrated in 50% and 75% ethanol and air-dried, followed by rehydration in 100 mM-Tris/HCl, pH 7.5. A CAT primer was dissolved at 100 ng/ml in 25% formamide/6 × SSC/1 × Denhart's solution with 100 µg of salmon sperm DNA/ml and annealed at 37 °C for 24 h to the fixed cells. Controls consisted of: (1) cultures with no primer, (2) a primer to HGH sequences (not expected to be present), and (3) NRK52E cells which had not been retrovirally infected. After annealing, excess primer was removed by three washes with 1 × SSC at room temperature and one 5 min wash at 55 °C. Primer extension was carried out at 37 °C for 2 h by adding a solution containing KCl (40 mM) Tris/HCl (10 mM, pH 8), actinomycin D (100 µg/ml), MgCl₂ (10 mM), dCTP, dGTP and dTTP (100 µM each), biotin-7-dATP (15 µM), DTT (3 mM), and AMV reverse transcriptase (200 units/ml). The coverslips were washed five times with 1 × SSC at 22 °C and with 0.1 × SSC at 55 °C for 5 min. Coverslips were rinsed with PBS, and blocked with PBS containing 0.1% crystalline BSA and with 300 µg of non-immune rabbit IgG/ml. Affinity-purified goat anti-(biotin IgG) (Zymed, 1:100) in PBS/0.1% BSA was added and incubated overnight at 4 °C. The coverslips were washed several times with PBS and affinity-purified biotinylated rabbit anti-(goat IgG) (1:100) in PBS/0.1% BSA was added and incubated overnight at 4 °C. After several washes with PBS, fluorescein-streptavidin (Zymed; 1:50 in PBS) was added for 30 min in the dark. The coverslips were extensively washed in PBS, mounted and examined by epifluorescence microscopy.

Cell culture

Both the NRK52E and ψ -2 cell lines were grown in Dulbecco's modified Eagle's medium containing glucose (25 mM), fetal bovine serum (5%), Nu-Serum (Collaborative Research; 5%), minimal essential medium (Eagle's) premix (Collaborative Research), Hepes (12.5 mM), NaHCO₃ (24 mM), 1 × MEM non-essential amino acids, penicillin (100 units/ml) and streptomycin (100 µg/ml) in 5% CO₂/air. Experimental media were serum-free and contained Hepes buffer (25 mM) instead of NaHCO₃.

RESULTS

The NRK52E cell line was selected as a renal cell model for the pH-sensitive induction of PEPCK mRNA for several reasons. Most importantly, this cell line is gluconeogenic and retains pH-sensitive ammoniogenesis; both are properties of the kidney which are pH-sensitive *in vivo* [9–11]. We have previously shown that PEPCK promoter/reporter constructs transiently expressed in these cells retain hormonal and pH-sensitivity of transcription from the PEPCK promoter [12].

We used PCR of first-strand reverse-transcribed mRNA from the NRK52E cell to semi-quantitatively assess the presence and level of PEPCK message in NRK52E cells. Although expressed at too low a level to be probed on Northern blots, a PEPCK PCR product is demonstrated in Fig. 2. For comparison, an equal amount of mRNA from the H4IIE rat hepatoma cell line, which expresses high levels of endogenous PEPCK mRNA, was reverse-transcribed, amplified and probed on a Southern blot (Fig. 2). Although the reverse-transcription/PCR procedure we followed was not designed to be quantitative, it is clear that endogenous PEPCK mRNA is present in the NRK52E cell line.

After transfection into ψ -2 cells and G418 selection, all four of the described retroviral constructs (Fig. 1) produced G418-

resistant ψ -2 cells. Of the four constructs, only pZH-f-PCP-NEO had a functional eukaryotic promoter driving NEO transcription. As a consequence, only the viral supernatants from the pZH-f-PCP-NEO-transfected ψ -2 cells were able to transduce G418 resistance to NRK52E cells at high titre, yielding ~10⁴ G418-resistant colonies/ml of viral supernatant from mixed cultures. The pZH-PCP-CAT/a construct resulted in a rare G418-resistant NRK52E cell after co-culturing with mitomycin-C-treated ψ -2 producer cells.

We evaluated the pH- and hormonal sensitivity of the ante-grade NEO construct. RNA was prepared from controls and NRK52E cells infected with ψ -2 pZH-f-PCP-NEO supernatant and selected in G418. Parallel cell cultures were exposed to 8-bromo-cAMP (0.1 mM), dexamethasone (10⁻⁸ M) or low pH (6.8) for 12 h in Hepes-buffered media. Primer extension analysis of the PEPCK-NEO chimeric message was performed using a primer whose 3' end terminated at the *Bgl*II site of the NEO cDNA.

Under basal conditions (i.e. without hormones or at low pH), the predominant primer extension product of RNA from NRK52E cells infected with pZH-f-PCP-NEO was initiated prematurely at +54 bases relative to the normal initiation site of PEPCK mRNA [19] (Fig. 3). Dexamethasone and 8-bromo-cAMP, agonists which are known to interact with regions of the PEPCK promoter used, produced a significant proportion of transcripts which were initiated correctly at +1. Similarly, exposure of cells to low pH also produced correctly initiated transcripts. This suggests that within the relatively fixed environment of the integrated enhancerless proviral cDNA, lowered pH is as effective as cAMP or glucocorticoids in generating correctly initiated transcripts from the PEPCK promoter.

Retroviral integration may occur at many locations in the genome of infected cells. However, with selection, as was done with NEO resistance in this population, clones with a limited number of integration patterns may predominate [6,20,21]. Thus, only clones with effective basal NEO transcription from the

Fig. 2. Demonstration of PEPCK mRNA in NRK52E cells

Poly(A)⁺ RNA from both H4IIE hepatoma cells and NRK52E rat renal cells was prepared and reverse-transcribed using random hexamers as primers. An aliquot of the first-strand cDNA was amplified using Taq polymerase and the primer pair 5'-CGGTTGGCCTTCCCTCTGGAACAC and 5'-GCATGATGACCTTGCCCTTATGCTC. PCR products were electrophoresed, transferred to nylon membranes and probed with a unique internal ³²P-labelled oligonucleotide (5'-GCTTCTCGAAGTCCTTCTTGACA). (a) Relative locations of the priming pair on the PEPCK RBA; (b) 1 and 24 h autoradiographic exposures of Southern blots of PCR products from H4IIE (lane 1) and NRK52E (lane 2) cells, which were probed with the labelled oligonucleotide.

Fig. 3. Primer extension of poly(A)⁺ RNA from G418-resistant NRK52E cells infected with pZH-f-PCP-NEO virus

NRK52E cells infected with virus from pZH-f-PCP-NEO-transfected ψ -2 cells were selected in G418 and resistant colonies were pooled. Confluent cells were exposed to control Hepes-buffered medium, pH 7.4 (A, B), pH 6.8 (C), 8-bromo-cAMP, 0.1 mM (D), dexamethasone, 10 nM (E) or 8-bromo-cAMP + dexamethasone (F) overnight. Poly(A)⁺ RNA was prepared, and primer extension with a primer whose 3' end was at the *Bgl*III site of the NEO gene was performed. Products were analysed on an 8% acrylamide sequencing gel.

PEPCK promoter would have been selected. In order to qualitatively evaluate the effectiveness of viral infection and expression in the absence of a selectable marker, we used the construct pZH-PCP-CAT/a, with the PEPCK promoter directing transcription of a CAT cassette in a direction antegrade to the viral LTR. After transfection and G418 selection of ψ -2 cells, we infected the NRK52E recipient cells by co-cultivation as described above, and then passaged the surviving NRK52E cells at least three times after all ψ -2 cells in parallel cultures had been eliminated.

In order to assess the fraction of recipient cells infected, we performed *in situ* primer extension analysis to determine the efficiency of expression. Using a primer complementary to the prokaryotic CAT sequence, primer-dependent reverse transcription was used to incorporate biotinylated dATP. We detected the biotin incorporated into the reverse-transcribed cDNA with a double-antibody technique combined with streptavidin-

fluorescein. Cells were plated and prepared for labelling so that separate single-cell-derived colonies were discernible. Using a primer specific for the CAT gene, the majority (> 80%) of the colonies inspected proved to be positive for CAT-specific primer extension products (Fig. 4). One notable feature of this technique is the significant degree of subcellular mRNA localization obtained. Most signal was present in a granular, asymmetrical perinuclear distribution (Figs. 4b and 4c). There was no nuclear fluorescence, excluding hybridization to genomic DNA. When either no primer or an irrelevant HGH primer was used, or when the CAT primer was used in uninfected cells, no fluorescence was observed (Fig. 4). The effective viral titre obtained and the efficiency of the internal promoter was sufficient to detect CAT primer-dependent reverse transcription products in most cells without further selection.

The expression of cDNA from internal promoters inserted in retroviral vectors may show a dependence on the direction in which the insert is cloned within the retroviral vector. Such directional dependence may result from several features of the retroviral life cycle. Features such as transcription terminators or polyadenylation signals may prevent full-length genomic transcription from the retroviral LTR [22,23]. The presence of known or cryptic splicing signals may produce unexpected or truncated transcripts. To evaluate expression of PEPCK promoter/reporter constructs whose transcriptional direction was opposite that of the retroviral LTR, two additional constructs were evaluated.

pZH-r-PCP-GH contained a genomic HGH clone with the PEPCK promoter, including both the introns and the native polyadenylation region. This construct could only confer NEO resistance to ψ -cells in the retrograde direction, because the polyadenylation signal would have prevented full-length viral transcripts. [This was tested with the forward construct, which yielded no G418-resistant ψ -2 cells (results not shown)].

An analogous retrograde construct using the CAT gene as a reporter was made. pZH-r-PCP-CAT included the same PEPCK promoter region with a CAT gene and the polyadenylation signal from SV40 (Fig. 1). ψ -2 cells were transfected with these retrograde constructs and selected in G418. Supernatant medium from ψ -2 producer cells transfected with either of the retrograde constructs did not transduce G418 resistance in NRK52E cells. Since a viral titre could not be determined for either of these

Fig. 4. Detection of retrovirally derived CAT transcripts at the single-cell level

NRK52E cells, infected by co-cultivation with mitomycin-C-treated pZH-PCP-CAT/a-transfected ψ -2 cells, were plated so that single-cell-derived colonies were still discernible, and fixed at this degree of confluence. *In situ* reverse transcription from a CAT-specific primer was performed incorporating biotin-7-dATP. The incorporated biotin was detected with a double antibody system terminating with streptavidin-fluorescein and photographed under epifluorescence illumination. (a) Control uninfected NRK52E cells, (b) NRK52E cells co-cultured with ψ -2 cells producing pZH-PCP-CAT/a, (c) detail of centre photo. The control and experimental photographs were taken with the same illumination and exposure time. The final magnification is $\times 300$ for (a) and (b) and $\times 1000$ for (c).

Fig. 5. Detection of CAT transcripts from pZH-r-PCP-CAT-infected NRK52E cells

NRK52E cells, infected by co-cultivation with ψ -2 cells stably transfected with pZH-r-PCP-CAT, were exposed to 100 μ M-8-bromo-cAMP (lane 1), to pH 6.8 medium (lane 2) or to control medium (lane 3) for 12 h. Poly(A)⁺ RNA from these cells was reverse-transcribed and amplified with primers to detect the PEPCK-CAT chimera. PCR products were electrophoresed, blotted to nylon membranes and probed with a CAT internal ³²P-labelled oligonucleotide.

Fig. 6. Detection of HGH transcripts from pZH-r-PCP-GH-infected NRK52E cells

The experimental conditions and design was the same as in Fig. 5. The cells used were co-cultured with ψ -2 cells transfected with pZH-r-PCP-GH. Lane 1, 100 μ M-8-bromo-cAMP; lane 2, pH 6.8 medium; lane 3, control medium.

constructs, NRK52E cells were infected by 24 h of co-cultivation with mitomycin-C-treated ψ -2 producer cells which had been grown to confluence. After co-cultivation and passage until no mitomycin-C-treated ψ -2 cells remained, the residual NRK52E cells were evaluated for evidence of retroviral infection. No CAT activity or secreted HGH was detectable in NRK52E cells co-cultured with pZH-r-PCP-GH- or pZH-r-PCP-CAT-transfected ψ -2 cells. Similarly, Northern blots using up to 10 μ g of poly(A)⁺ RNA from co-cultured NRK52E cells failed to detect CAT or HGH messages.

To pursue the expression of the reporter gene messages, PCR of reverse-transcribed RNA was again used. Poly(A)⁺ RNA was prepared from control cells, cells treated with 8-bromo-cAMP or cells exposed to low pH for 12 h. The RNA was reverse-transcribed with AMV reverse transcriptase using random priming, and the resultant cDNA was amplified using the primer strategy indicated in Figs. 5 and 6. Southern blots of the PCR

Fig. 7. Detection of transcripts initiating from the viral LTR

Poly(A)⁺ RNA from ψ -2 producer cells and NRK52E cells infected with pZH-f-PCP-NEO was reverse-transcribed and amplified using primer pairs designed to detect transcripts originating from outside the initiation sites of the internal promoter. Lane 1, virus-producing ψ -2 cell line; lane 2, NRK52E cell line infected with the virus pZH-f-PCP-NEO. Pictured is a 24 h radiograph of a Southern blot of PCR products probed with a unique ³²P-labelled NEO oligonucleotide. 5'SD, 5' splice donor site.

products were probed with unique internal ³²P-end-labelled oligonucleotides. Transcription of the CAT and HGH genes from pZH-r-PCP-CAT- (Fig. 5) and pZH-r-PCP-GH- (Fig. 6) infected cells was only detectable in cells treated with 8-bromo-cAMP. Although the PCR procedure was not quantitative, prolonged exposure of the blots failed to show an amplification product in the low-pH-treated cells. We also used a 25-mer 5' PCR primer beginning at +50 bases from the transcription start of the PEPCK promoter and extending to the *Bgl*II cloning site. Amplification using this primer would demonstrate a prematurely initiated transcript, as was found in the primer extension experiments on the NEO construct. Although this primer pair detected a transcript in 8-bromo-cAMP-stimulated cells, none could be found in control cells (results not shown).

The method we used for detection of transcripts of the inserted cDNA does not differentiate between transcription from the PEPCK promoter and transcription from an unexpectedly functional viral LTR (in the case of the HGH construct, the genomic and spliced sizes of the HGH product would be different). Reconstitution of a functional LTR could have come about through a rare recombination event with the integrated ψ -defective provirus in the packaging cell line [24]. Thus additional measures were necessary to confirm that we were not detecting genomic transcripts.

To verify the absence of full-length viral transcripts, reverse-transcribed RNA from both virally infected NRK52E cells and the corresponding ψ -2 producer cells was amplified using primers expected to only be contained in a genomic transcript originating from a functional 5' viral LTR. In the case of the HGH and CAT vectors, the specific primers annealing at the 5' splice donor and 3' splice acceptor regions would be expected to amplify sequences inserted into the *Bam*HI site and to be present in an unspliced, full-length genomic transcript. Although the expected PCR product was found in ψ -2 cells bearing pZH-r-PCP-GH, no such product was found in the infected NRK cells (Fig. 7). Similarly, amplification between the 5' splice donor and an internal NEO sequence demonstrated the expected hybridization product in the pZH-f-PCP-NEO-transfected ψ -2 cells, but not in the NRK52E recipients (Fig. 8). Not shown are similar amplifications of reverse-transcribed RNA from pZH-r-PCP-CAT using the 5' and 3' splice region primers, in which no full-length viral

Fig. 8. Detection of transcripts initiating from the viral LTR

The same procedure as detailed in Fig. 7 was used for ψ -2 and NRK52E cells infected with pZH-r-PCP-GH using the appropriate primers. Pictured is a 24 h radiograph of a Southern blot of PCR products probed with a unique ^{32}P -labelled HGH oligonucleotide. Lane 1, virus-producing ψ -2 cell line; lane 2, NRK52E cell line infected with pZH-r-PCP-GH. 5'SD, 5' splice donor site; 3'SA, 3' splice acceptor site.

transcripts were detectable in infected NRK52E cells. Thus the inserted cDNA transcription products detected by PCR did not arise through genomic transcription via a transcriptionally competent LTR, but were indeed derived from the infected construct containing an internal PEPCK promoter.

DISCUSSION

We have used a self-inactivating retroviral vector to deliver three reporter genes to a renal epithelial cell line. The NRK52E line expresses PEPCK mRNA at very low levels; however, it is capable of expression from transfected PEPCK enhancer constructs in a cAMP- and characteristically pH-sensitive manner [8]. The assay we used was either primer extension or a reverse transcription/PCR technique which would detect a correctly initiated transcript. In each case, the reporter genes (CAT, NEO and HGH) were under the transcriptional control of an enhancer/promoter fragment of the PEPCK gene. Enhancerless retroviruses yielded low levels of transcription from the PEPCK promoter, while there was no detectable transcription from the disabled retroviral LTR. However, the transcriptional orientation of the insert relative to the retroviral 5' splice donor and the LTR affected both the level and the responsiveness of transcription. Viruses containing the PEPCK-reporter chimera in an antegrade direction were expressed at higher levels than were promoter/reporter genes expressed in a retrograde direction. Most significantly, the forward constructs displayed hormone- and pH-sensitive initiation of transcription, in which a correctly initiated transcript appeared under circumstances known to induce transcription of the PEPCK gene *in vivo*. The retrograde constructs did display cAMP-sensitive transcription, but there was no transcript evident under basal conditions.

There have been a number of reports using retroviral vectors to achieve regulated expression from internal promoters. In most reported studies, a dominant selectable marker driven either by the Moloney LTR or by an internal promoter has been used to select a cell population expressing at least one virally transduced property. However, interactions between several promoters within a retroviral construct may be complex. Selection for one marker, e.g. G418 resistance, may result in a skewed population in which expression from a non-selected promoter is suppressed [25].

Hatzoglou *et al.* [6], reported regulated expression from the PEPCK promoter in a retroviral vector expressed in hepatoma cells which normally express PEPCK mRNA. Using a Moloney-based vector with functional LTRs but without internal splice sites, they found that the major transcript originated from the PEPCK promoter regardless of its position or orientation. In addition, the promoter retained the pattern of hormonal responsiveness seen with conventional transfection experiments. The vector they used would have produced a proviral cDNA with an intact 5' LTR promoter. In subsequent studies of promoter competition within the context of the same retroviral vector, these workers demonstrated a complex pattern of promoter competition between the PEPCK promoter and various viral promoters introduced between the functional Moloney LTRs. This pattern was dependent both on chromosomal integration sites in individual G418-resistant clones and on the nature and number of internal promoters.

Because of promoter competition between the LTR and internal promoters, there have been a number of reports using self-inactivating vectors whose integrated proviral cDNA contains deletions in the 5' LTR which arise when the U3 region of the 3' LTR of the plasmid form is duplicated. These proviral forms allow expression from an internal promoter without interference from LTR promoter activity. In two recent studies, these promoter-deficient vectors have been used to assess expression from both internal viral promoters and of the inducible interferon promoter in two cell lines. Engelhardt *et al.* [25] used enhancerless vectors with both internal SV-neo and the human β -interferon enhancer cDNA to express human β -interferon in murine interferon-producing cells. These studies did indicate that expression from a self-inactivating vector, in which the integrated proviral 5' LTR was disabled, was associated with an enhanced level of expression from an inducible promoter. Some of the retroviral constructs demonstrated inducible expression of human β -interferon; however, examination of several permutations of direction of transcription and orientation of the enhancer element failed to produce a general rule about the internal requirements for inducible expression.

Soriano *et al.* [26] also systematically examined the effect of both LTR inactivation and the presence of a partial *gag* sequence on expression of either the *neo* or the β -galactosidase gene from three constitutive internal promoters. Although differences in the level of expression were found, these authors could derive no general rule for predicting the effect of internal structure on expression.

In the present study, when the PEPCK chimeras were introduced in the antegrade direction relative to the Moloney LTRs, basal transcription was evident. The NEO construct yielded sufficient amounts of transcription products to confer G418-resistance and be seen on Northern blots (results not shown). Although the transcript abundance did not rise when hormonal agonists were applied, the primer extension analysis indicated that lowered pH, a physiological stimulus for renal PEPCK transcription *in vivo*, was associated with an increase in correctly initiated transcripts.

The antegrade CAT construct was also capable of expression in a large proportion of cells, as indicated by the *in situ* primer extension studies. The direct demonstration of transcribed elements in cells without selectable markers may be a useful application of this technique. In addition, the specificity and greater amplification derived from *in situ* primer extension, as opposed to conventional *in situ* hybridization, permits the reliable identification of non-abundant or even rare transcripts.

The position of the insert in the retroviral vector may influence the level of expression, beyond the fact that the NEO constructs were selected for NEO transcription with G418. Mulligan and

co-workers used a similar defective LTR vector to infect either embryonal or haematopoietic cells and noted that the presence of a viral splice donor and part of the *gag* coding sequence 5' to the promoter-bearing inserted sequence significantly increased the apparent viral titre from an internal promoter driving the NEO gene [27]. The vectors we used in our studies also contain the 5' splice donor and ~ 120 bases of the *gag* sequence. Although the titre of our non-selectable viral constructs could not be evaluated, it is possible that the direction of cloning relative to the 5' splice donor is important in expression from enhancerless vectors. It is also possible, as suggested by Kreigler *et al.* [28] that splice accepters (possibly cryptic) within the insert may have interacted with the splice donor to decrease the effectiveness of transcription.

Overall, the level of expression from these integrated viruses was lower than we had observed in this cell line using transient or stable transfection of similar constructs in which the PEPCK promoter directed transcription ([12]; results not shown). It is significant that the expression from the antegrade viruses is more representative of the low level of native PEPCK gene expression in these cells than is transient or stable expression from transfected plasmid vectors. The finding that the NEO construct correctly initiated in response to physiological stimuli, including lowered pH, indicates that the responsiveness of this promoter fragment in renal cells is retained in this delivery system.

Retroviral vector systems have proved to be versatile for a variety of applications in which reliable high-level expression of exogenous sequences, or long-term stable integration, is needed. In this study we demonstrate that enhancerless retrovirus-delivered chimeras retain cell-dependent transcriptional properties similar to those of the native gene donating the promoter. This finding even extends to the low level of transcription characteristic of the parent renal epithelial cell. However, the physiological responsiveness of the PEPCK promoter to glucocorticoids, cAMP and low pH is retained in this epithelial cell. Co-cultivation of renal epithelial cells with virus-producing ψ -2 cells resulted in detectable infection and expression by the majority of epithelial cells. The high frequency of detectable infection occurred even in the absence of G418 selection. The use of enhancerless retroviral vectors may thus allow a more realistic evaluation of transcriptional properties of a promoter chimera in renal epithelial cells than does conventional transfection.

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