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Hormonal Control of Interacting Promoters Introduced into Cells by Retroviruses*

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The interaction of promoters contained in a Moloney murine leukemia virus (MoMLV)-based retroviral vector was studied after infection of FTO-2B rat hepatoma and NIH 3T3 mouse fibroblast cells. Segments of the phosphoenolpyruvate carboxykinase (PEPCK) promoter-regulatory region, which are known from previous studies to confer responsiveness to hormones, were linked to the structural genes for bovine growth hormone, amino-3′-glycosyl phosphotransferase (*neo*), and herpes-virus thymidine kinase and inserted into a MoMLV-based retroviral vector. In vectors in which PEPCK was the only internal promoter, it was the major site of gene transcription. This dominant effect was independent of the orientation of the PEPCK promoter relative to the 5′ long terminal repeat of the provirus and was noted with as little as −174 base pairs of the 5′-flanking sequence. NIH 3T3 cells, which do not express the endogenous PEPCK gene, transcribed the transduced PEPCK-chimeric genes at the same high levels as was observed in hepatoma cells. When two promoters were present in the provirus, the expression of chimeric structural genes depended on the relative position and orientation of these genes as well as the type of cell infected by the retrovirus. Differential responses of proviral promoters in infected cells were also observed in the presence of hormones. Dibutyryl cyclic AMP increased the expression of genes linked to the PEPCK promoter in FTO-2B and NIH 3T3 cells, whereas glucocorticoids stimulated transcription from both the PEPCK promoter and the long terminal repeat in FTO-2B cells. The effect of these hormones on transcription of proviral promoters depended on their position relative to the 5′ long terminal repeat. In contrast, insulin uniformly inhibited transcription from the PEPCK promoter in a position-independent manner but only in hepatoma cells and not in fibroblasts. In clonally isolated FTO-2B cells infected with a retrovirus, the site of proviral integration was also a major factor determining the expression and hormonal regulation from the internal promoters. The data suggest that the hormonal regulation of the expression of genes contained in retroviral vectors depends on the type and position of the regulatory elements present in the provirus and the lineage of the infected cell.

The unique features of retroviruses make them excellent vectors for transferring genes into cells and animals to modify metabolic processes (1–6) or to correct defects in existing genes (7–10). However, the effective utilization of retroviral vectors for these purposes is limited by several factors including the complexity of the interaction between the provirus and the host cell genome (11–15). Although the chromosomal site of integration is an important determinant of the expression of the integrated provirus, integration usually occurs in transcriptionally active sites of the genome (16, 17). Moreover, the presence in the provirus of multiple transcriptional enhancers can influence expression of the genes contained in the provirus. Studies with a variety of genes that have been introduced into cells by retroviruses indicate that the transcriptional activity of a single promoter depends on its position (18–22) and association with a selectable marker gene in the provirus (23, 24).

In addition to the interaction of proviral promoters, there is a marked difference in the level of proviral gene expression associated with the type of cell or tissue infected. Variations in the activity of specific viral, as compared to internal, promoters have been reported in fibroblasts (25–28), hepatoma cells (29) or primary hepatocytes (7, 8, 10, 30), hematopoietic cells (31–36), F9 cells (37), and in undifferentiated embryonic choriocarcinoma cells (38–41). These differences in cell-specific response are due in part to the presence of transcription factors that control expression of genes introduced by the retrovirus. For example, Tsukiyama *et al.* (42) have proposed a mechanism of suppression of the MoMLV[1] LTR in embryonic choriocarcinoma cells in which the shortage of a transcriptional activator and the presence of negative regulatory proteins inhibited transcription from the LTR.

A major consideration in the design of retroviral vectors to be introduced into a specific tissue is the potential for regulation of the gene contained within the provirus. One strategy is to include a chimeric gene linked to a promoter containing transcriptional regulatory elements that are recognized by specific transcription factors in the target tissue. Among the transcriptional regulators shown to be effective *in vivo* are hormones (43, 44) or factors such as heavy metals (45, 46). In a previous study using virally infected hepatoma cells (29), we have shown that a segment (−550 to +73) of the phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK) promoter was the predominant site of transcription of the *neo* structural gene, when it was placed downstream from the 5′ LTR.

Several combinations of promoters and structural genes were introduced in the retroviral vectors used in the current study, including the PEPCK promoter, the SV40 early promoter, and the TK promoter. The PEPCK promoter (−450

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[1] The abbreviations used are: MoMLV, Moloney murine leukemia virus; PEPCK, phosphoenolpyruvate carboxykinase; LTR, long terminal repeat; TK, thymidine kinase; bp, base pair(s); kb, kilobase(s); bGH, bovine growth hormone; Bt_2cAMP, dibutyryl cyclic AMP.

to +73) contains a number of regulatory elements that direct expression of linked genes to the liver and kidney cortex of transgenic animals (47) and control its response to cAMP, insulin, and glucocorticoids (29, 47–51). Several of these regulatory elements have been characterized and the transcription factors which bind to these sequences identified (52, 53). Also, when the PEPCK promoter was transduced into hepatoma cells via a MoMLV retrovirus, it was highly expressed and regulated by hormones in a manner similar to the endogenous PEPCK gene (29).

The TK promoter contains a 109-bp 5′-flanking sequence of the TK gene, which includes a TATA box and two Sp1-binding sites. This promoter has been shown by Stewart *et al.* (54) to be strongly expressed, relative to the 5′ LTR of the Moloney murine cytomegalovirus-based retrovirus, in the livers of transgenic mice that were produced by infection of preimplantation embryos. Also, the relative expression of the TK promoter in cells infected *in vitro* depended on other promoters present in the provirus (23, 24, 54, 55). The SV40 early promoter (−281 bp of the 5′-flanking sequence) has six Sp1-binding sites as well as AP-1- and AP-2-binding domains, which lie within the 72-bp repeats of the SV40 enhancer. This promoter, when incorporated into the genome of transgenic animals, was not expressed in the liver (54) but was transcriptionally active when it was transduced into hepatoma cells in culture (29).

The MoMLV LTR is a strong promoter, containing three glucocorticoid regulatory elements, two of which are within the 75-bp repeat (56). The LTR is not responsive to glucocorticoids in NIH 3T3 cells infected with MoMLV virus (57), but was inducible by steroid hormones in FTO-2B hepatoma cells (29). When the MoMLV LTR was introduced into transgenic animals, it was not transcriptionally active in the liver (54). Ledley *et al.* (7) reported a low level of transcription from the LTR in primary hepatocytes transduced with LTR-based vectors, whereas Wilson *et al.* (30) demonstrated efficient expression of the LTR-driven β-galactosidase gene when it was introduced into primary hepatocytes.

Our long-term goal is to modify metabolic processes in the liver by introducing genes with retroviral vectors (58). The expression of these genes will be regulated by hormones that control transcription from the largely liver-specific PEPCK promoter. In order to optimize this system, we have analyzed the expression of the PEPCK promoter as well as several nonhepatic promoters in a variety of orientations within viral vectors. The relative activity of different promoter/enhancer elements within the provirus was studied by the quantitative analysis of the product (mRNA) of the proviral genes in homogeneous populations of cells. A complex pattern of promoter/promoter interaction between the various transcriptional elements present in the provirus and the host cell genome was observed.

EXPERIMENTAL PROCEDURES

Materials—All DNA-modifying enzymes and nucleotides were purchased from Boehringer-Mannheim and Pharmacia-LKB Biotechnology Inc. [α-^{32}P]dCTP (3,000 Ci/mmol) and [γ-^{32}P]ATP (7,000 Ci/mmol) were purchased from Du Pont-New England Nuclear. Restriction enzymes were used according to the specifications of the manufacturer. FTO-2B cells (59) were provided by Dr. K. Fournier, Fred Hutchison Cancer Center, Seattle, and the retroviral vector, pLJ (60), and Ψ2 (61) cells used in this study were a gift from Dr. Richard Mulligan, MIT, Boston. The gene for bovine growth hormone (bGH) (62) was from Dr. Fritz Rottman, Case Western Reserve University, Cleveland.

Construction of Retroviral Vectors—The recombinant retroviral vectors were modified from the parent vector, *pLJ* (60). Four types of retroviral vectors were used in this study.

Vectors containing one promoter at the 5′ end of the provirus were pLJneo, pLJ, pLJPP$^+$, pLJPCK(−2000)neo, pLJPCK(−550)neo, pLJPCK(−355)neo, and pLJPCK(−174)neo. The procedure for construction of pLJneo, pLJPP$^+$, and pLJPCK(−550)neo has been described previously (29). Retroviral vectors containing PEPCK promoter deletions were constructed by insertion of segments −2000 to +73, −355 to +73, and −174 to +73 into the *Bam*HI-*Hin*dIII site of pLJ, using a PUC19 polylinker (Fig. 3).

Vectors containing one promoter at the 3′ end of the provirus, inserted into the unique *Cla*I site, were pLJneoPbGH(+) and pLJneoPbGH(−). These vectors were constructed by inserting a chimeric PEPCK-bGH gene, containing −450 to +73 of the PEPCK promoter and the complete bGH structural gene (62), into the *Cla*I site of pLJneo in both forward and reverse orientations (Fig. 4).

Vectors containing two promoters in the provirus were pLJPCKneoPbGH(+), pLJPbGH(−), pLJPTK, and pLJTK. The vector pLJPCKneoPbGH(+) contained a chimeric PEPCK-bGH gene (−450 to +73 of the PEPCK promoter) that was inserted into the unique *Cla*I site of pLJPCK (29) in the same transcriptional orientation as the 5′ LTR (Fig. 4). pLJPbGH(−) contained a chimeric PEPCK-bGH gene (−450 to +73 of the PEPCK promoter) that was inserted into the unique *Cla*I site of pLJ in an orientation opposite to the transcription from the 5′ LTR (Fig. 6). pLJPTK contained a chimeric PEPCK-TK gene (−450 to +73 of the PEPCK promoter) linked to the TK structural gene (63) and inserted into the *Bam*HI site of pLJ by blunt ligation in the reverse transcriptional orientation to the 5′ LTR (Fig. 6). pLJTK contained the complete TK structural gene with 109 bp of the 5′-flanking sequence, inserted into the *Bam*HI site of pLJ by blunt ligation in the reverse transcriptional orientation to the 5′ LTR (Fig. 6).

Cell Culture and Generation of Retrovirus—DNA transfection, rescue of recombinant retrovirus, and infection of cells were performed as described previously (29). The titer of the retrovirus colony-forming units/ml generated by mass cultures of Ψ2 cells was determined by infection of NIH 3T3 cells: vLJ, 4×10^4; vLJneo, 2×10^4; vLJPCK(−550)neo, 8.5×10^6; vLJPP$^+$, 6.3×10^3; vLJPTK, 4×10^3; vLJTK, 3×10^2; vLJPbGH(−), 5×10^5; vLJPCKneoPbGH(+), 2×10^2; vLJneoPbGH(+), 10^2; vLJneoPbGH(−), 3×10^5. FTO-2B cells were grown in Dulbecco's modified Eagle's medium, supplemented with 5% calf serum and 5% fetal calf serum. NIH 3T3 and Ψ2 cells were grown in the same media containing 10% calf serum. Hormonal treatment of the infected cells was carried out in serum-free medium, in the presence of 0.5 mM Bt_2cAMP plus 1 mM theophylline, 1 μM dexamethasone, or 50 nM porcine insulin, as indicated in the various figures.

DNA Probes—For DNA/DNA or DNA/RNA hybridization experiments, the following DNA probes were used. PEPCK cDNA: 1.1-kb *Pst*I/*Pst*I fragment from the 3′ end of the PEPCK cDNA, pPCK10 (64); PEPCK: 620-bp *Bam*HI/*Bgl*II fragment from the 5′ end of the PEPCK gene (64); *neo*: 1.0-kb *Bgl*II/*Eco*RI fragment from the 3′ end of the *neo* gene; bGH: a 1-kb *Pst*I fragment containing the entire bGH cDNA; *TK*: a 1.2-kb *Bam*HI/*Hin*dIII fragment containing the entire *TK* gene; TAT: the cDNA for the tyrosine amino transferase gene (65); gapdh: the cDNA for the glyceraldehyde-3-phosphate dehydrogenase gene (66).

For S1 nuclease mapping of the PEPCKneo RNA shown in Fig. 3, the appropriate DNA fragments were 5′ end-labeled at the *Bgl*II site of the *neo* gene (see map in Fig. 1), using [γ-^{32}P]ATP. Details of the labeling procedure have been published previously (67).

RESULTS

Southern Analysis of Proviral DNA Transduced into Cells by Various Retroviruses—The integrity of the provirus was determined in mass cultures of infected cells. All of the proviruses were found to be intact except those containing the PEPCKbGH (PbGH)-chimeric gene, which had significant rearrangements or deletions. Proviruses that contained the PbGH gene in the same transcriptional orientation as the 5′ LTR (vectors 8 and 10) were intact in approximately 10% of the cells selected for expression of the *neo* gene. In contrast, proviruses containing the PbGH gene in a transcriptional orientation opposite to the 5′ LTR (vectors 9 and 11) were intact in 90% of the cells. We selected vectors 8 and 9 for detailed Southern analysis (Fig. 1). DNA was isolated from FTO-2B cells infected with these two viruses, digested with

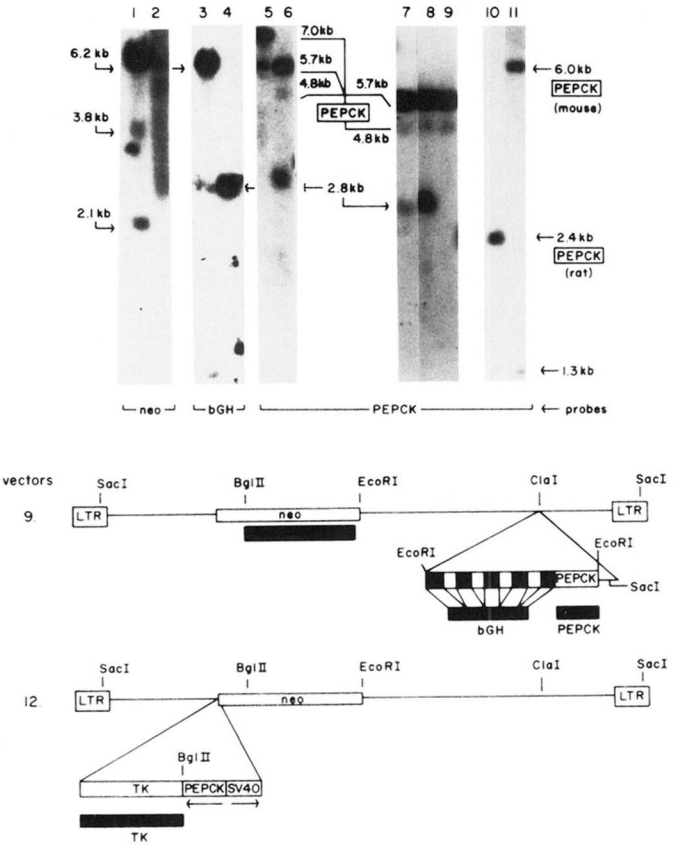

FIG. 1. **Analysis of the integrated provirus in mass cultures of cells infected with retroviruses.** DNA was isolated from mass cultures of FTO-2B cells infected with vector 9 grown in media containing G418. The DNA (20 μg) was digested with *Sac*I (*lanes 1, 3,* and *5*) or *Eco*RI (*lanes 2, 4, 6,* and *8*) and hybridized with *neo* (*lanes 1* and *2*), bGH (*lanes 3* and *4*), or PEPCK (*lanes 5, 6,* and *8*). DNA from FTO-2B cells infected with vector 8 was digested with *Eco*RI and hybridized with PEPCK (*lane 7*). *Lane 9* contains DNA from control, noninfected FTO-2B cells digested with *Eco*RI and hybridized with PEPCK. *Lanes 10* and *11* contained *Bgl*II-digested DNA that had been isolated from a mass culture of FTO-2B (*lane 10*) or NIH 3T3 (*lane 11*) cells infected with vLJPCKTK. The DNA was analyzed using a PEPCK probe. The size of proviral fragments is indicated in the figure. The *boxed* PEPCK represents DNA sequences from the endogenous PEPCK gene (rat, *lanes 5–10*; mouse, *lane 11*) that hybridize with the PEPCK probe. The diagram at the bottom of the figure is a map of the provirus, with the restriction sites used in this study and with the DNA hybridization probes (*dark boxes*).

*Sac*I or *Eco*RI, subjected to electrophoresis, and hybridized with three different probes (neo, PEPCK, and bGH (see diagram at the bottom of Fig. 1)).

*Sac*I digestion of genomic DNA isolated from FTO-2B cells infected with vector 9 removes virtually the entire provirus from the hepatic genome. We noted three proviral forms when *Sac*I-digested DNA was hybridized with the *neo* probe: a 6.2-kb DNA fragment, representing the intact provirus, and two deleted forms of the provirus, which were 3.8 and 2.1 kb in length (Fig. 1, *lane 1*). When a bGH or PEPCK probe was hybridized to the same DNA, only the 6.2-kb fragment was identified (Fig. 1, *lanes 3* and *5*). The DNA band at 7 kb (Fig. 1, *lane 5*) represents a fragment of the endogenous PEPCK gene. Comparison of the intensity of hybridization of the 6.2 kb band with the 3.8 or 2.1 kb bands indicated that the provirus was intact in 90% of the cells.

*Eco*RI digestion of the same DNA removes the intact, chimeric PbGH gene, whereas the remaining part of the provirus is contained within different size junction fragments from the host genome. Analysis of *Eco*RI-digested DNA using a bGH or PEPCK probe demonstrated the presence of a 2.8-kb segment of the provirus containing the complete PbGH gene (Fig. 1, *lanes 4* and *6*). The two additional fragments (5.7 and 4.8 kb) hybridized with the PEPCK probe (Fig. 1, *lane 6*) derived from the endogenous PEPCK gene. No specific bands hybridized with the neo probe (Fig. 1, *lane 2*), indicating the random integration of the provirus in the chromosomal DNA of the mass culture of infected FTO-2B hepatoma cells. DNA isolated from FTO-2B cells infected with vector 8 (the PbGH gene in this vector is in the same orientation relative to the 5′ LTR) contained mainly proviral forms that had the PbGH gene deleted. In order to identify the percentage of cells containing the intact PbGH gene, we performed Southern analysis of genomic DNA digested with *Eco*RI and hybridized with the PEPCK probe (Fig. 1, *lane 7*). A band at 2.8 kb was evident but it was less intense than the one identified with vector 9 (Fig. 1, *lane 8*), indicating that fewer cells contained the intact provirus (compare *lanes 7* and *8* in Fig. 1).

Another type of vector used in this study contained the PEPCK (vector 12) or TK (vector 13) promoters next to the SV40 promoter. Both promoters were maintained intact in the provirus of the mass cultures of FTO-2B or NIH 3T3 cells. Southern analysis of genomic DNA isolated from cells infected with vector 12 is presented in Fig. 1 (*lanes 10* and *11*). The predicted 1.3-kb fragment hybridized with the PEPCK probe after *Bgl*II digestion of genomic DNA isolated from FTO-2B (*lane 10*) and NIH 3T3 (*lane 11*) cells. Two additional fragments (2.4 and 6 kb) were also noted, due to hybridization to fragments of the endogenous PEPCK gene from the rat and mouse, respectively.

The integrity of the provirus was also examined using DNA isolated from single clones of cells infected with vectors 5 (*5–1* through *5–5* in Fig. 2*B*) and 9 (*9–1, 9–2, 9–4,* and *9–0* in Fig. 2*A*). Both the *neo* and PbGH genes were intact in the genomic DNA from three out of the four cultures of clonally isolated cells infected with vector 9 (Fig. 2*A*, *first* and *second panels*), whereas one clone (*9–0*) contained a deleted provirus of 2.1 kb, which was also present in the mass culture of FTO-2B cells (Fig. 1, *lane 1*). The mass culture of FTO-2B cells infected with vector 9 (Fig. 2*A*, *9-M*) is also presented for comparison. Analysis of *Eco*RI-digested DNA from these clones using a probe for the *neo* gene indicated random integration of the provirus into the host genome (Fig. 2*A*, *third panel*). The same analysis was performed using clonally isolated cells infected with vector 5. The provirus was intact in all of the clones (Fig. 2*B*, *first panel*) and was integrated into different sites in the genome (Fig. 2*B*, *second panel*).

The Effect of the Position of Promoters within the Provirus on Transcription and Hormonal Regulation of Gene Expression—A variety of retroviral vectors containing three promoters in different positions within the provirus was used to study the effect of promoter location and orientation on transcription and the interaction that these promoters have with each other.

The PEPCK Promoter within the Provirus—We noted previously that the PEPCK promoter (−550 to +73) inserted within the retroviral vector pLJ (vector 5) inhibited transcription from the 5′ LTR of the provirus after transduction into FTO-2B cells (29). This could be due to the transcriptional activation of the PEPCK promoter relative to the LTR by the binding of specific regulatory factors or to an epigenetic mechanism of transcriptional suppression (22–24) of the LTR due to selection for expression of a selectable gene such as

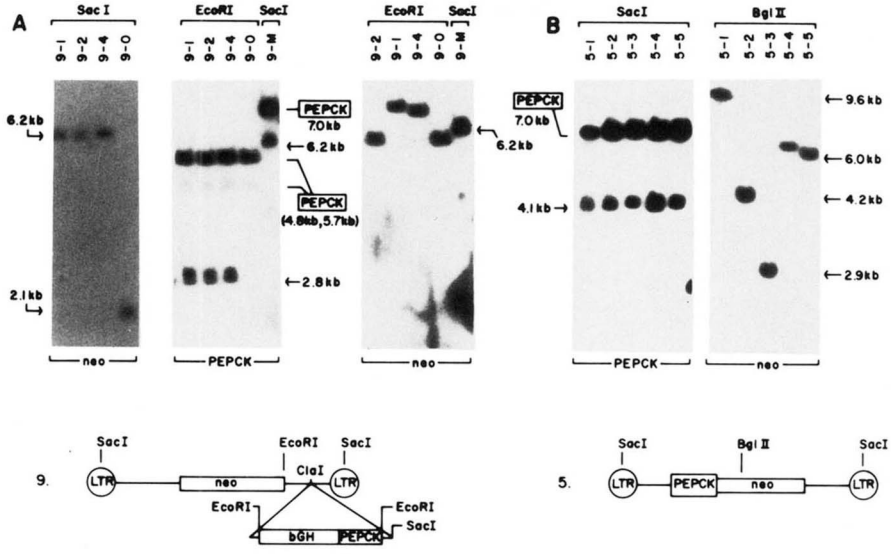

FIG. 2. **Southern analysis of DNA from clonally isolated cells infected with retroviruses.** DNA was isolated from two sets of clonally isolated cells; one set was infected with vector 9 (*panel A*: *9–1*, *9–2*, *9–3*, *9–4*, *9–0*) and the other infected with vector 5 (*panel B*: *5–1* through *5–5*). The DNA (20 μg) was digested with *Sac*I, *Eco*RI, or *Bgl*II and hybridized with the probes indicated at the bottom of the figure. *Sac*I digestion removes almost the entire provirus (6.2 kb, series of vector 9; 4.1 kb, series of vector 5), whereas *Eco*RI removes the entire PbGH gene (2.8 kb; *panel A*, PEPCK probe). The different size proviral junction fragments generated after digestion with *Eco*RI (neo probe; 9 series) or *Bgl*II (neo probe; 5 series) are shown in *panels A* and *B*, respectively. *Lane 9-M* represents Southern analysis of DNA isolated from mass cultures of cells infected with vector 9 (*panel A*). The *boxed* PEPCK indicates fragments from the endogenous PEPCK gene that hybridize with the PEPCK probe.

neo that is linked to the PEPCK promoter. In order to examine the first possibility, we determined which region of the PEPCK promoter was responsible for the previously observed inhibition of transcription (29). Segments of the PEPCK promoter (−2000 to +73, −350 to +73, and −174 to +73) linked to the *neo* gene and transduced into FTO-2B cells were tested. S1 nuclease mapping of the RNA isolated from FTO-2B cells infected with the viruses containing the deleted PEPCK promoter was performed using hybridization probes able to map transcription from both promoters simultaneously (Fig. 3). This analysis indicated that longer segments of the PEPCK promoter (−2000 to +73) totally suppressed transcription from the 5′ LTR (Fig. 3*A*). However, when the PEPCK promoter was deleted further (−350 to +73 and −174 to +73), transcription from the LTR increased, as compared to the PEPCK promoter (compare the relative hybridization intensities of the two bands in Fig. 3*B*). Thus, the segment of the PEPCK promoter from −350 to −174 contains sequences that are able to suppress transcription from the 5′ LTR of the provirus. The same result was observed when an internal fragment from the PEPCK promoter (−416 to −61) was linked to the SV40 promoter (vector 3) present in pLJ (vector 2); the PEPCK promoter inhibited transcription from both the LTR and the SV40 promoters (Fig. 3*C*). In order to more clearly demonstrate the relative level of transcription of the two promoters in the vectors, we used 25 μg of RNA from cells infected with vector 2 and 75 μg of RNA isolated from cells infected with vector 3 in the S1 analysis shown in Fig. 3*C*. With all segments of the PEPCK promoter, there was the expected induction of transcription by Bt_2cAMP (Fig. 3).

Next, we examined the expression of a second, nonselected gene (PbGH), which was positioned 3′ to the neomycin resistance gene. The PEPCK promoter was linked to the bGH structural gene and inserted into the *Cla*I site of pLJneo in both orientations relative to transcription from the 5′ LTR (vectors 8 and 9). Fig. 4 shows the level of transcription of bGH (PEPCK promoter) or *neo* (5′ LTR) in cells infected with vectors 8 and 9. Both promoters were transcriptionally active, but the level of transcription from the PEPCK promoter greatly exceeded that of the 5′ LTR when dexamethasone was added. Since the 5′ LTR responds very acutely to dexamethasone (29), the high level of transcriptional induction from the PEPCK promoter is probably due to its synergistic effect on the glucocorticoid regulatory elements present in the PEPCK promoter (50, 51). RNA shorter than the predicted full-length viral transcript was noted with both vectors (Fig. 4, *first panel*, vectors 8 and 9; the band is indicated with an *arrow*). This probably represents a spliced form of the full-length RNA. Cyclic AMP had no effect on transcription from the PEPCK promoter in both vectors (Fig. 4).

Individual clones of FTO-2B cells infected with vectors 5 and 9 were used to determine the influence of the integration site of the provirus on the relative level of transcription from the PEPCK promoter and the viral LTR. We examined 24 clones containing vector 5 and 12 clones containing vector 9. In only one of the 24 clones analyzed (only five are presented in Fig. 5*A*) did the LTR appear to be more transcriptionally active than the PEPCK promoter (clone 5–4). Dexamethasone stimulated transcription from the PEPCK promoter in all of the clones except for clone 5–4, in which transcription was induced from the LTR and not from the PEPCK promoter (Fig. 5*A*). When FTO-2B cells were infected with vector 9, the level of transcription from the two promoters in the 12 clones analyzed (five are presented), varied in each individual cell line, but transcription from both promoters was induced by dexamethasone. However, there are two basic patterns of response. Clones 9–1 and 9–2 had equal levels of transcription from both the PEPCK promoter and the LTR, whereas in cell line 9–4, dexamethasone induction from the PEPCK promoter was markedly induced relative to the LTR (Fig. 5*B*). Interestingly, in mass cultures of FTO-2B cells infected

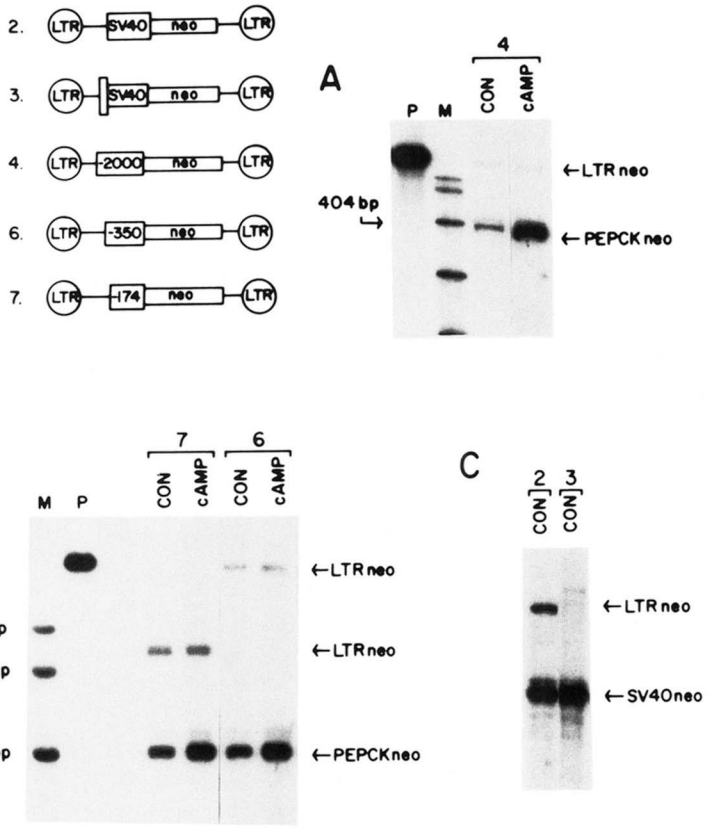

FIG. 3. **Analysis of the regions of the PEPCK promoter responsible for suppression of the activity of the 5′ LTR.** *Panels A* and *B*, S1 analysis of RNA (25 μg) isolated from FTO-2B cells infected with retroviruses 4–7. The appropriate DNA fragments were 5′ end-labeled at the *Bgl*II site of the *neo* gene (see Fig. 1) with [γ-^{32}P]ATP as described under "Experimental Procedures" and indicated in the figure (*P*). These DNA probes map the start site of transcription from the PEPCK promoter (*PEPCK-neo*) and RNA transcribed from the 5′ LTR (*LTRneo*). The cells were incubated for 4 h with the hormones indicated in the figure, before the analysis of RNA. *Panel C*, S1 analysis of RNA isolated from FTO-2B cells infected with retroviruses 2 and 3 (see Fig. 2). All conditions are the same as those described in *panel A*, except that the cells were incubated in media without serum before the isolation of RNA and hybridized with the appropriate DNA probes. For reasons of comparison, *lane 2* contains 25 μg of RNA, whereas *lane 3* contains 75 μg. These DNA probes map the start site of transcription from the SV40 promoter (*SV40neo*) and RNA transcribed from the 5′ LTR (*LTRneo*). *CON*, control.

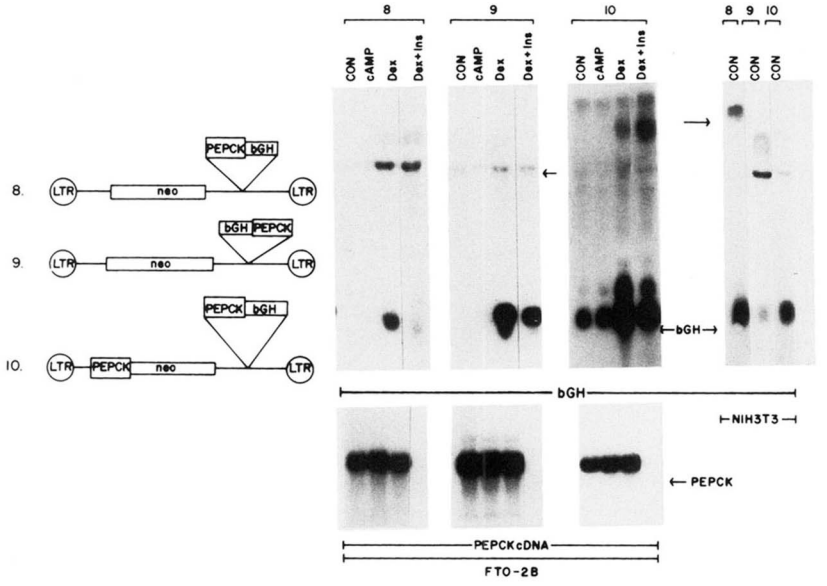

FIG. 4. **Hormonal regulation of transcription of the PEPCK-bGH gene transduced into FTO-2B and NIH 3T3 cells.** Northern analysis of RNA isolated from mass cultures of cells infected with retroviruses 8–10. The cells were incubated with the hormones indicated on the figure, and the RNA was isolated after 4 h. bGH was used as a hybridization probe. The same RNA blots as above were rehybridized with the PEPCK cDNA probe. *CON*, control; *Dex*, dexamethasone; *Ins*, insulin.

with this virus, three out of the four mass cultures exhibited the pattern of clone 9–4 (only one is presented in Fig. 4), suggesting that the differential response to dexamethasone was due to integration of the provirus into specific site(s) in the host cell genome.

Cyclic AMP is known to markedly induce transcription from the same segments of the PEPCK promoter used in this study, when introduced into FTO-2B cells by transfection (48, 49) and retroviral infection (29). However, the cyclic nucleotide had no effect on transcription from the PEPCK promoter in retroviral vectors in which it was placed at the 3′ end of the provirus (Fig. 4 and Fig. 5*C*). The inclusion of two copies of the PEPCK promoter in vector 10 resulted in the highest level of basal transcription of the bGH gene, but did not result in the greatest response to dexamethasone or Bt_2cAMP (Fig. 4). As expected, insulin inhibited the glucocorticoid induction of transcription originating from the PEPCK promoter in all vectors used in this study (Fig. 4). The endogenous PEPCK and TAT genes, which are expressed in FTO-2B cells and induced by cAMP and dexamethasone, were used in this study as controls for the effectiveness of the added hormones and to demonstrate that the RNA isolated from the hepatoma cells was intact (Figs. 4 and 5). In agreement with a previous study (29), we noted that the concentration of PEPCK mRNA was higher in cells infected with the various retroviruses, and in some cases the level of expression was as high as that noted after hormonal induction (Fig. 4, vectors 8–10). One out of five of the clonally isolated cell lines

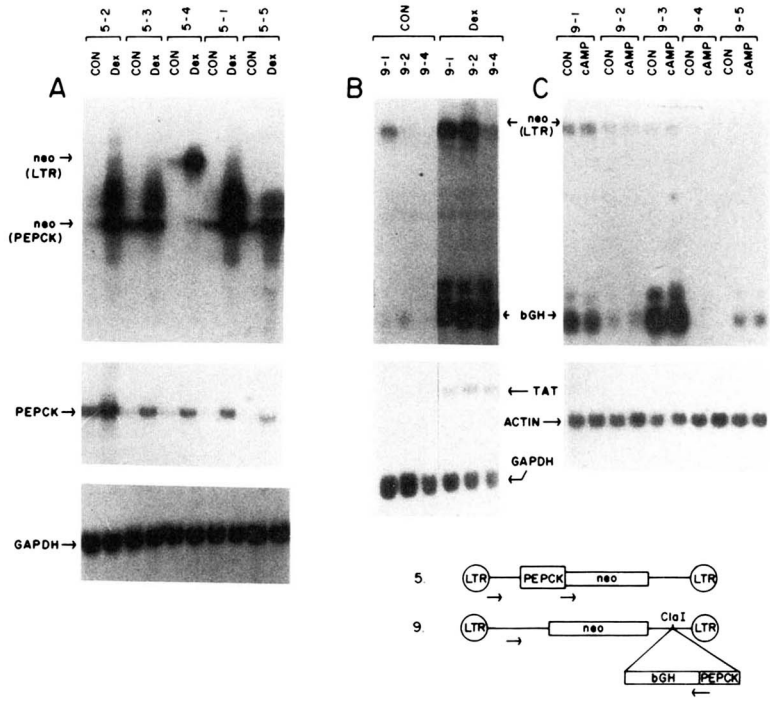

FIG. 5. **Interaction of promoters in single clones of FTO-2B cells infected with vector 5 and vector 9.** RNA was isolated from cells treated with dexamethasone or Bt_2cAMP for 4 h and analyzed by Northern blotting, using the probes indicted on the figure. Clones 5–1 through 5–5 were derived from cells infected with vector 5 (*panel A*), and clones 9–1 through 9–5 were derived from cells infected with vector 9 (*panel B*). A map of the two retroviruses used in this experiment is included at the bottom of the figure. *CON*, control; *Dex*, dexamethasone; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

had exceptionally high levels of PEPCK mRNA (Fig. 5, clone 5–2). The reason for this effect is not clear.

Two Different Promoters within the Provirus—The 5′ LTR was always transcriptionally active in the absence of additional promoters in the provirus (Fig. 6*A*, vector 1; note the 3.7 kb RNA transcript). A provirus containing the relatively strong SV40 promoter placed 450 bp downstream of the LTR (vector 2) suppressed transcription from the LTR, as shown by the 3.2 kb RNA band resulting from hybridization with the *neo* probe (compare this with the 3.7 kb RNA transcript from the 5′ LTR; Fig. 6*A*, vectors 1 and 2). As noted previously (Fig. 3), when a chimeric promoter containing a fragment of the PEPCK promoter (−416 to −61) was linked to the SV40 promoter (vector), transcription from both the SV40 promoter and the 5′ LTR was suppressed (compare vectors 2 and 3 in Fig. 6*A*). A pronounced suppression of total proviral transcription was noted when a chimeric gene containing the PEPCK promoter (−450 to +73) driving the TK structural gene was included in the virus at a position adjacent to the SV40 promoter, but in an orientation opposite to transcription from the LTR (Fig. 6*A*, vector 12). No suppression was noted from either the SV40 or TK promoters when the TK gene, under the control of its own promoter, was included at the same position in the provirus as the chimeric PEPCK-TK gene (Fig. 6*A*, vector 13). It should be noted that the TK promoter adjacent to the SV40 promoter was more transcriptionally active than the 5′ LTR (Fig. 6*A*, vector 13, TK probe) since the TK probe hybridized only with TK RNA transcribed from the TK promoter and not the full-length RNA transcribed from the 5′ LTR. These results indicate that the promoters in close proximity can interact, resulting in preferential inactivation or stimulation of transcription.

Transcription from both the PEPCK promoter and the 5′ LTR is stimulated by dexamethasone (29, 48–50). We next tested the effects of dexamethasone on the relative level of transcription from the promoters included in the provirus. As noted above, when the only promoter in the provirus was the 5′ LTR, dexamethasone induced the concentration of *neo* RNA 30-fold (Fig. 6*C*, vector 1). In proviruses where the SV40 promoter was placed 3′ to the 5′ LTR (vectors 2 and 3), dexamethasone stimulated transcription from the SV40 promoter and not from the LTR (Fig. 6*C*; neo probe). In contrast, dexamethasone caused a 30-fold induction from the 5′ LTR when the complete TK gene was inserted adjacent to the SV40 promoter, in an orientation opposite that of the LTR (vector 13), without enhancing transcription from the SV40 or TK promoters (Fig. 6*C*, vector 13; compare the pattern with neo and TK probes). Since transcription from the SV40 promoter is not induced by dexamethasone, enhancer elements in the 5′ LTR are responsible for the hormone-induced elevation in the levels of *neo* RNA in the cells infected with vector 2. This effect was not noted when the TK structural gene was inserted in the provirus (vector 13), perhaps due to the distance between the LTR and the SV40 promoter.

We also determined the effect of dexamethasone on proviral transcription when the PEPCK-TK structural gene was inserted into the virus adjacent to SV40 (vector 12). As mentioned above, the level of transcription from all promoters in this provirus was low. However, dexamethasone caused a 4-fold induction in the concentration of *neo* RNA transcribed from the SV40 promoter, indicating that promoter suppression is reversible in the presence of the hormone-responsive enhancer elements in the PEPCK promoter (Fig. 6*C*, vector 12, *neo* probe). Dexamethasone did not induce transcription from the 5′ LTR in this vector (Fig. 6*C*, vector 12, absence of a high molecular weight band with a neo probe), perhaps due to an interaction of glucocorticoid-responsive elements in the 5′ LTR and the PEPCK promoter, which resulted in a stimulation of transcription from the SV40 promoter rather than the LTR. In contrast, in FTO-2B cells infected with vector 11 (Fig. 6*A*, vector 11, bGH probe), dexamethasone induced transcription from the PEPCK promoter and not from the SV40 (Fig. 6*C*, vector 11, bGH probe), indicating cooperation of the glucocorticoid-responsive elements contained in the 3′ LTR and the PEPCK promoter in the observed stimulation of expression of the bGH gene.

Cell Type-specific Interaction of Proviral Promoters—A different pattern of promoter interaction between cell lines was

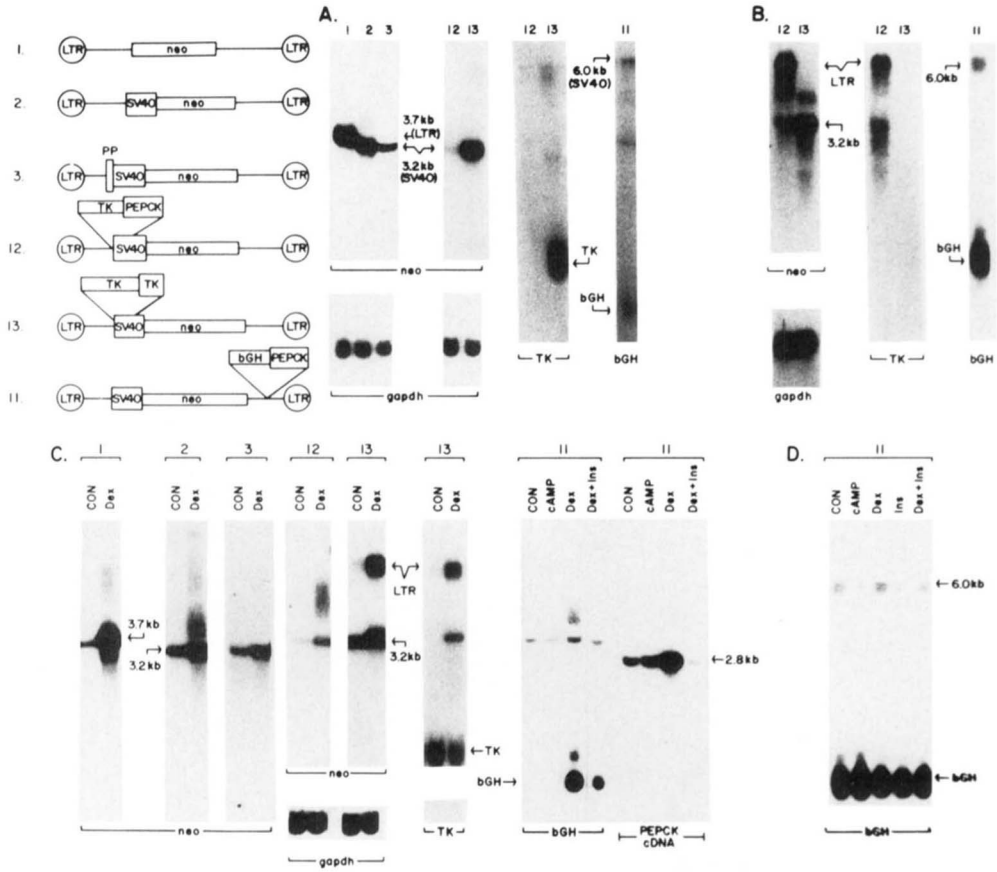

FIG. 6. **Expression and hormonal regulation of chimeric genes contained in a retrovirus transduced into FTO-2B and NIH 3T3 cells.** *Panel A,* Northern analysis of RNA isolated from the mass cultures of FTO-2B cells infected with retroviruses 1–3 and 11–13. RNA was isolated 4 h after the cells were placed in serum-free media, and 25 μg of RNA from each infected mass culture of cells was separated by electrophoresis and hybridized using *neo* as a probe. The RNA samples were analyzed at the same time and hybridized with the DNA probe indicated at the bottom of the figure. The Northern blot containing RNA from FTO-2B cells infected with virus containing vectors 12 and 13 was rehybridized with TK. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a control for the amount of RNA contained in each lane. *Panel B,* Northern analysis of RNA isolated from NIH 3T3 cells infected with retrovirus 11–13. The same analysis as above was performed using 25 μg of RNA from each mass culture of infected cells. The Northern blot containing RNA from vectors 12 and 13 was rehybridized with TK. *Panel C,* Northern analysis of RNA isolated from hormonally treated FTO-2B cells infected with retroviruses 1–3 and 11–13. Mass cultures of cells were incubated for 4 h with the hormones indicated in the figure, and the RNA was isolated and analyzed by Northern blotting using *neo,* TK, and bGH as hybridization probes. The Northern blot from vector 13 was rehybridized with TK. The Northern blot from vector 11 was rehybridized with PEPCK cDNA. *Panel D,* Northern analysis of RNA isolated from hormonally treated NIH 3T3 cells infected with retrovirus 11. RNA was isolated from mass cultures of infected cells and analyzed as outlined in *panel B.* RNA blots shown in *panels A, B,* and *C* were rehybridized with the *gapdh* probe. *Arrows* point out the expected RNA transcripts: *TK,* TK mRNA; *bGH,* bGH mRNA; *3.7 kb, neo* mRNA transcribed from the 5′ LTR; *3.2 kb, neo* mRNA transcribed from the SV40 promoter; *6 kb, neo* mRNA transcribed from the SV40 promoter; and *LTR, neo* mRNA from the 5′ LTR. A schematic representation of vectors 1–3 through 11–13 is presented at the side of the figure. *CON,* control; *Dex,* dexamethasone; *Ins,* insulin.

evident when comparing FTO-2B and NIH 3T3 cells infected with vectors 12 and 13. No promoter suppression was evident with vector 12, so the 5′ LTR was more active transcriptionally than the SV40 promoter (Fig. 6*B*, compare hybridization with neo and TK probes). In contrast, in NIH 3T3 cells infected with vector 13, the major promoter was SV40 (Fig. 6*C*), whereas in the same cells infected with vector 11, the PEPCK promoter predominated. The PEPCK promoter was dominant, even when no other promoter was included in the provirus of NIH 3T3 infected cells (Fig. 4, *second panel,* vectors 9 and 10). Dexamethasone and insulin had no effect on gene transcription from any of the promoters contained in virus transduced into NIH 3T3 cells, indicating the absence of specific hepatic factors (Fig. 6*D*, vector 11, bGH probe). Finally, the overall level of expression of the PEPCK-bGH gene in NIH 3T3 cells was much higher than noted in FTO-2B cells, despite the fact that the hepatoma cells express high level of the endogenous PEPCK gene (29), and PEPCK mRNA is not present in NIH 3T3 cells.

The relative level of expression of the PEPCK-bGH gene in the provirus as compared to the endogenous PEPCK in infected FTO-2B cells was determined. Both PEPCK and bGH mRNA share 73 bp of 5′-untranslated sequence, allowing us to directly compare the relative levels of mRNA originating from the PEPCK promoter linked to both genes. The concentration of PEPCK mRNA was almost 3 times higher than the level of bGH mRNA in hepatoma cells infected with vector 9 and treated for 4 h with dexamethasone (Fig. 7). Since PEPCK mRNA accounts for as much as 0.1–0.3% of hepatic mRNA after induction by starvation or hormones (43), the level of expression of chimeric genes containing the

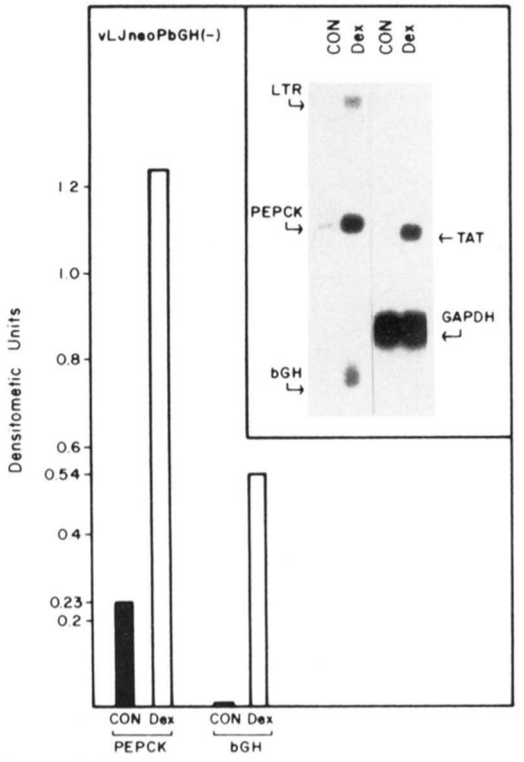

FIG. 7. **A comparison of the level of mRNA transcribed from the PEPCK promoter introduced into FTO-2B cells via retroviral transduction with RNA from the endogenous PEPCK gene.** RNA was isolated from hepatoma cells infected with vector 9 and analyzed by Northern blotting, using PEPCK as a probe. Cells were incubated for 4 h with dexamethasone, as indicated in the figure. The relative levels of PEPCK and bGH mRNA are expressed as densitometric units, after scanning a Northern blot such as the one presented in the *inset*. The Northern blot was rehybridized with *gapdh* and TAT. *CON*, control; *Dex*, dexamethasone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PEPCK promoter in hepatoma cells is high enough to potentially alter metabolic processes in these cells.

DISCUSSION

For retroviral vectors to be a truly useful tool for gene therapy, it is necessary to characterize the interaction between promoter enhancer elements contained in the provirus that are to be introduced into specific cell types. Since the viral LTR of the MoMLV-based retroviral vectors contain several enhancer elements (56), it is expected to have a major effect on transcription from surrounding promoters (21). This greatly complicates a simple prediction of the pattern of basal and hormonally regulated expression of genes within the LTRs. In addition, cell-specific factors and the site of integration of the provirus in the host cell genome can influence both basal expression from proviral promoters and their response to hormones and other factors (18, 32, 55). We have addressed each of these points in the present paper by using three internal promoters, PEPCK, TK, and the SV40 early promoter, and two cell lines, rat hepatoma cells and mouse fibroblasts. Understanding the functioning of proviral promoters in fibroblasts is important since these cells are commonly used as packaging cell lines for the generation of replication-incompetent retrovirus (68) and for the introduction of genes *in vivo* by infecting primary skin fibroblasts that are then grafted to the skin of host animals (25, 69).

Relative Position of Proviral Promoters—It is known from the work of Emerman and Temin (23, 24) that a promoter positioned 3′ to the 5′ LTR will suppress transcription from the LTR, when the promoter is linked to a selectable marker gene. They show that if two genes are included in the provirus, selection for one of these genes greatly reduces expression of the second. We have shown in this paper that the *relative position* of promoter/enhancer elements in the provirus greatly influences expression of the chimeric genes linked to these promoters, independent of selection for expression of that gene. We noted that the PEPCK promoter can efficiently transcribe either the selectable marker gene *neo* or the nonselectable bGH gene when it is placed within the LTRs in either transcriptional orientation (see Fig. 4). Transcriptional elements present in the PEPCK promoter are responsible for the promoter suppression of the 5′ LTR since deletions in the PEPCK promoter from −355 to −174 decreased the rate of transcription from the PEPCK promoter relative to the 5′ LTR. It is thus possible to carry out an analysis of the *cis* sequences and transcription factors that regulate the two promoters and are responsible for the relative transcription of proviral genes noted in this study. The region of the PEPCK promoter between −355 and −174 contains several CAAT/enhancer binding protein binding domains, one of which is essential for the expression of a chimeric PEPCK-bGH gene in the livers of transgenic mice[2] and for the cAMP-regulated transcription of the PEPCK promoter in hepatoma cells.[3] We are currently testing systematic mutations of individual regulatory elements in both the 5′ LTR and the PEPCK promoter for their effects on the relative transcription from these promoters.

Hormones may also influence the transcription from promoters contained in a provirus. Both the viral LTRs and the PEPCK promoter contain glucocorticoid regulatory elements that control transcription depending on the relative position of the promoters in the provirus. The greatest effect of glucocorticoids was observed when the PEPCK promoter was placed close to the glucocorticoid-responsive elements of the 3′ LTR. With promoters that lack hormone regulatory elements (such as the SV40 or TK promoters), induction by steroid hormone depended on the proximity of these promoter(s) to the viral LTRs (see Fig. 6*C*, vectors 2, 3, and 13) or the PEPCK promoter (Fig. 6*C*, vector 12).

It is also possible to create vectors in which two internal promoters are positioned in such a way as to allow enhancer interaction, thereby regulating transcription from both promoters. For example, the SV40/TK promoter cassette transcribed both linked genes at high levels (see Fig. 6*A*, vector 13). Despite the fact that selection in G418 was exerted on the SV40-*neo* gene, the TK promoter linked to its own structural gene remained transcriptionally active, perhaps due to the presence of strong enhancer elements in the SV40 promoter. This type of promoter-promoter interaction permits the design of retroviral vectors in which selection targeted to one gene does not interfere with the expression of another internal gene.

Effect of Proviral Position in the Host Cell Genome on Expression of Internal Genes—It is generally assumed that retroviruses integrate randomly into the host cell genome. However, several recent studies point to some specificity in the site selected for proviral integration, which favors transcriptionally active regions of the genome. A detailed analysis of the site of proviral integration of replication-competent Rous sarcoma virus was recently reported by Shih *et al.* (70), who estimated that 20% of the proviruses were targeted to a single location in the host cell genome. In this study, we have

[2] Y. Patel, unpublished observation.
[3] J. Liu, unpublished observation.

noted that the site of proviral integration influences the selection of promoter activity and hormonal regulation of gene expression in infected hepatoma cells. Two patterns of promoter activity were noted with clones isolated from infected FTO-2B cells (Fig. 5); in one, the internal PEPCK promoter was dominant, whereas in the other the LTR was equally expressed. This could be due to a deletion or mutation in one of the promoters resulting in a loss of transcriptional activity. However, this is unlikely since dexamethasone, which can stimulate transcription from both promoters, always induced expression from the PEPCK promoter, whereas induction from the LTR varied with each clone. This indicates that the site of proviral integration is responsible for the transcriptional response to hormones rather than inactivation of the promoters due to mutations. In addition, the integration site of the provirus could provide a growth advantage for a population of cells that have a similar pattern of expression of proviral genes, as noted in the mass cultures of infected cells (Figs. 4 and 5). This effect is *not* due only to selection of a marker gene, since in some of the mass cultures of infected hepatoma cells the dominant promoter was not linked to the selectable *neo* gene.

Cell Type-specific Regulation of Proviral Gene Expression—The PEPCK gene is expressed predominately in the liver, and also in a subset of mammalian tissues, including kidney cortex, adipose tissue, and the jejunum (71). The segment of the PEPCK promoter, linked to the bGH structural gene that was used in this study (−450 to +73), has been introduced previously into the germ line of transgenic mice and shown to express in the same tissues as the endogenous PEPCK gene (47). Since the PEPCK gene is expressed in a variety of hepatoma cell lines but not in fibroblast cells and since the PEPCK promoter contains elements necessary for tissue-specific expression, we expected differences in the level of expression from the PEPCK promoter in the various cell types infected with retrovirus. However, transcription from the PEPCK promoter dominates over the LTR in both rat hepatoma and mouse fibroblast cells (Figs. 3 and 6). This dominant effect in mouse fibroblasts is possibly due to enhancer elements in the LTRs that interfere with the binding of factors to the PEPCK promoter and that cause its transcriptional inactivation (72). This is supported by the observation that the PbGH gene was not expressed in NIH 3T3 cells, transfected with a pBR322-based plasmid containing the PbGH gene without viral sequences (data not shown). Additionally, there are differences between cell lines in the glucocorticoid induction of transcription from both the PEPCK promoter and the viral LTR. In FTO-2B hepatoma cells, dexamethasone markedly stimulates transcription from these promoters, whereas in NIH 3T3 cells there is no induction by the steroid hormone. The glucocorticoid receptor, needed for responsiveness to steroid hormones in these cells, appears to be present since it has been shown previously that transcription from the murine mammary tumor virus promoter, introduced into NIH 3T3 cells, was stimulated by glucocorticoids (57). Co-transfection of the glucocorticoid receptor with the retroviral vectors in NIH 3T3 cells did not alter transcription from either the LTR or PEPCK promoter in the presence of the hormone (data not shown). A more detailed understanding of the factors necessary for regulating transcription of complex promoters will be necessary before an entirely rational basis in designing retroviral vectors will be possible.

A final factor in evaluating the usefulness of retroviral vectors for introducing genes into cells is the level of expression of proviral genes relative to the endogenous genes of the infected cells. The PEPCK promoter, introduced into hepatoma cells in this study, was expressed at approximately 30% of the level of the endogenous PEPCK gene (Fig. 7), when the gene was induced by dexamethasone. The fact that transcription from the PEPCK promoter in a retroviral vector can be markedly depressed by insulin (29) also allows for modulation of gene expression. The predictable regulation of a gene introduced into cells by retroviruses is critical for their ultimate use in gene therapy.

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