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Regulation of Chimeric Phosphoenolpyruvate Carboxykinase Genes by the *trans*-Dominant Locus TSE1

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Extinction of phosphoenolpyruvate carboxykinase (PCK) gene expression in hepatoma × fibroblast hybrids is mediated by a *trans*-acting genetic locus designated tissue-specific extinguisher 1 (TSE1). To identify PCK gene sequences required for extinction, hepatoma transfectants expressing PCK-thymidine kinase (TK) chimeric genes were fused with TK^- fibroblasts and PCK-TK expression in the resulting hybrids was monitored. Expression of a PCK-TK chimera containing PCK sequences between base pairs −548 and +73 was extinguished in four of five hepatoma transfectants tested, although hybrids derived from one transfectant clone failed to extinguish PCK-TK expression. In contrast, crosses between hepatoma transfectants expressing the herpesvirus TK gene from its own promoter and TK^- fibroblasts produced TK^+ hybrids; extinction of the transfected TK gene was not observed. Thus, rat PCK gene sequences between base pairs −548 and +73 are sufficient for tissue-specific extinction in hybrid cells. Extinction of PCK-TK gene expression in transfectant microcell hybrids mapped specifically to human chromosome 17, the site of human TSE1.

Differentiated mammalian cells display a remarkable capacity for the selective expression of specific genes. A particular gene may account for a large fraction of total transcription in one cell type, yet be virtually silent in other cell lineages. These differences in gene activity are controlled, at least in part, by interactions between *trans*-acting factors and discrete sequence elements within target genes. By systematically mutating tissue-specific genes in vitro and introducing the altered constructs into living cells or organisms, both positive and negative elements involved in gene control have been defined (1, 2, 15, 16, 23, 32, 40). Specific protein factors that bind to those sequences have been identified and, in several cases, isolated and characterized (4, 20).

The regulation of tissue-specific gene expression in mammalian cells is obviously complex: individual genes are mosaics of discrete control elements, and multiple factors are required for regulated transcription. In view of this complexity, it is perhaps surprising that expression of tissue-specific genes is altered systematically in genetic crosses between different cell types. For example, tissue-specific gene activity is generally repressed in heterokaryons and hybrids formed by fusing cells of different types (11). In contrast, activation of previously silent genes can occur in polyploid heterokaryons (3), in hybrids with biased gene dosage (13), or in hybrid segregants (21, 43, 44). These systems provide a genetic approach for identifying factors that regulate expression of specific mammalian genes.

Virtually all liver-specific gene activity is repressed in intertypic hybrids formed by fusing highly differentiated rat hepatoma cells with other cell types (7). This extinction phenomenon has a specific genetic basis: dominant, *trans*-acting loci of the nonhepatic parental cells are responsible for extinction of particular liver genes. The first locus so defined, tissue-specific extinguisher 1 (TSE1), is a discrete genetic element that resides on mouse chromosome 11 or its human homolog, chromosome 17 (21). This locus extinguishes the expression of several liver genes, including those encoding tyrosine aminotransferase (*Tat-1*) and phosphoenolpyruvate carboxykinase (*Pck-1*) (26). Since the TSE1, *Tat-1*, and *Pck-1* loci reside on three different mouse chromosomes (27, 36), this regulation necessarily occurs in *trans*. Furthermore, expression of other liver genes is not affected by TSE1; rather, extinction of these genes is mediated by extinguisher loci that map to other mouse and human chromosomes (8, 37).

As a first step toward defining the mechanism of TSE1-mediated extinction, and to explore the relationship between extinction and other aspects of liver-specific transcriptional control, we sought to identify sequence elements of the *Pck-1* gene that are required in *cis* for extinction. We report that chimeric genes containing 5′-flanking sequences from the rat *Pck-1* gene are expressed in hepatoma transfectants, but extinguished in intertypic hybrids. Furthermore, we show that extinction of these chimeric genes was a specific genetic effect mediated by fibroblast TSE1.

MATERIALS AND METHODS

Cell lines and culture conditions. FTO-2B is a ouabain-resistant derivative of FT-2 (22), a thymidine kinase-deficient (TK^-) rat hepatoma line isolated from Fu5-5 (43). Ltn is a TK^- mouse L cell (Ltk^-) that contains a single integrated copy of the plasmid pTM (19), which renders the cells G418 resistant ($G418^r$). Microcell hybrid clones L(17n)C and L(17n)E are mouse L cells that contain a single human chromosome 17 in which a *neo*-containing retroviral vector [ZIPneoSV(X)1 (5)] is integrated, whereas L(17)A contains an unmarked human chromosome 17. These hybrids were constructed as described previously (14).

The cells were cultured in a 1:1 mixture of Ham F12 medium and Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (GIBCO Laboratories). Antibiotics were not used, and all cell lines were free of

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TABLE 1. Properties of the hepatoma transfectants[a]

Clone	TK gene	Copy no.	Relative plating efficiency in[b]:		TK activity (μU/mg)[c]
			HAT	BrdU	
PTK-1	PCK-TK	1	0.92	1×10^{-5}	120
PTK-2	PCK-TK	5	0.43	$<1 \times 10^{-5}$	110
PTK-3	PCK-TK	ca. 100	0.66	$<2 \times 10^{-5}$	ND[d]
PTK-4	PCK-TK	10–20	0.78	$<2 \times 10^{-5}$	73
PTK-5	PCK-TK	1	0.76	$<1 \times 10^{-5}$	76
HTK-1	TK-TK	2	0.96	2×10^{-5}	38
HTK-2	TK-TK	1	0.77	$<3 \times 10^{-5}$	17
HTK-3	TK-TK	1	0.50	$<1 \times 10^{-5}$	22
HTK-4	TK-TK	1	0.83	$<3 \times 10^{-5}$	31

[a] Clonal lines of rat hepatoma cells transfected with PCK-TK or TK-TK genes were isolated as described in the text. The presence of HSV TK coding sequences in genomic DNA of each transfectant clone was verified by Southern blot hybridization (Fig. 2 and 3; data not shown), and approximate copy numbers of the transfected sequences are indicated.

[b] The relative plating efficiency in medium containing HAT or BrdU indicates that each transfectant displays a HAT^r $BrdU^s$ phenotype, as expected for TK^+ cells.

[c] Herpesvirus-specific TK activity in cell extracts was quantitated as described in Materials and Methods.

[d] ND, Not determined.

mycoplasmas as judged by staining with the fluorochrome Hoechst 33258 (6).

Plasmids. Plasmids pOPF, pPCTK-6A, d5′*neo*548, d5′*neo*134, d5′*neo*tk, and pPCK10 (41, 47, 48) were obtained from R. Hanson, Case Western Reserve University, Cleveland, Ohio. The pKα-1 recombinant contains a 1.6-kbp insert of human α-tubulin cDNA (10).

Transfections. Plasmids were introduced into FTO-2B recipient cells by either calcium phosphate-DNA coprecipitation (45) or electroporation (38). For calcium phosphate transfection, the cells were seeded at 2×10^6 per 100-mm dish 24 h prior to transfection. Sterile, ethanol-precipitated DNA was dissolved in distilled water, and $CaCl_2$ was added to a final concentration of 250 mM. The DNA-$CaCl_2$ solution was added dropwise to an equal volume of HEPES-buffered saline solution (280 mM NaCl, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 1.5 mM sodium phosphate, [pH 7.1]) with air-bubble agitation. The coprecipitate was allowed to develop for 30 to 45 min at room temperature without agitation, and 1 ml was added directly to the culture medium of each 100-mm plate of recipient cells. The amount of DNA per dish was typically 0.1 to 1.0 μg of plasmid plus 20 μg of salmon sperm DNA carrier. The cells were incubated at 37°C for 24 h, and selection was applied.

For electroporation, log-phase cells were harvested, washed in ice-cold phosphate-buffered saline, and suspended in Ca^{2+}-, Mg^{2+}-free phosphate buffered saline at a concentration of 10^8 cells per ml. Linearized plasmid DNA was added to 40 μg/ml, and the suspension was incubated in a plastic cuvette with aluminum foil electrodes at 4°C for 10 min. An electric pulse was discharged across the electrodes by using an ISCO 494 power supply with a voltage setting of 2,000 V and a current of 0.9 mA. The cells were incubated on ice for 10 min, and 4×10^6 cells were seeded into each of a series of 100-mm dishes. Selection was applied after 24 h.

The TK^+ phenotype was selected in medium containing hypoxanthine, aminopterin, and thymidine (HAT), while *neo* gene expression was selected in the presence of 500 μg of G418 per ml. In either case, transfectant clones were picked after 2 to 3 weeks by using glass cloning cylinders.

Cell fusion. Mixed monolayers of rat hepatoma transfectants and mouse Ltn cells (ca. 2×10^6 of each in a 25-cm^2 flask) were treated with 1 ml of 50% (wt/wt) polyethylene glycol 1540 (J. T. Baker Chemical Co.) for 1.0 min, rinsed three times with serum-free medium, and incubated in complete, nonselective medium for 24 h. The cells were distributed into 5 to 10 100-mm plates, and selection was applied.

Microcell fusion. L(17)A, L(17n)C, or L(17n)E donor cells were micronucleated by exposure to 0.06 μg of colcemid per ml for 48 h. The cells were harvested and seeded onto plastic "bullets" to which concanavalin A had been covalently linked, as described previously (17). The cells were enucleated by centrifugation at 28,000 × *g* at 28 to 32°C in serum-free medium containing 10 μg of cytochalasin B per ml. Isolated microcells were pooled, suspended in serum-free medium containing 100 μg of phytohemagglutinin P (Difco Laboratories) per ml, and added to a 70 to 80% confluent monolayer of hepatoma recipient cells in a 25-cm^2 flask. After 10 to 15 min at 37°C, the microcells had agglutinated to the recipient monolayer, and fusion was induced as described above for cell-cell hybridization.

DNA blot hybridization. High-molecular-weight cellular DNA (31) was digested to completion with *Eco*RI (New England BioLabs, Inc.) and electrophoresed through 0.7% agarose gels in 0.04 M Tris-acetate–2 mM EDTA (pH 8.0). The DNA was transferred to nylon hybridization membranes by standard techniques (9). The blots were prehybridized for 5 min to several hours at 42°C in buffer consisting of 0.5 M sodium phosphate, 1 mM EDTA, 7% sodium dodecyl sulfate, 1% bovine serum albumin (fraction V), and 50% formamide (pH 7.2). Hybridization was carried out for 17 to 24 h at 42°C in fresh buffer containing 1×10^7 to 8×10^7 cpm of randomly primed ^{32}P-labeled DNA probe (specific activity, 0.5×10^9 to 1.0×10^9 cpm/μg). The filters were washed sequentially in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for 15 min at room temperature, 0.1× SSC–0.1% sodium dodecyl sulfate for 15 min at room temperature, and 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C with two changes at 30-min intervals. The washed blots were exposed to Kodak XAR or XRP film for 1 to 5 days with a single intensifying screen.

RNA blot hybridization. Cytoplasmic RNA was extracted (12) and fractionated on 1.2% agarose–formaldehyde gels. The RNA was transferred to nylon membranes by capillary transfer, and the blots were baked (for 2 to 4 h at 80°C) and UV cross-linked by standard techniques (9). Prehybridization, hybridization, and autoradiography were performed as described above for DNA blot hybridization.

TK activity assays. TK activity in soluble cell extracts was

TABLE 2. Relative yield of HAT- and BrdU-resistant hybrids in transfectant × fibroblast fusions[a]

Hybrid cross	No. of hybrids challenged[b]	Fraction of hybrids that were[c]:	
		HAT^r	$BrdU^r$
PTK-1 × Ltn	237	<0.004	0.50
PTK-2 × Ltn	193	0.02	0.67
PTK-3 × Ltn	16	<0.06	1.00
PTK-5 × Ltn	40	0.38	0.03
HTK-1 × Ltn	10	0.60	<0.10
HTK-2 × Ltn	63	0.63	0.33
HTK-3 × Ltn	40	0.38	0.03
HTK-4 × Ltn	60	0.15	0.46

[a] Rat hepatoma transfectants (TK^+ Oua^r) were fused with mouse Ltn fibroblasts (TK^- $G418^r$), and portions of each fusion mixture were plated in medium containing ouabain plus G418, ouabain plus G418 plus HAT, or ouabain plus G418 plus BrdU. After 3 to 4 weeks, the plates were rinsed with phosphate-buffered saline, fixed with methanol, and stained with 1% crystal violet in 25% methanol.

[b] The number of hybrids surviving ouabain plus G418 selection, and hence the number of clones challenged with each selective medium, is indicated for each hybrid cross.

[c] The fraction of HAT^r hybrids was determined by dividing the total number of clones surviving ouabain plus G418 plus HAT selection by the number obtained in medium containing ouabain plus G418 alone; the fraction of $BrdU^r$ hybrids was calculated in a similar manner.

quantitated as described by Mayo et al. (33). Subconfluent monolayers were washed three times with ice-cold saline and harvested by scraping. The cells were suspended in buffer consisting of 10 mM Tris hydrochloride (pH 7.2), 10 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 10 mM NaF, 50 mM γ-aminocaproic acid, and 10 mM 2-mercaptoethanol. After sonication on ice, the homogenate was centrifuged at 15,000 × *g* for 30 min at 4°C. Cleared supernatant (20 μl) was added to 100 μl of buffer containing 150 mM Tris hydrochloride (pH 7.5), 10 mM ATP, 10 mM $MgCl_2$, 25 mM NaF, 10 mM 2-mercaptoethanol, and 5 μCi of [^{3}H]thymidine (25 Ci/mmol; ICN Radiochemicals). Aliquots were removed after 30 and 60 min and spotted onto DEAE-paper disks. The filters were washed three times in 95% ethanol, and radioactivity was quantitated by liquid scintillation counting. In parallel reactions, 100 μM 5-iododeoxycytidine, a specific inhibitor of herpesvirus TK, was included in the assay mix. In this assay, 1 U of enzyme activity catalyzes the formation of 1 μmol of thymidylic acid per min at 37°C. The protein content of the cell homogenates was quantitated by the method of Lowry et al. (28).

RESULTS

Properties of the parental cells. Plasmid pOPF contains a 2,400-base-pair (bp) *Bam*HI-*Eco*RI fragment of the herpes simplex virus (HSV) type I genome that includes the TK gene and its promoter (19). Plasmid pPCTK-6A was derived from pOPF by replacing the 800-bp *Bam*HI-*Bgl*II HSV TK promoter fragment with a 621-bp *Bam*HI fragment from the 5′ end (bp −548 to +73) of the rat phosphoenolpyruvate carboxykinase (PCK) gene (46). Both plasmids convert TK^- hepatoma cells to the TK^+ phenotype upon transfection; in addition, TK expression in pPCTK-6A transfectants is cyclic AMP and glucocorticoid inducible (46), as is the chromosomal PCK gene.

Transfectant clones expressing HSV-TK activity were prepared by introducing pOPF (TK-TK transfectants) or pPCTK-6A (PCK-TK transfectants) into TK^- FTO-2B rat hepatoma recipients by either calcium phosphate coprecipi-

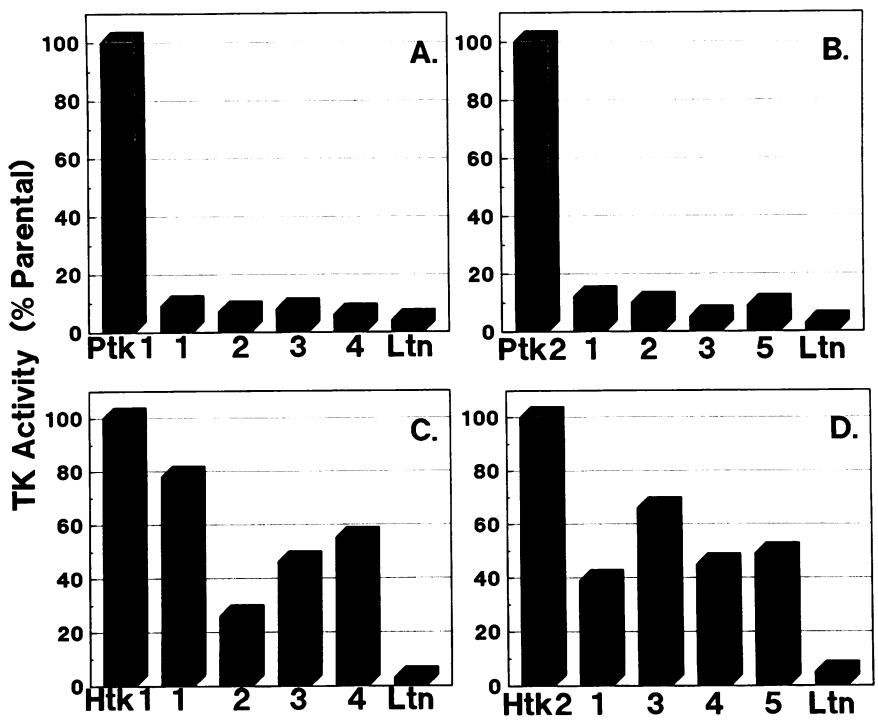

FIG. 1. TK activities of transfectant × fibroblast hybrids. PCK-TK transfectants Ptk-1 (A) and Ptk-2 (B) and TK-TK transfectants Htk-1 (C) and Htk-2 (D) were fused with Ltn mouse fibroblasts, and hybrids were selected in medium containing ouabain plus G418. HSV TK activities of isolated hybrid clones are expressed relative to parental transfectant levels, which were set at 100%. Absolute HSV TK activities of Ptk-1, Ptk-2, Htk-1, and Htk-2 are presented in Table 1.

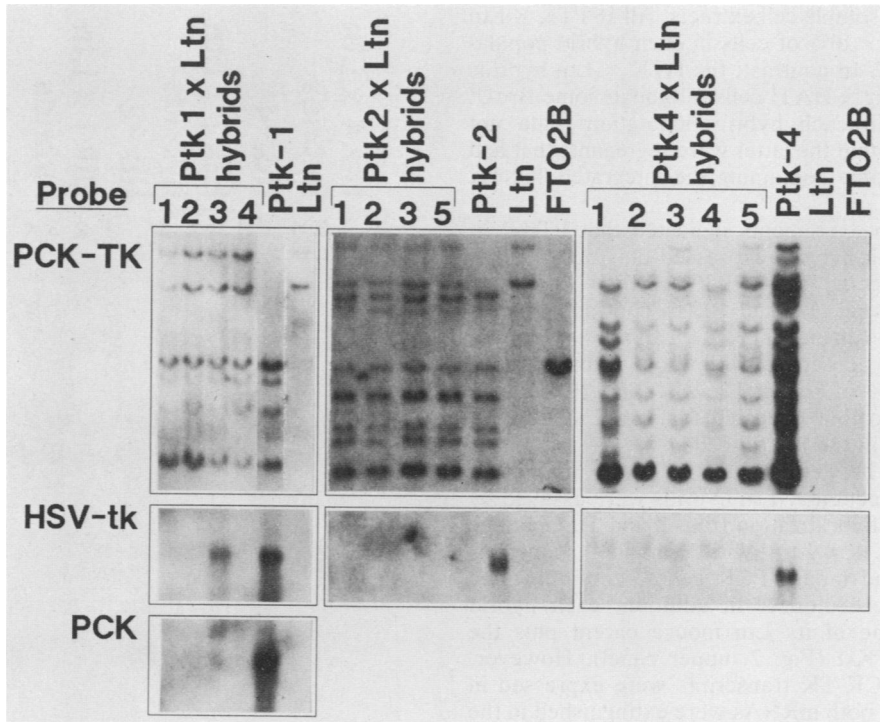

FIG. 2. Retention and expression of PCK-TK gene sequences in Ptk × Ltn hybrids. The three top panels show Southern blots of *Eco*RI-digested genomic DNAs from Ptk-1× Ltn (left), Ptk-2 × Ltn (center), and Ptk-4 × Ltn (right) hybrids that were probed with labeled inserts from plasmids pPCTK-6A (Ptk-1 and Ptk-2 hybrids) or pOPF (Ptk-4 hybrids). The pPCTK-6A probe hybridized with the chromosomal PCK genes of the rat (FTO-2B) and mouse (Ltn) parental cells as well as with transfected PCK-TK sequences. The pOPF insert hybridized specifically with HSV TK sequences of transfected PCK-TK genes. The lower panels show Northern (RNA) blots of cytoplasmic RNAs from Ptk-1 × Ltn (left), Ptk-2 × Ltn (center), and Ptk-4 × Ltn (right) hybrids that were probed with labeled pOPF (HSV TK probe) or pPCK10 (PCK probe) to detect HSV TK and PCK transcripts, respectively. A 1.4-kilobase HSV TK mRNA was expressed in PCK-TK hepatoma transfectants Ptk-1, Ptk-2, and Ptk-4 but not in mouse Ltn fibroblasts or Ptk × Ltn hybrids.

tation or electroporation. In TK-TK transfectants, the HSV-TK gene is expressed from the viral TK promoter. In contrast, PCK-TK transfectants express a chimeric PCK-TK mRNA that encodes HSV TK enzyme but includes 73 bp of noncoding sequence from the rat PCK gene at its 5′ end. Expression of this mRNA in PCK-TK transfectants is driven by the rat PCK promoter.

The properties of five PCK-TK and four TK-TK transfectants are summarized in Table 1. Most of the clones contained one or a few copies of the transfected TK gene, although PTK-3 and PTK-4 were multicopy transfectants. As expected, each transfectant clone plated with high efficiency in medium containing HAT but failed to survive bromodeoxyuridine (BrdU) selective challenge for the TK^- phenotype. HSV TK enzyme activity varied from 70 to 120 μU/mg among the PCK-TK transfectants, whereas TK-TK transfectants expressed 20 to 40 μU/mg, consistent with the relative strengths of the PCK and HSV TK promoters.

Chimeric gene extinction in whole-cell hybrids. Extinction of liver gene expression in hepatoma × fibroblast hybrids is a large effect: steady-state levels of hepatic mRNAs are generally reduced 500- to 1,000-fold (7). Therefore, hybrids between PCK-TK hepatoma transfectants and TK^- fibroblasts would be expected to display a TK^- phenotype ($BrdU^r$, HAT^s) if expression of the PCK-TK chimera were extinguished. In contrast, if TK-TK gene expression were not subject to extinction, the expected hybrid phenotype would be HAT^r, $BrdU^s$.

Clonal PCK-TK or TK-TK hepatoma transfectants (TK^+ Oua^r) were fused with TK^- mouse Ltn fibroblasts (TK^- $G418^r$), and hybrids were selected in medium containing ouabain and G418. Portions of the fusion mixture were also challenged with selective medium containing ouabain, G418, and either HAT or BrdU. The relative yields of HAT^r and $BrdU^r$ hybrids in these crosses are summarized in Table 2. Virtually all hybrids generated by fusing PTK-1, PTK-2, or PTK-3 PCK-TK transfectants with Ltn cells were $BrdU^r$; <2% of these hybrid clones survived in HAT. The PTK-5 transfectant behaved differently: most of its hybrid progeny were HAT^r, and very few of them (<3%) survived in BrdU. Thus, it appeared that PCK-TK gene expression in three of four PTK transfectants tested was extinguished when the cells were fused with fibroblasts. Since PTK-5 contained the same chimeric gene in an apparently unrearranged form (data not shown), it seems likely that a position effect at the site of integration precludes PCK-TK extinction in this clone. Fusions between TK-TK transfectants and Ltn cells gave predominantly HAT^r hybrids. These data suggested that PCK-TK gene expression was extinguished in most PTK × Ltn hybrids, but that TK-TK expression in analogous HTK × Ltn clones was not. To more fully document these apparent differences, individual hybrid clones were isolated and characterized.

Hybrids between mouse Ltn cells and PCK-TK transfectants PTK-1 through PTK-4 and TK-TK transfectants HTK-1, HTK-2, and HTK-4 were isolated without selective pressure on their TK phenotypes, i.e., in medium containing ouabain plus G418. The TK phenotypes of isolated hybrid clones were determined by challenging the cells with medium containing either HAT or BrdU and by quantitating

HSV TK activities in soluble cell extracts. All 16 PTK × Ltn hybrids were BrdU^r; <10% of cells in each hybrid population survived in HAT. In contrast, the HTK × Ltn hybrids contained predominantly HAT^r cells, although some BrdU^r cells were present in each hybrid population (data not shown). We presume that the latter were segregants that had lost the rat chromosome containing the integrated TK-TK gene.

Enzyme activity measurements indicated that PTK-1 × Ltn and PTK-2 × Ltn hybrids expressed only 5 to 10% as much herpesvirus-specific TK activity as their respective transfectant parents (Fig. 1A and B). In contrast, the TK activities of HTK-1 × Ltn and HTK-2 × Ltn hybrids were 26 to 78% of parental activities, with most hybrid clones expressing about 50% of parental levels (Fig. 1C and D). This value is close to that expected for simple dilution of parental TK enzyme in the hybrid cells.

Retention of HSV TK gene sequences and expression of TK mRNAs in transfectant × Ltn hybrids were assayed by DNA and RNA blot hybridization (Fig. 2 and 3). Genomic DNA from each PTK-1 × Ltn hybrid contained sequences from both the rat and mouse PCK genes, confirming the hybrid nature of these clones. In addition, each hybrid retained the *neo* gene of its Ltn mouse parent plus the PCK-TK gene of PTK-1 (Fig. 2, upper panel). However, whereas PCK and PCK-TK transcripts were expressed in PTK-1 parental cells, both mRNAs were extinguished in the intertypic hybrids (Fig. 2, lower panels). Similar results were obtained for the PTK-2 × Ltn and PTK-4 × Ltn hybrid families: in each case the hybrids retained PCK-TK gene sequences but failed to transcribe them into stable RNAs (Fig. 2). PTK-5 × Ltn hybrids, which were atypical in that most of them were HAT^r (Table 2), both retained and expressed the PCK-TK chimeric gene (data not shown).

The HTK × Ltn hybrids all retained the transfected TK-TK genes (Fig. 3, upper panel). These clones were extinguished for PCK mRNA expression, but they continued to express 1.4-kilobase HSV TK-specific transcripts (Fig. 3, lower panels). These observations indicate that expression of the PCK-TK chimera was extinguished in hepatoma × fibroblast hybrids but that expression of a control TK-TK gene was not.

Chimeric gene extinction in microcell hybrids. Extinction of PCK gene expression in hepatoma × fibroblast hybrids is mediated by a discrete genetic locus (TSE1) that resides on mouse chromosome 11 and human 17 (21, 26). To determine whether PCK-TK chimeric genes were specifically extinguished by TSE1, we prepared microcell hybrids retaining that single human chromosome.

L(17n)C or L(17n)E donor cells (25) were micronucleated by prolonged (48-h) mitotic arrest, and chromosomes were transferred to PCK-1, HTK-1, or HTK-2 recipients by microcell fusion. Hybrids retaining human chromosome 17 were obtained by selecting for the G418^r phenotype encoded by a viral vector that had integrated into chromosome 17 (29). The microcell hybrids produced in these crosses allowed us to determine whether the PCK-TK extinction phenotype mapped specifically to human chromosome 17.

Expression of PCK, TK, and α-tubulin mRNAs in parental cells and microcell hybrids was assayed by RNA blot hybridization (Fig. 4). PTK-1, HTK-1, and HTK-2 parental cells expressed all three mRNAs. PTK-1-derived microcell hybrids were extinguished for both PCK and PCK-TK mRNA expression (Fig. 4), and HSV-TK enzyme activity was also extinguished in these clones (Fig. 5A). In contrast, neither HSV TK mRNA (Fig. 4) nor enzyme activity (Fig. 5B) was affected when human chromosome 17 was introduced into TK-TK transfectants. Thus, extinction of PCK-TK expression in PTK-1 mapped specifically to human chromosome 17.

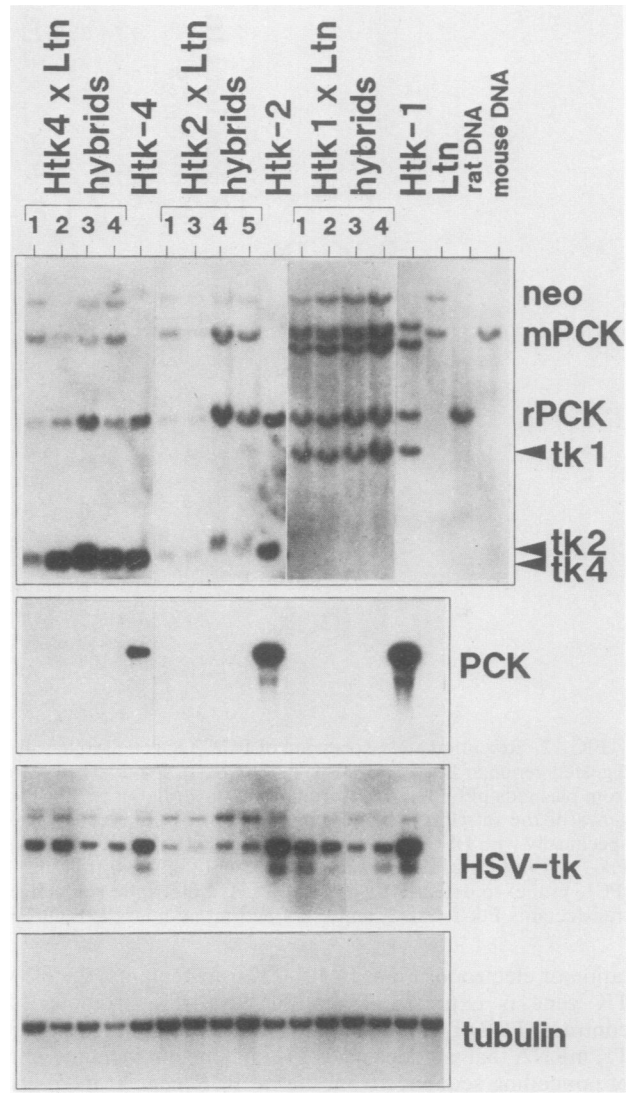

FIG. 3. Retention and expression of TK-TK gene sequences in Htk × Ltn hybrids. The top panel shows a Southern blot of *Eco*RI-digested genomic DNAs from Htk-1 × Ltn, Htk-2 × Ltn, and Htk-4 × Ltn hybrids that was probed with labeled plasmid pPCTK-6A. Hybridizing fragments from the mouse PCK (mPCK), rat PCK (rPCK), *neo*, and transfected TK-TK genes (tk1, tk2, and tk4) were detected under these conditions. The lower three panels show Northern blots of cytoplasmic RNAs from the same cell lines probed with labeled pPCK10, pOPF, or pKα-1 to visualize PCK, HSV TK, and α-tubulin transcripts, respectively.

Deletion analysis in microcell hybrids. The results described above indicate that DNA sequences of the rat PCK gene between bp −548 and +73 can confer TSE1 responsiveness on a heterologous reporter. However, although PCK-TK expression was extinguished in four of five transfectants tested, it was refractory to extinction in one transfectant (PTK-5). This suggests that integration into particular chromosomal sites may preclude extinction. Therefore, more precise localization of TSE1-responsive elements within the PCK promoter requires an assay in which position

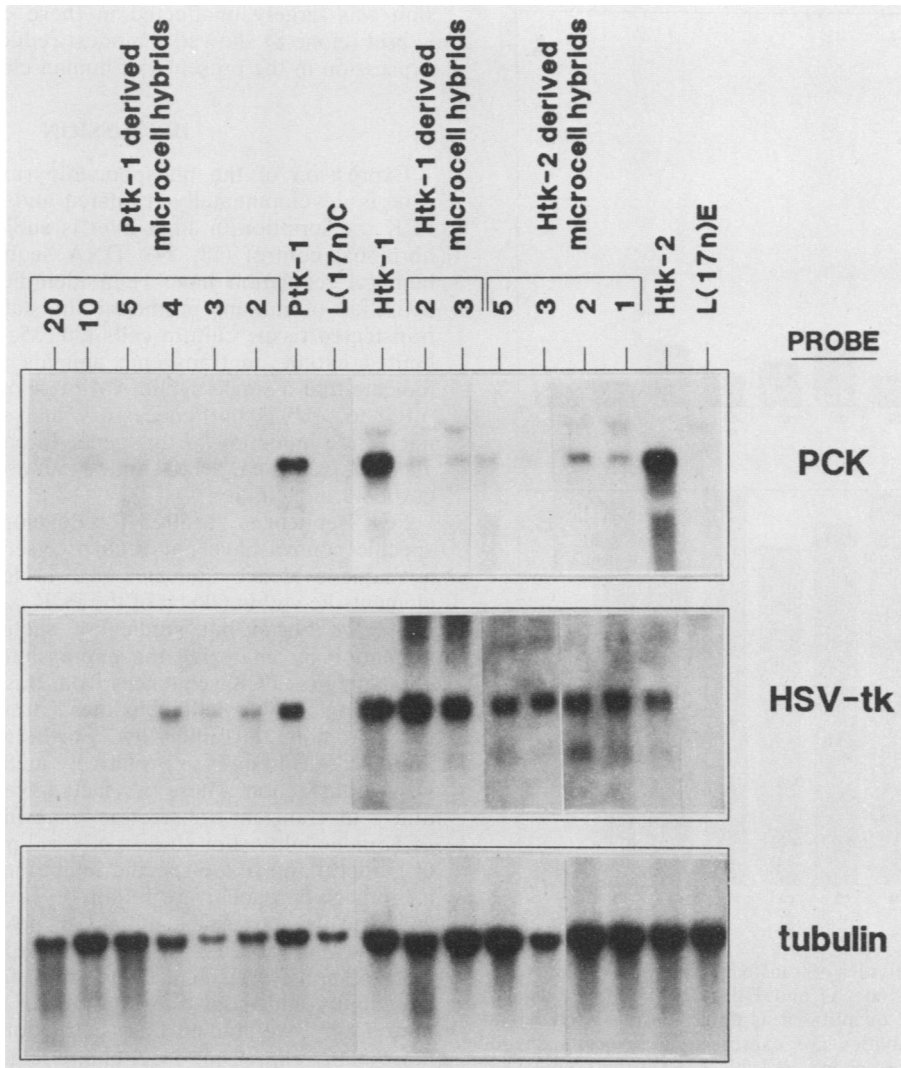

FIG. 4. Expression of PCK, HSV TK, and α-tubulin mRNA in transfectant microcell hybrids. Northern blots of cytoplasmic RNAs were probed with labeled pPCK10 (top), pOPF (middle), or pKα-1 (bottom) to visualize PCK, HSV TK, and α-tubulin mRNAs, respectively, expressed by parental and hybrid cells. HSV TK mRNA expression was extinguished in human chromosome 17-containing microcell hybrids derived from PCK-TK transfectant Ptk-1, but not in microcell hybrids from TK-TK transfectants Htk-1 or Htk-2.

effect artifacts can be recognized. Accordingly, we transferred human chromosome 17 into transfectant polyclones and isolated individual microcell hybrids so that a number of different integrations could be tested in a single experiment. Furthermore, we compared PCK-TK expression in primary hybrids retaining TSE1 and in TSE1$^-$ backselectants to directly assess the effects of human TSE1. To test this system, we analyzed transfectant polyclones containing a −548 to +73 PCK-*neo* reporter.

Plasmid d5′*neo*548 was introduced into FTO-2B rat hepatoma recipients by electroporation, and G418^r transfectants were selected. The transfectant clones were pooled and used as recipients in microcell fusions with L(17)A donors, and human TK$^+$ microcell hybrids were selected in HAT. PCK-*neo* mRNA expression was compared in primary hybrids retaining human chromosome 17 and in TK$^-$ (BrdU^r) backselectants from which chromosome 17 had been removed. In this experiment, each microcell hybrid has the PCK-*neo* chimera integrated into a different chromosomal site (data not shown).

PCK, *neo*, and α-tubulin mRNA expression in six d5′*neo*548-containing microcell hybrids (Δ−548 hybrids) and their respective backselectants was assayed by RNA blot hybridization (Fig. 6). PCK mRNA expression was extinguished in each primary microcell hybrid (left lanes within brackets) but reexpressed in backselectants (right lanes within brackets). Steady-state levels of *neo* mRNA were reduced 5- to 10-fold in two microcell hybrids (Δ−548 hybrid clones 1 and 6) compared with backselectants, two hybrids (clones 2 and 3) showed 2- to 3-fold reductions in *neo* mRNA levels, and two clones (4 and 5) failed to show PCK-*neo* extinction. Thus, PCK-*neo* extinction phenotypes were apparent in only a subset of the hybrid clones.

Hybrids derived from transfectant polyclones containing d5′*neo*134, which includes PCK promoter sequences between bp −134 and +73, and d5′*neo*tk, a control plasmid in which *neo* is expressed from the HSV TK promoter, were analyzed in a similar fashion (Fig. 6). PCK mRNA expression was extinguished in each microcell hybrid but reexpressed in its backselectant. In contrast, *neo* mRNA expres-

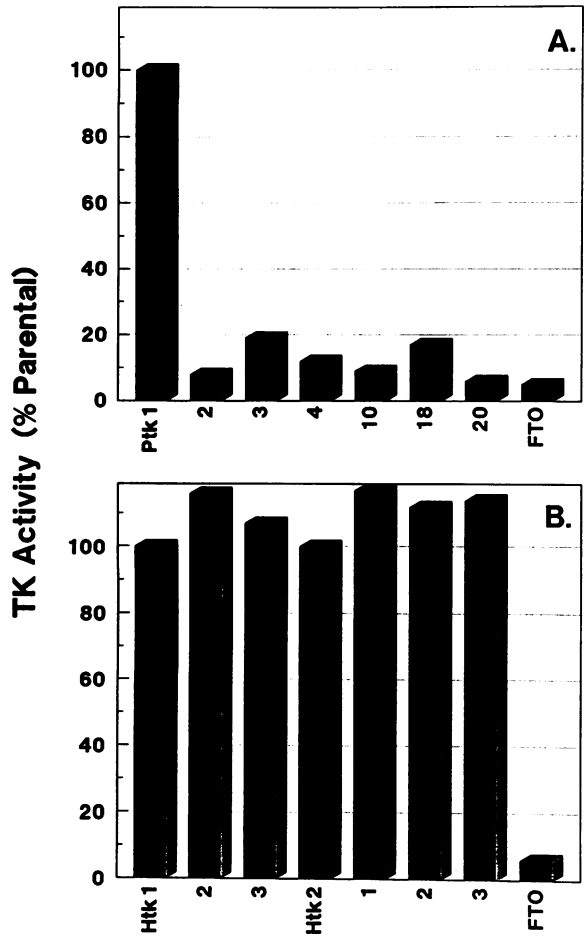

FIG. 5. HSV-TK activities of transfectant microcell hybrids. TK activities of Ptk-1-derived (A) and Htk-1- or Htk-2-derived (B) microcell hybrids were quantitated as described in Materials and Methods. Relative activities are expressed as a percentage of parental levels, which are shown in Table 1. FTO represents TK^- FTO-2B rat hepatoma parental cells.

sion was largely unaffected in these clones. One Δ−134 hybrid (clone 8) showed a modest reduction in *neo* mRNA expression in the presence of human chromosome 17.

DISCUSSION

Expression of the phosphoenolpyruvate carboxykinase gene is developmentally regulated and tissue specific, and PCK transcription in adult liver is subject to acute, multihormonal control (18, 24). DNA sequences required for humoral regulation have been identified by assaying the activities of mutant promoters in stably and transiently transfected tissue culture cells (30, 35, 41) and, to a more limited extent, in transgenic animals (34). These studies indicate that a single cyclic AMP-responsive element at bp −100 to −80 is both necessary and sufficient for cyclic nucleotide induction of this gene. In contrast, two distinct elements, at bp ca. −1200 and −450, are involved in glucocorticoid inducibility.

PCK sequences required for developmental and tissue-specific control have not been precisely defined, although available evidence indicates that at least some of these elements lie within 600 bp of the PCK cap site (34). Accordingly, we began our studies of sequences required for extinction by analyzing the expression of chimeric genes containing rat PCK sequences from this region.

Although highly inducible, basal transcription from the PCK promoter is rather low. Furthermore, rat hepatoma lines of the H4 family are relatively inefficient recipients for DNA transfection. These two facts have thus far limited the utility of transient-transfection experiments to studies of PCK inducibility. For studies of basal promoter function or of humoral and tissue-specific inhibition of promoter activity, other approaches are required. Thus, Quinn et al. (39) analyzed the expression of mutant promoters in heterologous CV-1 fibroblasts and provided evidence that the cyclic AMP-responsive element is important for both cyclic AMP inducibility and basal transcription. In the experiments reported here, we studied PCK extinction in clonal transfectants stably expressing PCK chimeric genes.

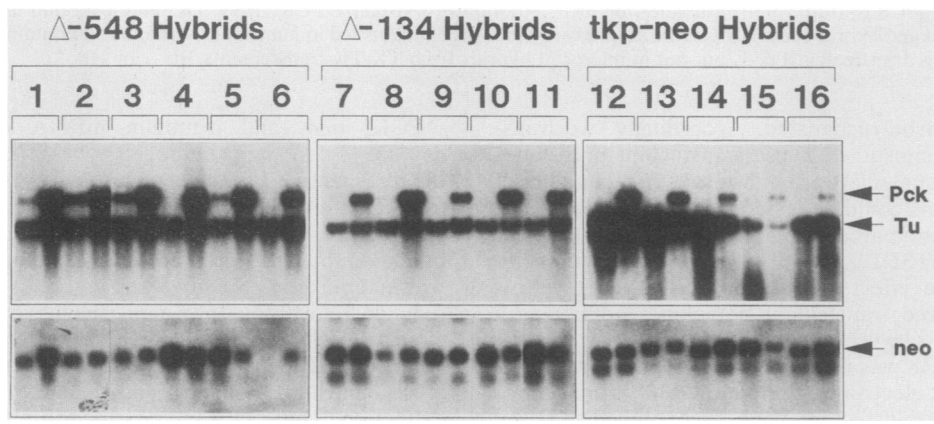

FIG. 6. PCK, *neo*, and α-tubulin mRNA expression in microcell hybrids and backselectants. FTO-2B (TK^-) rat hepatoma cells were transfected with plasmids d5′*neo*548, d5′*neo*134, or d5′*neo*tk, and G418-resistant cells were selected. Polyclonal mixtures of transfected cells were fused with microcells prepared from L(17)A donors, and TK^+ microcell hybrids retaining human chromosome 17 were selected in HAT. The Δ−548 hybrid series contains the d5′*neo*548 PCK-TK chimera (PCK sequences from bp −548 to +73bp), the Δ−134 hybrids contain a bp −134 to +73 PCK-TK chimera, and the tkp-neo hybrids contain a control *neo* gene whose expression is driven by the HSV TK promoter. Each isolated hybrid clone was challenged with BrdU, and a TK^- backselectant that had segregated chromosome 17 was obtained. Northern blots of cytoplasmic RNAs from primary hybrids and backselectants (lanes within brackets; hybrids on the left, backselectants on the right) were probed with labeled pPCK10 plus pKα-1 (upper panels) or d5′*neo*tk (lower panels) to detect PCK, tubulin, and *neo* mRNAs, respectively; their positions are indicated by arrows.

Expression of a bp −548 to +73 PCK-TK chimera was extinguished in four of five hepatoma transfectants tested, and extinction of this gene mapped specifically to the chromosome that encodes human TSE1. In one exceptional transfectant, PCK-TK gene activity was refractory to extinction. This transfectant (PTK-5) contained a single integrated PCK-TK chimera whose restriction endonuclease digestion pattern showed no evidence of deletion or rearrangement within the transfected sequences. Thus, it appears that the PCK-TK gene of PTK-5 is integrated into a chromosomal site that precludes extinction, perhaps by virtue of proximity to a strong enhancer. Furthermore, several of the bp −548 to +73 PCK-*neo* transfectants displayed similar properties. These observations indicate that chromosomal position effects can play dominant roles in dictating expression patterns of transfected genes, a factor which limits the utility of stable transfectants for precise mapping of TSE1-responsive elements.

In attempts to control position effect variations on gene expression, we assayed TSE1-mediated extinction of chimeric genes integrated at multiple chromosomal sites. These experiments indicate that PCK sequences between bp −548 to +73 are sufficient for TSE1-mediated extinction. However, the magnitude of PCK-TK extinction varied from clone to clone, and residual gene activity was apparent in most extinguished hybrids. Thus, we cannot exclude the possibility that other sequences within the PCK gene contribute to TSE1-mediated extinction. Finally, although PCK-*neo* genes containing PCK sequences between bp −134 and +73 showed little evidence of extinction, it is possible that these (small) chimeras were particularly susceptible to chromosomal position effects that precluded extinction. Thus, it seems prudent to leave open the question of whether PCK sequences between bp −134 and +73 are involved. Resolution of this issue, particularly acute in view of the documented cyclic nucleotide effects on both basal expression (39) and extinction (42), will probably require the development of transient assay systems in which reproducible regulation of the PCK promoter can be obtained.

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