Hormonal Regulation of Chimeric Genes Containing the Phosphoenolpyruvate Carboxykinase Promoter Regulatory Region in Hepatoma Cells Infected by Murine Retroviruses*

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Hepatoma cells were infected with replication-incompetent murine retroviruses containing the selectable gene for amino-3'-glycosyl phosphotransferase (neo) and/or the nonselectable gene for bovine growth hormone (bGH). Expression of these genes was controlled by the promoter regulatory region of the gene for the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK) from the rat, which contains hormone and tissue-specific regulatory elements. Expression of the transduced PEPCK-neo gene was stimulated by BtCAMP and glucocorticoids and inhibited by insulin. The amount of RNA which initiated within the retroviral 5′ long terminal repeat (5′ LTR) was inhibited when internal promoters were present in the retroviral vector. When no internal promoter was present, expression from the 5′ LTR was higher and stimulated by glucocorticoids, due to the presence of a glucocorticoid regulatory element in the 5′ LTR. Infection of cells with retroviruses altered the basal expression and hormonal regulation of the endogenous PEPCK gene, but had no effect on the expression of the tyrosine aminotransferase gene, which is regulated in a similar manner by cAMP and glucocorticoids. A segment of the PEPCK promoter acted as a hormonally regulated enhancer, bringing the SV40 early promoter under the control of BtCAMP. A second, nonselectable gene (PEPCK-bGH), contained in the retroviral vector together with PEPCK-neo, was expressed and regulated appropriately when introduced into hepatoma cells. The proviruses were initially integrated randomly into the host cell genome, but after prolonged selection for expression of the transduced PEPCK-neo gene, cells were selected which contain a predominant site(s) of integration. Among populations of cells, however, the predominant site(s) of proviral integration was different. The selection of cells with a specific site of integration from a population was accelerated by the presence of PEPCK promoter sequences in the provirus. Despite the need to better characterize their effects on the host cell, retroviruses appear to be versatile tools for the specific introduction of regulated genes into cells.

The liver plays a central role in metabolic processes including the synthesis and storage of body fuels, the metabolism and modification of drugs, toxins, and waste products, and the synthesis of serum proteins. There are a variety of liver-specific genetic disorders, resulting from single gene defects affecting one or more of these pathways, making the liver an important target for gene transfer and genetic modification. Retroviral infection has been used to efficiently transfer single copies of intact genes into a variety of mammalian cell lines (for review see Ref. 1). A series of highly efficient retroviral vectors have been developed, which use the long terminal repeats (LTRs) of the Moloney murine leukemia virus. The viral gag, pol, and env genes can be expressed in trans in packaging cell lines, such as 2/2 cells (2), to produce defective retroviruses capable of a single round of infection of murine cells. Recombinant retroviruses have been used to transfer genes into primary cultures of pluripotent bone marrow stem cells (3–9), fibroblasts (10–13), and keratinocytes (14), and often these genes were expressed. However, when these cells were transplanted into animals, the level of expression of the gene of interest was found to be low in some cases (3–7), or the transplanted cells lost the ability to express the gene (13–15). More information on the behavior of genes introduced into different cell types by retroviral infection is required in order to maximize the expression of transferred genes.

In order to study the properties of genes transferred into hepatoma cells by retroviral infection, we have designed a series of recombinant retroviral vectors, containing a strong, liver-specific promoter which is regulated by hormones. We have used the promoter regulatory region of the gene for the cytosolic form of P-enolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK) to drive the expression of two linked genes: the gene for the selectable marker amino-3'-glycosyl phosphotransferase (neo) and the gene for bovine growth hormone (bGH). A 548-bp segment of the PEPCK promoter regulatory region was used in these studies since it contains elements capable of directing expression of genes specifically in the liver (16), as well as hormonal regulatory elements for cAMP (17–20), glucocorticoids (18–21), and insulin (20). From these experiments we conclude that: 1) genes introduced into hepatoma cells were expressed at high levels from the PEPCK promoter and regulated by hormones; 2) transcription from the 5′ LTR was inhibited in the presence of an internal

*This work was supported by Grants DK21859 and DK24451 (to R. W. H.) from the National Institutes of Health and by a grant from the American Diabetes Association (to H. C. K.). Funds were also provided by the Thomas A. Edison Program of the State of Ohio and the Pew Charitable Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Supported by the Metabolism Training Program Grant AM07319 from the National Institutes of Health.

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1The abbreviations used are: LTRs, long terminal repeats; PEPCK, phosphoenolpyruvate carboxykinase; neo, amino-3'-glycosyl phosphotransferase; bGH, bovine growth hormone; bp, base pair(s); kb, kilobase pair(s); DMEM, Dulbecco's modified Eagle's medium; BtCAMP, dibutyryl cyclic AMP.
promoter contained in the provirus; 3) the expression and regulation of the endogenous PEPCK gene was altered by retroviral infection; 4) two genes introduced into cells by the retrovirus were expressed at high levels and regulated by hormones; and 5) the predominant site of proviral integration in the genome was noted in cells grown for 3 months under selection conditions, suggesting clonal overgrowth.

EXPERIMENTAL PROCEDURES

Materials—All DNA modifying enzymes, nucleotides, and dextran sulfate were purchased from Boehringer Mannheim and Pharmacal, LKB Biotechnology Inc. [α-32P]dCTP (3000 Ci/mmole), [γ-32P]dCTP (7000 Ci/mmole), and GeneScreen Plus were purchased from Du Pont-New England Nuclear. Restriction enzymes were used according to the specifications of the manufacturer. Nitrocellulose membrane (BA-85) was from Schleicher & Schuell. All media, sera, and G418 were from Gibco Laboratories. The other reagents used in this study were of the highest purity available.

Thymidine kinase-deficient rat hepatoma (FTO-2B) cells, provided by Dr. Keith Fournier, Fred Hutchinson Cancer Center, Seattle, WA, are well differentiated with hepatocyte morphology, and express liver-specific proteins, such as PEPCK and albumin (17, 32). Mouse hepatoma (Hepa 1-6C) cells, from Dr. Gretchen Darlington, Baylor College of Medicine, Houston, TX, are less differentiated and do not express PEPCK (23). The FTO-2B (2) and the retroviral vector pLJ (also called the herpes virus SV40 promoter) were a generous gift from Dr. Mulligan, Whitehead Institute, M. I. T., Cambridge, MA. The cDNA for tyrosine aminotransferase (25) was provided by Dr. Gunther Schuts, German Cancer Research Center, Heidelberg, Federal Republic of Germany. The gene for bGH (26) was from Dr. Fritz Rottman, Case Western Reserve University, Cleveland, OH.

Construction of Recombinant Retrovirus Vector pLJ—Four recombinant vectors were derived from the parent vector, pLJ (Fig. 1), which contains a chimeric gene composed of the SV40 early promoter (including the 72-bp enhancer repeats), ligated to the neo gene, and inserted between the Moloney murine leukemia virus LTRs. The viral splicing sites have been inactivated by site-specific mutations. Transcripts initiating in the 5' LTR or the SV40 promoter are polyadenylated within the 3' LTR, producing RNAs of 3.9 and 3.5 kb, respectively. The vector contains a polaera early region gene for more efficient replication during transient expression and a pBR322 origin of replication. ψ sequences needed for viral encapsidation are included in the vector, allowing rescue of infectious virus after transfection of FTO-2B cells, a retroviral packaging cell line, which contain the gag, pol, and env genes stably integrated into their genome (2). A further description of this retroviral vector is presented by Korman et al. (24).

pLJ(-SV40)—The SV40 promoter sequences were removed from pLJ by digestion with BamHI and HindIII. These DNA ends were made blunt with the Klenow fragment of DNA polymerase I and ligated. Transcription initiating within the 5' LTR and terminating in the 3' LTR would generate a 3.4 kb RNA.

pLJPCK—The SV40 promoter DNA sequences were removed from pLJ by digestion with BamHI and HindIII and replaced by the BamHI-BglII fragment from the PEPCK promoter regulatory region (positions -548 to +73). The transcriptional orientation of the PEPCK promoter regulatory region was the same as the 5' LTR. Two transcripts should be produced by this provirus, one of 4.2 kb which initiates in the 5' LTR and one of 3.2 kb from the PEPCK promoter. Both transcripts terminate in the 3' LTR.

pLPCKbGH—A chimeric gene containing an EcoRI-BglII fragment of the PEPCK promoter regulatory region (positions -481 to +73), linked to the entire structural gene for bGH (26), was ligated into the unique ClaI site of pLJPCCK by the use of a polylinker flanked by ClaI sites (27). The transcriptional orientation of the chimeric PEPC-bGH gene was opposite to the 5' LTR. Three transcripts should be produced by this provirus, one of 7.5 kb RNA, initiating in the 5' LTR and terminating in the 3' LTR; 2) a 6.2 kb RNA initiating in the PEPCK promoter and terminating in the 3' LTR; and 3) a 1-kb transcript of the PEPC-kbGH gene, initiating in the PEPCK promoter and terminating in the polyadenylation signal of the bGH gene, after processing and splicing.

Fig. 1 contains a schematic representation of these vectors.

Cell Culture, DNA Transfection, and Rescue of Recombinant Viruses—NIH3T3 and FTO-2B cells were grown in Dulbecco's modified MEM, supplemented with 10% fetal calf serum. FTO-2B and Hepa-6C cells were grown in DMEM supplemented with 10% fetal calf serum. Transfection of DNA into the cells was carried out by calcium phosphate precipitation (28). To rescue recombinant retrovirus, approximately 2 × 10^6 FTO-2B cells (seeded 24 h earlier) were transfected with 10 μg of retroviral vector DNA, and colonies containing stably integrated recombinant DNA were obtained by selection in media containing 1 mg/ml of G418. After the cells reached confluence, they were grown in DMEM media supplemented with 10% calf serum, for 48 h. The media containing the retrovirus was removed and filtered through 0.45-μm Nalgene filter. The titer of retrovirus in the medium produced by the FTO-2B cells was measured by the ability to transmit G418 resistance to NIH3T3 cells 3 weeks after infection. To infect cells, 1 ml of the media containing the virus was added to 5 × 10^6 NIH3T3 cells in a 100-mm dish and incubated for 5 h at 37 °C, in the presence of 5 μg/ml polybrene. DMEM, supplemented with 10% calf serum was added, the cells incubated for 2 days, and then split into media containing 1 mg/ml G418 (29). Transfected FTO-2B cells and infected NIH3T3 cells were grown in media with G418, containing 10% calf serum, whereas growth of infected FTO-2B and Hepa-1-6C cells in G418 required the addition of 5% fetal calf serum to the medium.

DNA Probes—The following probes used for DNA/DNA or DNA/RNA hybridization experiments: PEPCK cDNA, 1.1-kb PstI-PstI fragment from the 3' end of the PEPCK gene, pPKC10 (30); 5'-PEPC, 620-bp BamHI-BglII fragment from the 5' end of the PEPCK gene (30); neo, 1 kb, BglII-EcoRI fragment from the 5' end of the neo gene (Fig. 1A); TAT cDNA, 600-bp PstI-PstI fragment which includes the 3' end of the cDNA (25). All DNA probes were labeled using [α-32P]dCTP, by the method of random oligo-primer as described by the manufacturer (Boehringer Mannheim, Inc). The specificity of the DNA probe labeled in this manner was determined by standard methods and results were published previously (17).

Isolation and Analysis of Cellular DNA and RNA—Isolation of genomic DNA and total cellular RNA were carried out by standard procedures which have been described in detail previously (17). Southern and Northern blotting analysis, agarose gel electrophoresis, and Southern blot analysis were carried out by standard methods (31, 32).

Hormonal Treatment of Cells—Cells were changed to medium without serum and incubated for 0 or 24 h in the presence of 0.5 mM Btz-AMP plus 1 mM theophylline, 1 μM dexamethasone, or 50 nM porcine insulin or without hormones, as indicated in the various figures.

Measurement of bGH—The concentration of bGH released into the incubation medium by cells infected by retrovirus containing the PEPCK-bGH gene was determined by an enzyme-linked immunosorbent assay (16).

RESULTS

Viral Production by FTO-2B Cells—Infectious retrovirus was produced from the recombinant retroviral vectors (Fig. 1) after transfection into FTO-2B cells. The cells were selected in media containing G418, and surviving clones containing stably integrated plasmid DNA were expanded and characterized by standard procedures which have been described in detail previously (17). Southern and Northern blotting analysis, agarose gel electrophoresis, and Southern blot analysis were carried out by standard methods (31, 32).

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**A**

**Fig. 1. Retroviral vectors.** A, pLJ (DOL-), is a Moloney murine leukemia virus-based retroviral vector containing the SV40 early promoter, linked to the structural gene for neo. The dotted box represents the polyoma early region gene. Numbers refer to sizes of the DNA fragments produced by digestion with the restriction enzymes shown in the figure. This figure was redrawn from one provided by Dr. Richard Mulligan and details on the construction of the vector is provided by Korman et al. (24).

B, pLJ-derived retroviral vectors containing the PEPCK promoter regulatory region. The arrows beneath each vector refer to the direction of transcription from internal promoters. A detailed description of the construction of these vectors is contained under "Experimental Procedures."

**Table I**

<table>
<thead>
<tr>
<th>Retroviral vector</th>
<th>Titer of 0.5 cells</th>
<th>cp/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLJ</td>
<td>4 x 10^4</td>
<td></td>
</tr>
<tr>
<td>pLJ(SV40)</td>
<td>2 x 10^4</td>
<td></td>
</tr>
<tr>
<td>pLJPCK</td>
<td>8.5 x 10^6</td>
<td></td>
</tr>
<tr>
<td>pLJPP*</td>
<td>6.3 x 10^6</td>
<td></td>
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<tr>
<td>pLJPCkBGH</td>
<td>5 x 10^7</td>
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determined after infection by recombinant retrovirus. Cells were infected with vLJPCk and selected in G418-containing media until resistant colonies appeared (3 weeks). Genomic DNA was isolated from the combined colonies (approximately 5 x 10^6 cells) and analyzed by Southern blotting after digestion with appropriate restriction endonucleases. Southern blots were hybridized with the neo or 5' PEPCK probes. Infected cells contained the intact provirus, since after ScaI digestion, only the predicted 4.1-kb fragment hybridized with either probe (Fig. 24). In uninfected cells, no DNA hybridized with the neo probe, whereas the 5' PEPCK probe hybridized only to the endogenous PEPCK gene.

In order to determine the site(s) of integration of the provirus in the host cell genome, we digested the same DNA with two restriction enzymes: BglII, which cuts once within the provirus, and BamHI, which is not present in the provirus. Digestion with either enzyme should yield multiple bands of various sizes which hybridize with the neo DNA probe, after analysis by Southern blotting. No specific bands were observed when DNA from infected cells was digested with either of the two restriction endonucleases and hybridized to the neo probe. The only specific bands which hybridized with the 5' PEPCK probe corresponded to the endogenous PEPCK gene (Fig. 2A). Thus, the pLJPCk provirus was integrated randomly into the genome of FTO-2B cells after 3 weeks of selection in media containing G418.

The mixed population of infected cells, 3 weeks after infection, was grown in medium containing G418 for an additional

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Fig. 2. Analysis of the pattern of integration of the provirus into the genome of infected cells. A, FTO-2B cells were infected with vLPCK and selected for 3 weeks in a medium containing G418. Surviving cells (approximately 5 × 10⁶ cells) were pooled, and the genomic DNA was isolated. Control cells were not infected. Approximately 20 μg of genomic DNA was digested with SacI, which cuts once in each of the LTRs, yielding an intact provirus. The DNA was also digested with BglIII, which cuts once within the provirus, and with BamHI, a restriction site not included within the provirus. The DNA fragments were separated by electrophoresis on 0.8% agarose gel and hybridized with either a neo or 5' PEPCK probe. E, the FTO-2B cells from the experiment shown in the left panel were grown in culture medium containing G418 for an additional 2 or 3 months (as indicated at the top of the figure). The DNA was extracted from these cells, digested with SacI and BglII, and analyzed by Southern blotting using either neo or the 5' PEPCK DNA probes. The arrows indicate the size of the DNA fragments which hybridize with the DNA probes. The 2.4- and 7-kb bands represent fragments from the endogenous PEPCK gene which hybridize with the 5' PEPCK probe. A drawing of the provirus, indicating the sites of restriction endonuclease digestion, is included at the bottom of the figure, together with the predicted size fragments generated from the digestion. The DNA probes are shown as boxes in the drawing: open box, 5' PEPCK probe; dark box, neo probe.

2 and 3 months. Genomic DNA was isolated, digested with the SacI and BglIII, and analyzed by Southern blotting, using the neo and 5' PEPCK probes. Predominant bands were detected in DNA from the same population of cells which had a random pattern of proviral integration when analyzed at 3 weeks. After BglIII digestion, predominant DNA bands of 6.2 kb (neo probe) and 9.2 kb (5' PEPCK probe) were observed in one population (Fig. 2B). Additional DNA bands, which hybridized with these probes, were also observed. The intensity of these additional bands decreased between 2 and 3 months in culture, whereas the predominant bands (6.2 and 9.2 kb) became more intense. The provirus was contained in a 4.1-kb segment of the genome as demonstrated by SacI digestion (Fig. 2B), and the appropriate restriction sites were maintained within the provirus (data not shown), demonstrating that there were no gross rearrangements. The intensity of this 4.1-kb band did not change in cells cultured over this period.

Proviral integration in three separate populations of cells derived from individual infections with vLPCK (approximately 5 × 10⁶ individual colonies in each population) was analyzed as described above. DNA from infected cells was digested with BglIII, analyzed by Southern blotting, and hybridized with the neo probe. In DNA from cells in culture for 2 months, we noted specific bands which were different for each population of infected cells. In one population of infected cells (infection I in Fig. 3), we analyzed the pattern of proviral integration at 2 and 6 months after the cells were placed in culture. After 2 months, we noted a predominant DNA band at 7.8 kb, together with other, less intense bands at 5.6 and 4.4 kb. After 6 months in culture, these low abundance bands were less intense than at 2 months, whereas the predominant band at 7.8 kb was more intense. A different set of predominant bands were noted in two other populations of infected cells: 6.2 and 4.2 kb in infection I1 and 17, 5.6, and 3.7 kb in infection I11 (Fig. 3). Thus, in multiple, separate populations of infected cells, the growth of individual cells results in predominant DNA bands, reflecting the position of the provirus in the host cell genome after clonal expansion. These bands were different for each population of cells tested.

The pattern of proviral integration was determined in cells infected with vLJ and vLJ(-SV40), which lack the PEPCK promoter regulatory region. A mass culture of infected cells, maintained in G418 for 3 months, had the intact provirus integrated in a random manner (Fig. 4). However, after prolonged selection (more than 4 months), these cells contained a low percentage of specifically integrated provirus (data not shown). Thus, FTO-2B cells infected with all retroviruses...
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![Pattern of integration of vLJPCK into the genome of separate populations of FTO-2B cells. DNA was isolated from FTO-2B cells infected with vLJPCK, digested with BglII, and analyzed by Southern blotting. I, II, and III represent three separate populations of infected cells maintained in culture for 2 or 6 months. The size of the DNA fragments hybridizing with the neo probe are indicated in the figure. Experimental details are provided in the legend to Fig. 2.](image)

tested contained predominant sites of proviral integration after prolonged growth in G418, probably due to selection and clonal expansion.

**Regulation of Gene Expression of the pLJPCK Provirus by Bt$_2$cAMP Dexamethasone and Insulin**—The expression and hormonal regulation of the chimeric PEPCK-neo gene in FTO-2B cells infected with vLJPCK was analyzed by Northern blotting. An RNA transcript of 3.2 kb hybridized with the neo probe. This would be expected if transcription initiated at the PEPCK promoter and the newly synthesized RNA was polyadenylated within the 3' LTR (Fig. 5). Treatment of the cells with Bt$_2$cAMP plus theophylline or dexamethasone increased the expression of the PEPCK-neo gene by 6- to 7-fold after 4 h of treatment. Insulin, when added together with Bt$_2$cAMP, blocked the induction of neo gene expression by both the cyclic nucleotide and by dexamethasone. If the cells were pretreated with insulin for 2 h before the addition of Bt$_2$cAMP and then harvested 2 h later, the level of neo RNA was lower than that noted in control cells. We did not detect a 4.2-kb species by Northern blotting, which would be expected if transcription initiated at the 5' LTR. However, a low level of transcription from the 5' LTR was detected when the RNA was analyzed by S1 nuclease mapping (data not shown).

**Expression of the Endogenous PEPCK and Tyrosine Aminotransferase in Cells Infected with vLJPCK**—The effect of hormones on the expression of the endogenous PEPCK gene in FTO-2B cells infected with vLJPCK was also measured using the identical Northern blot. As predicted, a 2.8-kb RNA hybridized with the PEPCK cDNA probe (Figs 5 and 6). However, the basal concentration of PEPCK mRNA in infected cells was very high, compared with uninfected cells (Fig. 6). There was a marked induction of the endogenous PEPCK gene by both Bt$_2$cAMP and dexamethasone in cells which were not infected with the virus (Fig. 6). However, in cells infected with vLJPCK, the endogenous PEPCK gene was only slightly increased (less than 2-fold) by the addition of Bt$_2$cAMP or dexamethasone. These cells were responsive to Bt$_2$cAMP or dexamethasone since the PEPCK-neo gene, introduced into FTO-2B cells by infection, was induced by both compounds. Insulin addition caused a marked decrease in endogenous PEPCK mRNA in infected cells treated with these hormones (Fig. 5). The amount of RNA in each lane was identical, based on hybridization of the blots with ¿-tubulin (data not shown). We have also noted that insulin decreased the basal level of PEPCK gene expression in cells infected with retrovirus, in the absence of Bt$_2$cAMP or dexamethasone (data not shown).

Since infection of FTO-2B cells with vLJPCK was associated with a marked increase in the basal concentration of PEPCK RNA, we measured the level of mRNA for tyrosine aminotransferase, a gene regulated by cAMP and glucocorticoids in a manner similar to PEPCK (25). The infection of
addition of BtZcAMP decreased and dexamethasone increased expected manner (Fig. 6).

The multiple bands which hybridize with the PEPCK cRNA probe may be the precursor RNAs noted previously (64). Details on the procedure for the extraction and analysis of RNA and the concentrations of the hormones used in this experiment are provided under "Experimental Procedures." cAMP, BtZcAMP; Dex, dexamethasone; INS, insulin; CON, control (no added hormones).

FTO-2B cells with this virus had no effect on the basal level of tyrosine aminotransferase mRNA and the addition of BtZcAMP or glucocorticoids to the cells stimulated the expression of the endogenous tyrosine aminotransferase gene in the expected manner (Fig. 6).

Expression and Regulation of Proviruses Which Did Not Contain PEPCK Sequences—Cells were infected with vLJ(-SV40) and vLJ to determine the contribution of the PEPCK sequences to the hormonal regulation of transduced genes and their effect on the expression of the endogenous PEPCK gene. These proviruses have either no internal promoter (vLJ(-SV40)) or the SV40 promoter (vLJ) linked to the neo gene. Two identical Northern blots were hybridized with either a neo or a PEPCK cDNA probe. In vLJ(-SV40) infected cells (Fig. 7A), the neo probe hybridized with a 3.4-kb RNA which corresponded to the full-length viral transcript containing neo. Treatment of cells with BtZcAMP caused a small increase in neo RNA, whereas insulin did not significantly alter neo gene transcription. In contrast, the addition of dexamethasone to the infected cells caused a more than 10-fold induction of gene transcription from the 5′ LTR. Thus, dexamethasone has the potential to control the expression of a structural gene included in the provirus by altering transcription from the 5′ LTR, probably due to the presence of a glucocorticoid regulatory element in the LTR (33). In cells infected with vLJ (Fig. 7B), transcription from the 5′ LTR (3.9-kb RNA) was undetectable and the internal SV40 promoter (3.2-kb RNA) was expressed at high levels. The addition of BtZcAMP decreased and dexamethasone increased the level of this 3.2-kb RNA. Insulin, on the other hand, had no effect on SV40-neo gene expression. Based on these observations, the marked stimulatory effect of BtZcAMP and the negative effect of insulin on expression of the neo gene in cells infected with vLJPCK appears to be dependent on the PEPCK promoter regulatory element. The effect of dexamethasone may be due to sequences in the 5′ LTR or in the PEPCK promoter. The negative effect of BtZcAMP on SV40-neo expression is possibly the result of a negative element for CAMP present in the SV40 promoter. The basal level of expression of the endogenous PEPCK gene was increased after infection of FTO-2B cells with vLJ, and treatment with BtZcAMP and dexamethasone did not cause a further increase in the expression of this gene (Fig. 7B). The endogenous gene was expressed and regulated in cells infected with vLJ(-SV40) (Fig. 7A), in a manner identical to uninfected cells (Fig. 7B). Insulin decreased the expression of the endogenous PEPCK gene in cells infected with either virus. These results suggest that both SV40 and PEPCK sequences can alter the expression of the endogenous PEPCK gene.

The Expression and Regulation of Proviruses Containing a Chimeric PEPCK-SV40 Promoter Regulatory Element—A segment of the PEPCK promoter regulatory region (positions -416 to -61) was linked to the 5′ end of the SV40-neo gene (pLjPP", Fig. 1B) and introduced by infection into FTO-2B and Hepa 1-6C cells (Fig. 8). This segment of the PEPCK gene can act as a glucocorticoid- and cAMP-regulated enhancer element when linked to a heterologous gene (18). The
SV40 early promoter containing its own enhancer sequences is a strong promoter in many cell types and is capable of driving the expression of linked genes in retroviral vectors (24). FTO-2B or Hepa 1-6C cells infected with vLJPP+ expressed the neo gene from the SV40 promoter (Fig. 8). The basal level of neo gene expression in vLJPP+ infected cells was lower than noted in cells infected with vLJ (Fig. 8, compare first two lanes). The addition of Bt2cAMP to infected FTO-2B cells caused a marked induction in the levels of neo RNA transcribed from the SV40 promoter, but the cyclic nucleotide inhibited transcription from the same chimeric gene, when infected into Hepa 1-6C cells. The reason for these differences in the regulation of transcription of this chimeric gene in two different cell lines is not clear. However, Hepa 1-6C cells are less differentiated than the FTO-2B cells and do not express the endogenous PEPCK gene. Finally, the basal level of expression of the endogenous PEPCK gene is not altered by infection of FTO-2B cells with vLJPP+ (Fig. 8).

Infection of Hepatoma Cells with a Retrovirus Containing Two Genes between the LTRs—The usefulness of the pLJ system for introducing a second, nonselected gene into cells was tested by infecting FTO-2B and Hepa 1-6C cells with vLJPCKbGH, which contains two internal chimeric genes (Fig. 1). The provirus was integrated into the genome of these cells and the PEPCK-neo gene was expressed and regulated as described above for cells infected with vLJPC (data not shown). In order to determine the level of expression of the PEPCK-bGH gene included within the LTRs of the pLJPC provirus, we measured the concentration of PEPCK-bGH mRNA in infected cells. Low levels of this mRNA were detected by Northern blot analysis of total RNA from cells treated for 24 h with various hormones (data not shown). Quantitative S1 nuclease analysis of the RNA from these cells demonstrated that PEPCK-bGH mRNA initiated at the correct transcription start site within the PEPCK promoter (Fig. 9). The pattern of regulation of gene expression by Bt2cAMP, insulin, and dexamethasone in both FTO-2B and Hepa 1-6C cells was the same as that noted previously for PEPCK-neo (Fig. 5). Both cell lines synthesized and released considerable quantities of immunoreactive bGH into the culture medium (Fig. 10). The amount of bGH produced by Hepa 1-6C cells is six times that from FTO-2B cells (Fig. 10), even though the level of PEPCK-bGH mRNA was only 2-fold higher in Hepa 1-6C cells (Fig. 9). The addition of Bt2cAMP to both FTO-2B and Hepa 1-6C cells increased the concentration of bGH in the medium after 24 h, whereas dexamethasone was effective only in FTO-2B cells. Insulin, added together with Bt2cAMP, had little effect on the secretion of bGH by either cell line. These differences in the response to hormones between the level of bGH mRNA and bGH in the medium, noted in Figs. 9 and 10, may be due to effects on bGH synthesis, processing, and release by the cells. A lack of correlation between mRNA levels and protein secretion of human Factor IX in cells infected with virus containing Factor IX cDNA has been observed (13).

DISCUSSION

Retroviral vectors represent a valuable means of introducing genes into the genome of cells and animals (1). However, the expression and regulation of only a limited number of
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Fig. 8. Effect of regulatory elements in the PEPCK promoter regulatory region on the expression of the SV40/neo gene introduced into hepatoma cells by retroviral vectors. FTO-2B and Hepa 1-6C cells were infected with vLJ or vLJ (as indicated in the figure) and grown in medium containing G418 for 2 months. The cells were treated for 4 h with the hormones indicated in the figure, the RNA isolated, and analyzed by Northern blotting. CON refers to cells not treated with BtCAMP; CAMP is BtCAMP. The DNA probe used for Northern analysis was the neo probe or the PEPCK cDNA, as indicated. The 3.2-kb RNA is the transcript from the PEPCK promoter and the 2.8-kb band is mRNA for the endogenous PEPCK gene. The drawing at the bottom of the figure represents the provirus and shows the position of the PEPCK promoter regulatory region in the retroviral vector. The experimental conditions for hormonal treatment of the cells are described in the legends to Figs. 5 and 6.

Expression of Chimeric Genes Introduced into Cells with Retroviral Vectors—The usefulness of retroviral vectors for the introduction of genes into cells and animals depends on the ability of the provirus to express multiple genes included between the LTRs. For some vectors, the inclusion of an internal promoter linked to a selectable gene greatly reduces the promoter activity from the 5' LTR, whereas with other vectors, the internal promoter is less active transcriptionally than the LTR. In general, the activity of an internal promoter is dependent upon whether it is driving the expression of the selectable gene included in the retroviral vector. The negative effect of one promoter upon another ("promoter suppression") has been analyzed by Emerman and Temin (34, 35) using LTRs from avian and murine retroviruses, together with the promoter for the Herpes virus thymidine kinase gene. Transcription from the LTRs of both viruses was suppressed when the thymidine kinase promoter was included in the provirus. This suppression was not related to the strength of the thymidine kinase promoter or to the distance between the promoters but was inversely related to the DNase I sensitivity of the chromatin surrounding the integrated provirus.

When vectors which contain the PEPCK promoter regulatory region linked to the neo gene were transfected into $2$ cells, both the 5' LTR and the PEPCK promoters were active and high-titer virus was produced. However, when FTO-2B cells were infected with the virus, the PEPCK promoter was expressed at much higher levels than the 5' LTR. This was...
observed for all viruses containing sequences from the PEPCK promoter regulatory region. Thus, the PEPCK promoter, when driving the expression of a selectable neo gene is more active than the 5' LTR of the provirus. The 5' LTR was also transcriptionally active in NIH3T3 and normal rat kidney cells infected with these viruses, as demonstrated by Northern blot analysis. Since the 5' LTR is extremely responsive to glucocorticoids in the absence of an internal promoter, it may be possible to exploit this effect to produce high-titer retrovirus in packaging cell lines which are sensitive to glucocorticoids, even when strong, internal promoters are present in the provirus.

**Hormonal Regulation of Chimeric Genes Introduced into Cells with Retroviral Vectors**—An attractive feature of this system of gene insertion into cells via retroviral vectors is the possibility of including chimeric genes containing regulatable promoters in the vectors. The PEPCK promoter regulatory element is an excellent candidate for use with retroviral systems, since it is a strong promoter, especially in liver and kidney, which can be acutely regulated by various hormones (16-21). We have shown in this study that retroviral vectors containing 547 bp of 5'-flanking sequence of the PEPCK promoter, a region known to contain cAMP, glucocorticoid (17-19), and tissue-specific regulatory elements (16), conferred hormonal responsiveness to linked genes. The effect of hormones was noted even in a retroviral vector such as pLJPCkBGH, which contains two PEPCK promoters, both of which were regulated by hormones.

Transcription from the proviral SV40 promoter in the two hepatoma cell lines (FTO-2B and Hepa 1-6C) infected with vLJ (a virus containing no PEPCK sequences), was decreased by the addition of Bt2cAMP. However, a segment of the PEPCK regulatory region (between positions -416 and -61), when linked to the SV40 promoter, acted as a positive regulatory element in FTO-2B cells. Clearly, the presence of the cAMP regulatory element from the PEPCK promoter can override the negative effect of Bt2cAMP on the transcription of the SV40 promoter, but only in FTO-2B cells, since the same gene was negatively regulated by Bt2cAMP in Hepa 1-6C cells. Despite the inducibility of the SV40 promoter by Bt2cAMP when PEPCK regulatory sequences were present (vJPPP⁺ infected cells), the basal level of transcription from the chimeric PEPCK-SV40 promoter was markedly reduced, relative to the SV40 promoter. Thus, the chimeric PEPCK-SV40 promoter has potential usefulness in studies requiring a strong promoter, which has a low level of basal transcription but which can be markedly and rapidly induced by cAMP. This type of “on-off” mechanism might have application for chimeric genes introduced into animal tissues, where the expression of the linked structural gene must be rigorously controlled.

Previously, we reported that a chimeric PEPCK-neo or PEPCK-TK gene, containing segments of the PEPCK promoter regulatory region of varying length (we have used fragments from positions -2000 to +73) was not responsive to insulin when transfected into FTO-2B cells (17). In these studies, the effect of insulin was measured by its ability to block the induction of gene transcription by Bt2cAMP in stably transfected cells. The endogenous PEPCK gene, present in FTO-2B cells is responsive to insulin and was used as a control for the effectiveness of the hormone. In contrast, when the chimeric PEPCK-neo gene was introduced into cells by retroviral infection, insulin had a marked inhibitory effect on the ability of Bt2cAMP and dexamethasone to increase transcription. When the same retroviral vector, containing the chimeric gene, was transfected into FTO-2B cells, no effect of insulin on gene transcription was noted. This suggests that the insulin regulatory element is contained within -547 to +73 bp of the promoter regulatory region of the PEPCK gene, a finding which is in agreement with a study

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3 M. Hatzoglou and R. W. Hanson, unpublished data.

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C. R. Vandenbark, E. Park, M. Hatzoglou and R. W. Hanson, unpublished observations.
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by McGrane et al. (16) using transgenic mice. In this study, the expression of a chimeric PEPCK-bGH gene, containing the same region of 5' flanking DNA from the PEPCK gene, was markedly suppressed by feeding the animals a diet high in carbohydrate. Recently, Magnuson et al. (20) showed that insulin can block the induction by glucocorticoids and cAMP of a chimeric PEPCK-CAT gene (positions -600 to +73 of the PEPCK gene), which was expressed transiently in H41IE hepatoma cells. Differences in the expression of chimeric genes introduced into cells by transfection as compared to infection have been noted by other investigators (38). These differences in the response of hormonal regulatory elements introduced into cells by different techniques underlines the importance of the method of gene transfer on the regulated expression of the transgene as well as the cellular and chromosomal environment.

Effect of Retroviral Infection on the Expression of the Endogenous PEPCK Gene—Another interesting finding noted in this study is the stimulatory effect of retroviral infection on the basal level of expression of endogenous PEPCK gene in FTO-2B cells. This effect did not require the presence of PEPCK gene sequences in the provirus and was observed with all of the chimeric genes introduced by infection, except for vLJFP and vL2(-SV40). One common element between these two vectors is the low level of expression of the neo gene in infected cells containing either virus (compare Figs. 7 and 8). A high level of expression of the product of the neo gene, amino-3'-glycosyl phosphotransferase, could alter factors which regulate transcription of the PEPCK gene. The high basal level of the endogenous PEPCK gene is probably not due to an increase in intracellular cAMP caused by retroviral infection, since infection of FTO-2B cells with vLJFP did not alter the basal level of expression of the tyrosine aminotransferase gene. The effect was observed with viruses which do not contain PEPCK sequences, making it unlikely that factors which negatively regulate transcription of the PEPCK gene are removed by binding to proviral sequences. Whatever the mechanism, it is clear that retroviral infection can alter expression of the endogenous PEPCK gene in hepatoma cells.

Specificity of Proviral Integration—Recent studies have shown that there are a limited number of genomic sites for retroviral integration (40, 42, 44, 47, 48). Here, we demonstrate that proviral integration was initially random in FTO-2B cells, but when these cells were maintained in culture for 2 months cells were selected which contained the provirus in a predominant location. The ability to select cells which have the provirus integrated in a specific location appeared to be a general property of retroviruses containing the neo gene. However, the presence of the PEPCK promoter regulatory region in the recombinant retroviruses greatly accelerated this selection. When PEPCK sequences were present, the selection process and clonal expansion occurred within 2 months. In contrast, it took 4 months for this selection to occur in cells infected with virus where PEPCK sequences were absent, and the percentage of cells demonstrating this specificity was low. We have infected a variety of transformed cells, including the hepatoma cell lines FTO-2B (rat) and Hepa i-6C (mouse), NIH3T3 (fibroblast) cells, as well as liver cells in the intact animal by microinjection of infectious retrovirus into fetal rats in utero. Only the hepatoma cells and the livers of the animals injected with the retrovirus demonstrated specificity in the pattern of proviral integration. Battula and Temin (46) reported the integration of spleen necrosis virus in infected chicken fibroblasts early and late after infection. They found that proviral integration into the host cell genome was random in acutely infected cells, but in chronically infected cells, the virus appeared to be integrated at a single site in the genome. Our observations are compatible with these findings. The ability to detect nonrandom integration events may depend on the cell line and retroviral vector used, as well as the time after infection at which integration is studied.

It is probable that a clonal expansion of a subset of hepatoma cells, containing the provirus at a specific position, resulted in the ultimate integration pattern noted after 2 months of growth. Integration of the provirus at one or more of these sites may result in the overproduction of the neo gene product. This may provide selective advantages for these cells, since they are grown in the presence of high concentrations of G418. A small population of infected cells could divide at a rate exceeding that of other cells containing the provirus, resulting in clonal outgrowth. Barklis et al. (40) found that the site of integration of the provirus was important for the expression of transduced neo genes in embryonic carcinoma cells. The provirus was in two distinct chromosomal locations in 30% of the selected cell lines. This suggests that the site of integration is important for proviral expression, possible because it is an actively transcribed region of chromatin. Alternatively, a growth factor may also be induced by viral infection, providing a selective advantage for a subpopulation of cells. Extensive cloning and sequencing of a number of these integration sites will be required to explain these results, as was recently reported for the integration sites of Rous sarcoma virus in chicken cells (48).

Gene Transfer into Hepatic Cells—The liver is an important organ for gene transfer, both for metabolic studies and for the correction of genetic diseases. Retroviral transduction has been used to introduce genes into hepatoma cells (49) and primary hepatocytes (50–52). Progress in the transplantation of hepatocytes into animals (53) may provide an approach to gene transfer into animals. Recently, we have used retroviruses to directly infect fetal hepatocytes in vivo by injection of infectious retrovirus into the peritoneal cavity of fetal rats in late development. The provirus was integrated into the liver and the chimeric PEPCK-neo gene included in the virus was expressed and regulated by hormones. The efficient transfer of genes by retroviral transduction, coupled with the high level of expression and hormonal regulation of genes linked to the PEPCK promoter regulatory region, may provide an ideal means to alter gene expression and metabolic processes in the liver.

Acknowledgments—We are indebted to Dr. Richard Mulligan for generously providing us with the retroviral vector pLd (DOL+) prior to its publication. We also wish to acknowledge the excellent technical assistance of Yasobitra Patel and the advice provided during the course of these studies by Drs. Thomas Wagner and Fritz Rottman. We are especially indebted to Drs. David Samols, Hsiao-Jien Kuang, and William Roesler for reviewing the manuscript and to Josh Bloom for assaying GGH.

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