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Regulated expression of foreign genes in mammalian cells under the control of coliphage T3 RNA polymerase and *lac* repressor

(expression units/stable integration/microinjection/firefly luciferase)

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ABSTRACT **Systems that stringently regulate the expression of individual genes within a complex genetic background have contributed greatly to the analysis of gene function. In this report the development of a highly regulated expression system in mammalian cells is described in which transcription of a foreign gene is mediated by the bacteriophage T3 RNA polymerase under the control of the *Escherichia coli lac* repressor. Rabbit kidney cell lines have been established that constitutively express the phage RNA polymerase and *lac* repressor. The two bacterial proteins regulate the transcription of the coding sequence of the firefly luciferase, which has been placed under the control of a T3 promoter/*lac* operator fusion. In the presence of the inducer isopropyl β-D-thiogalactoside, efficient T3 polymerase-dependent transcription is observed, which is tightly repressed in the absence of inducer. Translation of the T3 transcripts can be mediated by vaccinia virus functions. The demonstration that a specific transcription activity can be regulated over a range of several orders of magnitude in higher eukaryotic cells by using a highly specific and nontoxic inducer has broad implications for a variety of studies.**

New insights into gene function have been gained in a variety of systems by selectively controlling the activity of individual genes at the level of transcription. Ideally, this is achieved with transcription signals that can be tightly controlled by effectors supplied from the outside. In prokaryotes negative transcription controls allow regulation of expression over a range of >3 orders of magnitude, but no comparable system is presently available for the study of gene function in higher eukaryotic cells.

Eukaryotic transcription initiation signals that are subject to regulation, such as the metallothionein promoter (1, 2) or the mouse mammary tumor virus promoter (3, 4), are relatively leaky under noninduced conditions and show rather modest levels of induction. On the other hand, promoters that are highly inducible—e.g., by glucocorticoid hormones (5)—require the presence of hormone receptors in the cell. More importantly, as in the case of heat shock promoters (6, 7), the induction of transcription in these systems generates pleiotropic effects that may obscure the phenotype of the gene under study.

Therefore it has been examined whether prokaryotic elements can be exploited for the regulation of gene activity in higher eukaryotic cells. An elegant approach in which the simian virus 40 (SV40) early promoter is controlled by the *Escherichia coli lac* operator/repressor/inducer system has been described (8–10). The ≤60-fold reversible repression reported (possibly underestimated due to the low sensitivity of the chloramphenicol acetyltransferase assay used to monitor reporter gene expression) may sufficiently reduce the activity of genes in some studies; in others, however, such a repression factor may not be enough.

Here we describe the development of a simple, tightly regulated transcription system that functions in mammalian cells and is composed entirely of prokaryotic elements. The genes that encode the coliphage T3 RNA polymerase and the *E. coli lac* repressor were inserted into the genome of rabbit kidney cells (RK13), where they are stably maintained and constitutively expressed. Both gene products are functional and control transcription from a phage T3 promoter fused to a *lac* operator. The activity of the T3 RNA polymerase is regulated over a several hundred-fold range by the activity of the *lac* repressor, and this effect can be reversed by the addition of isopropyl β-D-thiogalactoside (IPTG) to the culture medium. This finding demonstrates that repression factors comparable to those observed in prokaryotes can be achieved in mammalian cells.

MATERIALS AND METHODS

Plasmids. *pUHD11-1 and pUHD12-1.* The parent plasmid of these constructs, pUHD10-1, is composed of the origin of replication and the β-lactamase gene of pUC18 (positions 2686 to 626; ref. 11), with a *Hin*dIII cleavage sequence introduced at the *Pvu* II site; a SV40 early polyadenylylation signal isolated from pNEO5′ (12); the polylinker sequence of pUC18 (positions 451 to 423); and the human cytomegalovirus IE promoter (P_{CMV}), which was removed from pRR23 (a gift of B. Fleckenstein, University of Erlangen, F.R.G.) as a 745-base-pair (bp) fragment containing 75 bp of the transcribed region (13) and modified to contain flanking *Xho* I and *Eco*RI sites. The *lacI* gene of *E. coli*, in which the start codon was converted from GTG to ATG, was inserted into the polylinker as a *Bam*HI fragment to yield pUHD11-1. In an analogous way gene *1* of coliphage T3 was inserted as a 2735-bp *Kpn* I–*Bam*HI fragment (positions 135 to 2807; ref. 14) to yield pUHD12-1. A gene *1* sequence that ensures the translational start at the original codon was a gift of H. Stunnenberg (European Molecular Biology Laboratory, Heidelberg, F.R.G.). The overall structure of both plasmids is shown in Fig. 1.

pUHR11-1 and pUHR11-2. Plasmid pUHR11-2 contains the same β-lactamase gene and origin of replication as pUHD11-1. The T3 promoter/*lac* operator sequence $P_{T3/O+14}$ was synthesized as a 49-bp *Xho* I–*Asp*718 fragment: TCGAG-TAATTACCCTCACTAAAGGGAATTGTGAGCG-GATAACAATTTCG (5′ → 3′). The sequence of the 21-bp operator, centered around position +14 (underlined; +1

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Abbreviations: SV40, simian virus 40; IPTG, isopropyl β-D-thiogalactoside; rlu, relative light units.
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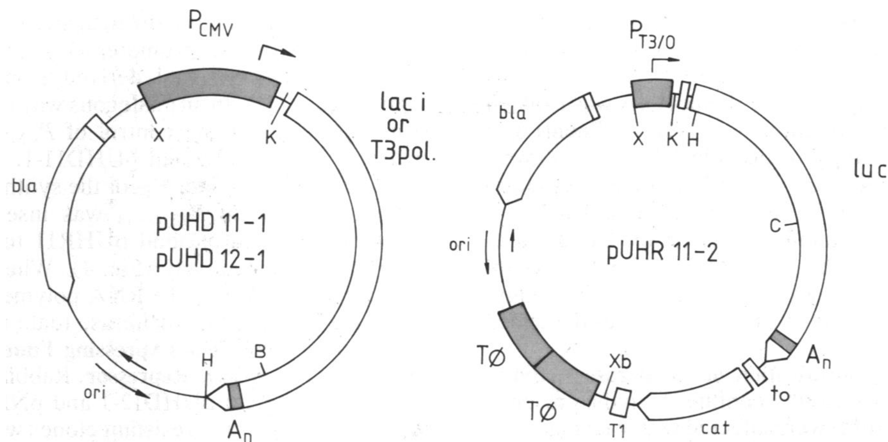

FIG. 1. Structure of plasmids pUHD11-1, pUHD12-1, and pUHR11-2. All three plasmids contain the ColE1 replicon (*ori*) and the β-lactamase gene (*bla*). (*Left*) The human cytomegalovirus promoter P_{CMV} directs the transcription of either the *lacI* gene (pUHD11-1) or the phage T3 RNA polymerase gene (pUHD12-1). A_n denotes a SV40 polyadenylylation signal. Plasmids pUHD11-1 and -2 were used to construct the stable cell lines RK-T3 and RK-T3i. (*Right*) Plasmid pUHR11 contains the coding region of the firefly luciferase gene (luc) fused to a SV40 polyadenylylation signal (A_n). Transcription of the luciferase sequence is initiated either by P_{T3}, a phage T3 promoter (pUHR11-1, not shown), or by $P_{T3/O+14}$ ($P_{T3/O}$), a promoter/*lac* operator sequence (pUHR11-2). P_{T3}-specified transcription is terminated at two phage T7 termination signals (T_ϕ). The transcription terminators t_0 and T_1 as well as the chloramphenicol acetyltransferase gene (cat) and the *E. coli* ribosomal binding site (□) are elements of a transcription unit used for luciferase synthesis in *E. coli*. Cleavage sites for restriction enzymes: X, *Xho* I; K, *Kpn* I; H, *Hin*dIII; C, *Cla* I; B, *Bam*HI; Xb, *Xba* I.

being the transcriptional start site), has been shown to tightly control the promoter in the presence of repressor (15). The coding sequence for the firefly (*Photinus pyralis*) luciferase gene was isolated as a *Hin*dIII–*Bam*HI fragment from pSV2AL-Δ5′ (16) and, together with the polyadenylylation signal of the SV40 large tumor (T) antigen gene, was inserted into pDS5 (17). The region spanning from the *Kpn* I to the *Xba* I site of the pDS5 derivative was ligated to a fragment composed of $P_{T3/O+14}$, the *ori* region, and the tandemly arranged T7-specific terminators (18), resulting in pUHR11-2 (Fig. 1). In plasmid pUHR11-2 the luciferase gene is fused to an *E. coli* ribosomal binding site, which provides an ATG initiation codon. This results in an N-terminal fusion of 18 additional amino acids due to a polylinker sequence; however, this does not affect the activity of the luciferase. The overall structure of pUHR11-2 is shown in Fig. 1. Exchange of $P_{T3/O+14}$ with the unregulated promoter sequence P_{T3} results in pUHR11-1.

Chemicals and Enzymes. Restriction enzymes, T3 RNA polymerase, *E. coli* DNA polymerase I Klenow fragment, and S1 nuclease were purchased from Boehringer Mannheim. D-Luciferin and luciferase from *P. pyralis* were from Sigma, and hygromycin B and the neomycin analogue G418 were purchased from Sigma and GIBCO, respectively.

Cell Culture and Construction of Cell Lines. Rabbit kidney cells (RK13) were grown in Dulbecco's modified Eagle's medium (DMEM, from GIBCO) supplemented with penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively) and 10% fetal bovine serum (Boehringer Mannheim) at 37°C and 5% CO_2. Cells growing in 10-cm plates at 80% confluence were transfected with 10 μg of plasmid DNA (as a calcium phosphate precipitate; ref. 19) in 4 ml of DMEM without serum. After 2 hr the medium was removed and the cells were incubated for 4 min at room temperature with 4 ml of phosphate-buffered saline containing 10% (vol/vol) dimethyl sulfoxide. For transient expression the cells were washed and kept for 3 hr at 37°C and 5% CO_2 in DMEM with 10% fetal bovine serum.

To establish stable cell lines expressing the T3 RNA polymerase, cells were transfected with 10 μg of a mixture of pUHD12-2 and pNEO5′ (15:1, wt/wt). Forty-eight hours after transfection, the cells were trypsinized and split at a ratio of 1:10. Antibiotic G418 (500 μg/ml) was added and resistant colonies were selected in DMEM. Resistant clones were expanded and one clone that was positive for T3 RNA polymerase activity *in vitro* was subcloned by limiting dilution in nonselective medium, resulting in the RK-T3 cell line.

To establish a cell line expressing both T3 RNA polymerase and *lac* repressor, 10 μg of a 15:1 mixture of pUHD11-2 and pHM24 (carrying the hygromycin B-resistance gene; ref. 20) was used to transfect RK-T3 cells. Resistant cells were selected in DMEM containing hygromycin B (500 μg/ml). RK-T3i clones were obtained upon limiting dilution of resistant colonies as described above.

"Western Blot" Analysis of Nuclear and Cytoplasmic Extracts of RK-T3 Cells. Cytoplasmic and nuclear extracts were prepared from cells grown to 80% confluence in 10-cm plates. Cells of one plate suspended in 400 μl of phosphate-buffered saline containing 1 mM dithiothreitol were frozen and thawed three times, and nuclei and cell debris were separated from the cytoplasmic extract by sedimentation (10 min, 10,000 × *g*). The sediment was resuspended and aliquots of this suspension as well as of the cytoplasmic extract were mixed with sample buffer and subjected to SDS/PAGE (21). The proteins were electrophoretically transferred to nitrocellulose (22), and the resulting Western blots were probed with rabbit polyclonal antibodies directed against T3 RNA polymerase and subsequently developed with goat anti-rabbit IgG alkaline phosphatase conjugate (Promega) as described by the supplier.

Gel Retardation Assay. Aliquots (10 μl) of cytoplasmic cell extracts were mixed with 10 μl of binding buffer (20 mM Tris·HCl, pH 7.8/2 mM $MgCl_2$ containing 10 μg of calf thymus DNA and 0.02 pmol of ^{32}P-labeled *lac* operator DNA. After 15 min at 20°C the samples were subjected to electrophoresis as described by Fried and Crothers (23).

Analysis of *in Vitro* and *in Vivo* Transcripts. *In vitro* transcription assays were performed at 37°C for 10 min in 20-μl mixtures containing 20 mM Tris·HCl (pH 7.9); 20 mM KCl; 6 mM $MgCl_2$; 2 mM spermidine; 4 μg of yeast tRNA; 300 μM ATP, GTP, and UTP; 50 μM CTP (1 μCi of [α-^{32}P]CTP, 3000 Ci/mmol, Amersham; 1 μCi = 37 kBq); and 100 ng of supercoiled pUHR11-2. The reaction was started by adding 1 μl (1/400th) of the cytoplasmic extract or, as control, 8 units of T3 RNA polymerase (Boehringer Mannheim). For repression of the transcriptional activity, 100 ng of *lac* repressor was added per assay and induction was achieved with 500 μM IPTG. Transcription products were analysed by PAGE [4%

polyacrylamide, 8.3 M urea, TBE buffer (90 mM Tris/90 mM boric acid/2 mM EDTA, pH 8.0), 20 V/cm] and visualized by autoradiography.

For the *in vivo* transcripts, total cellular RNA was isolated from rabbit kidney cells (10-cm plates, 80% confluent) 6 hr after transfection with 10 μg of plasmid DNA (see above). One-tenth of the total RNA sample was hybridized in solution to 0.2 pmol of the 95-bp ^{32}P-end-labeled *Hin*dIII–*Xho* I fragment of pUHR11-2 containing $P_{T3/O+14}$, and S1 nuclease-resistant material was subjected to PAGE (8% polyacrylamide, 8.3 M urea, TBE buffer, 20 V/cm) and autoradiography. RNA isolation and S1 mapping were carried out as described (24).

Microinjection and Quantification of Luciferase Activity. Rabbit kidney cells grown to 80% confluence in the presence or absence of IPTG (10 mM) were infected with vaccinia virus strain WR (multiplicity of infection, 5) 2 hr prior to microinjection. After 45 min with virus, cells were washed and then incubated in DMEM containing 10% fetal bovine serum. Solutions containing plasmid DNA (5 μg/ml) with or without IPTG and various amounts of purified *lac* repressor were transferred into the cytoplasm of 200 individual cells by the automated microinjection system described by Ansorge and Pepperkok (25). After incubation for 7 hr, cells were washed and then suspended in 100 μl of extraction buffer (0.1 M potassium phosphate, pH 8/1 mM dithiothreitol). Cytoplasmic extracts were prepared as described above. Aliquots (10 μl) were assayed for luciferase activity as described (16). Relative light units (rlu) were determined with a Biolumat LB 9500 luminescence analyzer (Berthold) using the integral mode of measurement (10 sec).

RESULTS

Construction of Expression Units. The coliphage T3 RNA polymerase gene (gene *1*), trimmed to initiate and terminate translation at the original codons, was inserted into plasmid pUHD10-1, where it is flanked upstream by P_{CMV}, the human cytomegalovirus IE promoter (13), and downstream by a polyadenylylation signal derived from the SV40 large T antigen gene (12). In an analogous way the *lacI* gene of *E. coli* was placed under the control of P_{CMV}. The two resulting plasmids, pUHD12-1 and pUHD11-1, are shown in Fig. 1. The phage T3 promoter P_{T3} or the synthetic T3 promoter/*lac* operator sequence $P_{T3/O+14}$ was inserted in front of the luciferase gene in plasmid pUHR11 to yield pUHR11-1 or pUHR11-2, respectively (Fig. 1). When introduced into *E. coli* cells that express T3 RNA polymerase, these plasmids give rise to functional luciferase (data not shown).

Rabbit Kidney Cells Expressing Functional T3 RNA Polymerase and *E. coli lac* Repressor. Rabbit kidney cells (RK13) cotransfected with pUHD12-1 and pNEO5′ (12) were challenged with G418 and resistant clones were examined *in vitro* for T3 RNA polymerase activity. One of six positive clones was subjected to further screening, and several secondary clones were obtained. One cell line (RK-T3) selected from such clones was probed for the expression of T3 RNA polymerase by Western blot analysis with rabbit polyclonal antibodies directed against purified T3 RNA polymerase (Fig. 2*A*). The activity of T3 RNA polymerase was assayed in crude cell lysates by using plasmid pUHR11-2 as a template for *in vitro* transcription. Transcription was specifically initiated at $P_{T3/O+14}$ as judged from the length of the transcripts and from the efficient repression by a 30-fold excess of *lac* repressor over operator (Fig. 2*B*, lanes 7 and 8). The effect of *lac* repressor was reversed upon the addition of IPTG. Thus, as shown previously with the phage T7 RNA polymerase gene integrated into the yeast genome (26), these prokaryotic proteins are fully functional and their genes are stably maintained in eukaryotic cells.

S1 nuclease analysis of RNA isolated from RK-T3 cells that had been transfected with linearized pUHR11-2 or derivatives of this plasmid demonstrated that the T3 RNA polymerase initiates transcription *in vivo* at the $P_{T3/O+14}$ promoter

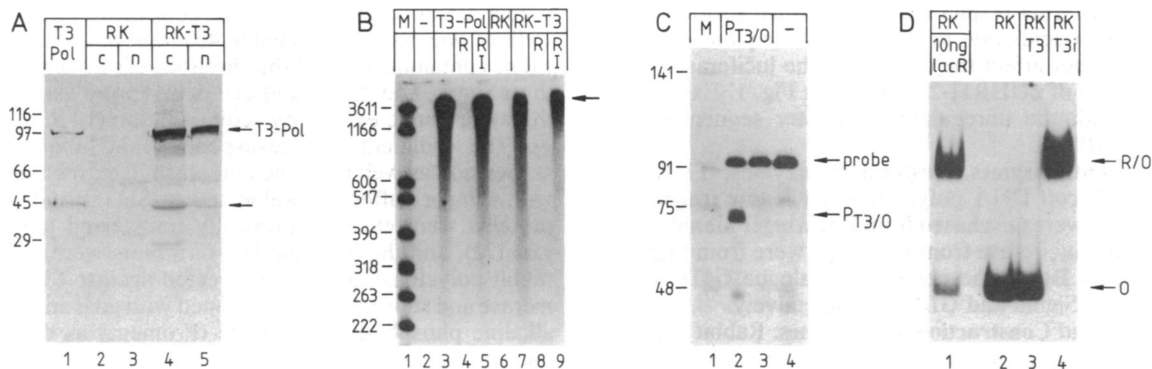

FIG. 2. Phage T3 RNA polymerase and *lac* repressor in rabbit kidney cells. (*A*) Western blot analysis of cytoplasmic (c) and nuclear (n) extracts of RK13 and RK-T3 cells. T3 RNA polymerase was identified by rabbit polyclonal antibodies raised against purified T3 RNA polymerase. An immunoreactive 100-kDa polypeptide in the extracts from RK-T3 cells (lanes 4 and 5) was indistinguishable from isolated T3 RNA polymerase (lane 1). No T3 RNA polymerase was observed in extracts of RK13 cells (lanes 2 and 3). The 46-kDa immunoreactive polypeptide (arrow) is most likely a degradation product of T3 RNA polymerase. Sizes (kDa) and positions of standard proteins are indicated at left. (*B*) T3 RNA polymerase activity in RK-T3 cells. *In vitro* transcripts of the expected length (≈4000 nucleotides) were obtained with cytoplasmic extracts from RK-T3 but not from RK13 cells when pUHR11-2 was used as template (lanes 7 and 6, respectively). The transcriptional activity from RK-T3 cells was repressed by *lac* repressor (R, lane 8), and induction of the repressed state was achieved by IPTG (R/I, lane 9). Corresponding results were obtained when RK-T3 extracts were replaced by T3 RNA polymerase purified from *E. coli* (lanes 2–5). M denotes molecular size standards (lane 1; lengths in nucleotides at left). Aliquots of the *in vitro* assays were subjected to PAGE (4% gel, 30:0.8 acrylamide/*N*,*N*′-methylenebisacrylamide weight ratio; 8.3 M urea, TBE buffer) and autoradiography. (*C*) S1-nuclease analysis of *in vivo* synthesized P_{T3}-specified RNA. RK-T3 cells were transfected with linearized pUHR11-2 (lane 2), with a supercoiled pUHR11-2 derivative containing no phage terminator (lane 3), or with pUHR11-0, which lacks a T3 promoter (lane 4). The S1-resistant material was analyzed by PAGE and autoradiography. The positions of the 95-nucleotide *Hin*dIII–*Xho* I fragment (probe) and the 70-nucleotide protected fragment corresponding to the correct initiation site at promoter $P_{T3/O+14}$ are indicated. Lengths of size markers (lane 1) are given at left. (*D*) DNA-binding activity of *lac* repressor from RK-T3i cells. Cytoplasmic extracts of RK13, RK-T3, and RK-T3i cells were incubated with a ^{32}P-end-labeled 95-bp DNA fragment containing $P_{T3/O+14}$ and subjected to PAGE. Only the extract from RK-T3i cells (lane 4) retarded the migration of the operator-containing DNA. The position of the *lac* repressor/operator complex (R/O) and of the free DNA (O) are indicated. As control, purified *lac* repressor was mixed with an extract of RK13 cells (lane 1).

(Fig. 2C). The short autoradiographic exposure time (90 min) that was required to monitor the P_{T3}-specified RNA in our S1 assays indicates a high abundance of these transcripts. The S1 signal obtained with a supercoiled template containing no T3-specific transcriptional terminator was significantly lower (Fig. 2C, lane 3). This could be due to readthrough around the plasmid or to reduced activity of the T3 promoter in a supercoiled template.

To obtain RK cell lines that constitutively express both T3 RNA polymerase and *lac* repressor, RK-T3 cells were transfected with a mixture of pUHD11-1 (Fig. 1) and pHM24, which confers hygromycin B resistance (20). Clones derived from this transfection were examined for the simultaneous presence of T3 RNA polymerase and *lac* repressor. Gel retardation experiments (Fig. 2D) showed that the cell line RK-T3i contains functional *lac* repressor. This cell line, used for the experiments described below, contains $\approx 10^6$ molecules of *lac* repressor and $\approx 8 \times 10^5$ molecules of T3 RNA polymerase per cell, as estimated from Western blot analysis.

Synthesis of Active Luciferase in RK-T3 and RK-T3i Cells. Although large amounts of T3 RNA polymerase-specified transcripts were produced in RK-T3 cells upon transfection with pUHR11-1 or pUHR11-2, no luciferase activity was detected in the cells. By contrast, when RK-T3 cells were infected with vaccinia virus prior to the transfer of the plasmid, extremely high luciferase activities were monitored. This demonstrates that T3-specific transcripts are not translated in uninfected cells under our conditions and that eukaryotic viral functions are required for this step. The remarkable specificity and sensitivity of the luciferase assay enabled us to use the automated microinjection system described by Ansorge and Pepperkok (25), which allowed rapid assay of intracellular T3 promoter activities under well-defined experimental conditions. In a typical experiment, 200 vaccinia virus-infected RK-T3 cells were injected with 120–600 copies of pUHR11-1 per cell (see below and Table 3). After 7 hr, 10^4–10^5 rlu were monitored in 1/10th of the extract, at background values of 50–80 rlu as measured in RK-T3 cells containing a luciferase construct that lacked a T3 promoter (Table 1).

Regulated Expression of the Luciferase Gene in RK-T3 and RK-T3i Cells. Coinjection of pUHR11-2 with purified *lac* repressor into vaccinia virus-infected RK-T3 cells resulted in a decrease of intracellular luciferase activity and, at an estimated concentration of 15–60 nM *lac* repressor inside the cell, luciferase activity was close to background (Table 1).

Table 1. Effect of coinjected *lac* repressor on T3-specific transcription in RK-T3 cells

	Luciferase activity, rlu × 10^{-3}								
	pUHR11-1 (P_{T3})			pUHR11-2 ($P_{T3/O+14}$)			pUHR11 (no T3 promoter)		
Exp.	–	R	R+I	–	R	R+I	–	R	R+I
1	ND	ND	ND	12	1	20			
2	138	42	43	8	0.1	4			
				9	0.3	6			
3	42	14	6	56	0.2	15	0.05	0.06	0.07
				14	0.08	6			
				8	0.1	8			

Two hundred vaccinia virus-infected RK-T3 cells in IPTG-free or IPTG-containing medium (I) were microinjected with pUHR11-1, carrying P_{T3}, with pUHR11-2, carrying $P_{T3/O+14}$, or with pUHR11, lacking any T3 promoter sequence. Some solutions contained, in addition to plasmid, IPTG (I; 50 mM) and various amounts of *lac* repressor (R): Exp. 1, none (–) or 100 μg/ml; Exp. 2, none (–) or 200 μg/ml; Exp. 3, none (–) or 800 μg/ml. Cells injected with IPTG were supplied at the same time with IPTG in the medium. Luciferase activity was determined 7 hr after injection, from 1/10th of the cell extract. ND, not done.

IPTG reversed this effect when it was coinjected with *lac* repressor into RK-T3 cells supplied at the same time with 10 mM IPTG in the medium (Table 1). The same results were obtained when the cells were grown in IPTG-containing medium for 6 hr (data not shown). The luciferase activity was not affected by coinjection of the *lac* repressor when the luciferase gene was controlled by P_{T3} (pUHR11-1; Table 1).

Since the *lac* repressor concentrations of our RK-T3i cell lines are also in the 60 nM range, luciferase activity was efficiently repressed when pUHR11-2 (containing $P_{T3/O+14}$) was injected. The presence of 10 mM IPTG in the medium reversed the effect of the *lac* repressor to a large extent. No effect of the *lac* repressor was observed when pUHR11-1 (containing P_{T3}) was injected into RK-T3i cells (Table 2). Thus, the activity of $P_{T3/O+14}$ is efficiently regulated in RK-T3 and RK-T3i cells by *lac* repressor and IPTG.

Calibration of the Microinjection System. To estimate the amount of material transferred into RK13 cells by microinjection, commercially available luciferase was included as a marker in solutions containing either plasmid DNA alone or DNA as well as *lac* repressor and IPTG. Two hours after microinjection, cytoplasmic extracts were prepared and the luciferase activities of the extracts were compared with those of correspondingly diluted standard solutions. As shown in Table 3, highly reproducible volumes of a given solution could be transferred. However, significant differences were observed between different solutions, even with identical pressures and injection times. These differences were probably due to differences in viscosity and particle content. Thus, at a pUHR11-2 concentration of 5 μg/ml, between 120 and 600 copies of the plasmid were transferred into the cytoplasm of a RK13 cell, depending upon the composition of the particular solution (Table 3).

DISCUSSION

We have shown that gene *1* of coliphage T3, encoding the phage RNA polymerase, as well as the *lacI* gene of *E. coli*, encoding the *lac* repressor, can be stably integrated into a mammalian cell under conditions where the two proteins are produced in active form. With transcription controlled by the human cytomegalovirus promoter P_{CMV}, the steady-state levels of RNA polymerase and *lac* repressor are $\approx 8 \times 10^5$ and

Table 2. Control of T3 RNA polymerase specific transcription in RK-T3i cells

	Luciferase activity, rlu × 10^{-3}			
			RK-T3i	
Promoter construct	RK-13	RK-T3	–IPTG	+IPTG
pUHR11-1 (P_{T3})	ND	46	58	51
		0.07*		
pUHR11-2 ($P_{T3/O+14}$)	0.07	33	0.2	10
	0.07	10	0.07	2
	0.06	27	0.1	3
		23	0.1	4
		33	0.08	4
			0.07	6
			0.1	3
			0.06	9
(Mean ± SD)	0.069	25 ± 9	0.1 ± 0.04	5 ± 3

A solution of plasmid pUHR11-2 (5 μg/ml) was injected into the cytoplasm of RK13, RK-T3, and RK-T3i cells. Two hundred cells were injected per experiment and induction of transcription in RK-T3i cells was achieved by growing the culture in the presence of 10 mM IPTG. Luciferase activity was monitored after 7 hr, from 1/10th of the extract. Values given are rounded numbers. The mean values and the deviations were calculated from the original numbers. *RK-T3 cells not infected with vaccinia virus.

Table 3. Determination of the volumes transferred into the cytoplasm of RK-T3 cells by microinjection

Luciferase solution	Luciferase activity, rlu	Deviation from the mean, %	Injected volume, pl
	132.337		
A	203.978	25	1
	225.543		
	18.008		
B	16.935	9	0.16
	15.017		

Solutions containing luciferase as an assayable marker were injected into the cytoplasm of 200 RK13 cells per experiment. Luciferase activity was determined 2 hr later, from 1/10th of the extract. Solution A contained luciferase at 10 mg/ml and pUHR11-2 at 5 μg/ml; solution B contained luciferase at 5 mg/ml, pUHR11-2 at 5 μg/ml, *lac* repressor at 400 μg/ml, and 50 mM IPTG.

$\approx 10^6$ molecules per cell, respectively. Rabbit kidney cell lines producing either RNA polymerase alone (RK-T3) or both RNA polymerase and repressor (RK-T3i) have been established.

A high intracellular activity of the phage enzyme was demonstrated by the large amounts of RNA specified by P_{T3}, a phage T3 promoter, or its derivative $P_{T3/O+14}$, a fusion between P_{T3} and the *E. coli lac* operator. Both promoter constructs control the coding sequence of the firefly luciferase, whose activity can be monitored in a highly specific and sensitive assay. However, this activity was detected only when RK-T3 or RK-T3i cells were infected with vaccinia virus prior to transfection. Thus, viral functions, such as capping of RNA, seem to be required for the translation of T3-specific transcripts.

The remarkable sensitivity of the luciferase assay has allowed us to exploit the advantages of the automated microinjection technique (25) for transient-expression studies: (*i*) injection of well-defined volumes into individual cells (Table 3) and (*ii*) the ability to deliver simultaneously to the same cell several reactants in defined concentrations, such as operator-containing DNA and repressor (Table 1). Signals that are 100–1000 times the background value can be obtained from just 20 RK-T3 cells injected with 120–600 copies of the luciferase coding sequence controlled by P_{T3}.

This assay system has enabled us to rapidly examine the effect of *lac* repressor on the expression of luciferase controlled by $P_{T3/O+14}$. Coinjection of repressor with various DNA constructs indicated that highly specific repression was achieved and that, at intracellular repressor concentrations of 15–60 nM, luciferase activity was close to background. This repression was largely prevented by IPTG (Table 1).

Extremely low luciferase activity was observed in the RK-T3i cell line when pUHR11-2 (containing $P_{T3/O+14}$) was injected in absence of IPTG (Table 2). The average value derived from eight independent experiments is 103 rlu. When the background value of 69 rlu monitored in RK13 cells containing pUHR11-1 (but no T3 RNA polymerase) is subtracted, the repressed level of luciferase activity in RK-T3i cells is 34 rlu. Comparison of this value to the luciferase activity measured in pUHR11-2-containing RK-T3 cells gives a calculated repression factor of 735 (25,038 rlu/34 rlu; Table 2). Comparison of the luciferase activity generated in RK-T3i cells by pUHR11-1 and by pUHR11-2, respectively, indicates a 1700-fold difference (57,900 rlu/34 rlu; Table 2) depending on the presence or absence of the *lac* operator. Moreover, based on the repressed level of 34 rlu, a 150-fold induction of luciferase activity is achieved when RK-T3i cells are grown in medium containing IPTG.

The above estimates for repression and induction factors show that the activity of a defined gene can be tightly controlled in a mammalian cell by inducers of the *E. coli lac* operon. More accurate data on the repression factor and an even more efficient induction of transcription are expected in cell lines that contain the luciferase expression unit stably integrated and that produce lower levels of repressor as well as T3 RNA polymerase.

A more general application of the T3 RNA polymerase/*lac* repressor system, which most likely will function in a wide spectrum of cells and even in whole organisms, requires that both prokaryotic proteins are located in the cell nucleus and that translation of T3-specific transcripts is achieved independent of a viral infection. Preliminary experiments have shown that both conditions can indeed be met (U.D., unpublished data). Moreover, the extremely efficient and tightly controllable synthesis of $P_{T3/O+14}$-specified transcripts shows that this system is suitable for regulating gene expression in higher cells via antisense RNA.

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