

Bacterial Virus Gene Expression in Human Cells

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Human fibroblasts, from a patient with congenital lack of α -D-galactose-1-phosphate uridyl transferase activity, have been infected with transducing bacteriophage that harbours either wild type or defective transferase gene. Infection only by the former phage initiates transferase synthesis.

If the genetic code is truly universal it might be possible to make genetic changes in mammalian cells by introducing a specific gene by bacteriophage transduction^{1,2}. Human fibroblasts from a patient with galactosemia served as a model system to test for such a possibility. Galactosemia is the result of an autosomal recessive inborn error of metabolism in which there is a congenital absence of the enzyme α -D-galactose-1-phosphate uridyl (GPU) transferase activity^{3,4}.

The viruses which were tested in this system were lambda (λ_{C1857} , $\lambda_{C1857S7}$)⁵⁻⁷ and two transducing viruses derived from λ : the first, λ pgal₈C₁₈₅₇(K⁺T⁺E⁺), contains the complete galactose operon (ref. 8, and personal communication from D. Court and S. Adhya), and the second, λ pgal₈C₁₈₅₇(K⁺T⁺E⁻), contains the galactose operon with a mutation that renders the transferase gene inactive (made for us by M. E. Gottesman).

To obtain a functional enzyme from a bacterial virus, a mammalian cell would have to transcribe at least part of the phage genome into mRNA and then translate this into protein. Transcription was demonstrated in this study by the discovery of λ -specific RNA in cells exposed to either λ virus or λ DNA. GPU-transferase activity was also detected after exposure of the cells to λ pgal T⁺ virus or its DNA.

Transcription was studied by using galactosemic human fibroblasts (CCL-72 obtained in the sixteenth passage from the American Type Culture Collection). In each experiment, flasks infected with λ virus and control flasks were labelled with radioactive uridine 24 h before the cells were collected. When RNA was extracted and hybridized with denatured λ DNA on cellulose nitrate filters, the production of λ -specific RNA reached a maximum of 0.2% of labelled total RNA within 4.5 days after infection (Fig. 1). Cultures exposed to λ have remained at this level through two 3:1 subcultures for longer than 40 days. A similar result was obtained when the cells were exposed to λ DNA. Less than 0.005% of the labelled RNA extracted from the control cells was bound to the λ DNA filters. As a control for any contaminant which might be homologous with *E. coli*, the labelled RNA from both the λ infected and non-infected cells was hybridized with *E. coli* DNA filters. The binding was less than 0.005%.

Transferase activity in fibroblasts was assayed by a modification of the method of Russell¹²⁻¹⁴. In this assay, galactose-1-phosphate (Gal-1-P) is converted to UDP-galactose (UDP-Gal) by the transferase enzyme and by using ¹⁴C labelled

Gal-1-P it is possible to follow this reaction (Fig. 2). This activity was linear with time in the conditions used and proportional to the amount of cell lysate used (Fig. 3). All activity was lost by boiling the cell lysate. Uninfected galactosemic cells had an activity ≤ 0.2 nmol of UDP-Gal/60 min/mg of protein (Table 1). No difference in enzyme activity could be detected between uninfected galactosemic fibroblasts and fibroblasts infected with λ virus or λ pgal T⁽⁻⁾, with a mutation in transferase structural gene. The λ pgal T⁺ DNA-infected cells had enzyme levels which were generally greater than the λ pgal T⁺ whole virus-infected cells. Infected cell cultures have

Table 1 Assays for Transferase Activity

Cell strain	Exposure	Multiplicity of infection	Time after exposure	Transferase* activity
Normal fibroblasts				
Rat embryo fibroblasts	None	—	—	46.0
VMK	None	—	—	23.2
WI-38	None	—	—	6.3
Galactosemic fibroblasts (CCL-72)				
(a) Uninfected	None	—	—	<0.2
(b) Infections with transferase negative virus or DNA				
λ virus		4×10^5	96 h	<0.2
λ pgal (K ⁺ T ⁺ E ⁺) virus		4×10^5	96 h	<0.2
λ pgal (K ⁺ T ⁺ E ⁺) phage p.f.u. equivalents †		6×10^4	96 h	<0.2
(c) Infections with transferase positive virus or DNA				
λ pgal (K ⁺ T ⁺ E ⁺) virus		4×10^5	96 h	2.0
λ pgal (K ⁺ T ⁺ E ⁺) virus		4×10^5	52 days	10.1
λ pgal (K ⁺ T ⁺ E ⁺) phage p.f.u. equivalents †		6×10^4	96 h	15.6
DNA				

* Galactose-1-phosphate uridyl transferase activity is expressed in terms of nmol of UDP-Gal formed in 60 min/mg of protein at 37° C.

† $1 \mu\text{g}$ of λ DNA = 2×10^{10} p.f.u. equivalents.

Rat embryo fibroblasts were prepared as a primary culture from Holtzman rat embryos (donated by V. Huebner), VMK is a primary cell strain from African green monkey kidney cells (prepared by the cell biology section of the Division of Biologics Standards) and WI-38 is a normal human diploid cell line (CCL-75, passage 16, from the American Type Culture Collection). The cells were grown, infected and assayed for transferase activity as described in Figs. 2 and 3. λ_{C1857} virus was grown vegetatively on *E. coli* N205 (a spontaneous gal⁺ revertant of strain W3102 gal K² and carries recA allele from strain 152 gal⁺ of Meselson)¹⁷. λ pgal(K⁺T⁺E⁺) was also grown on N205. λ pgal(K⁺T⁺E⁺) was grown vegetatively on N205 and also on N580 (a transferase negative strain, W3104)¹⁸. These viruses were purified and banded in CsCl prior to use⁹. Virus DNA was prepared as described in Fig. 3.

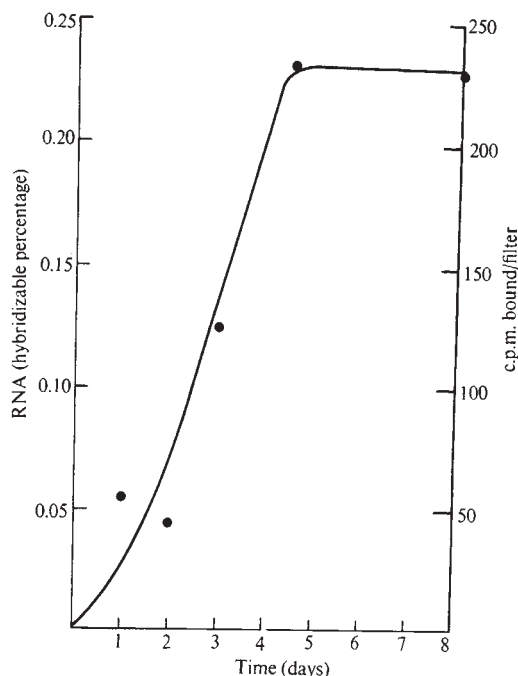


Fig. 1 Appearance of λ -specific RNA after infection of CCL-72 human galactosemic fibroblasts with $\lambda_{C1857S7}$ virus at a multiplicity of infection (m.o.i.) of 1×10^5 λ plaque forming units (p.f.u./cell). The data presented are in c.p.m. above background as well as hybridizable percentage which is defined as c.p.m. bound to filter-scintillation system background (20 c.p.m.)/total c.p.m. input. Hybridization with RNA extracted from uninfected cells gave ≤ 5 c.p.m. above background. Cells were cultured in Eagle's minimal essential media (EMEM) containing sodium bicarbonate (1 g/l.), foetal calf serum (10%), penicillin (100 U/ml.), streptomycin (50 μ g/ml.), and glutamine (0.17%) in 10 cm Petri dishes. This mixture was used for all tissue culture unless otherwise indicated. $\lambda_{C1857S7}$ was heat-induced from *E. coli* M5107 (given by E. Signer)^{6,7}, precipitated with polyethylene glycol, and banded in a gradient of CsCl⁹. The virus was dialysed in 0.015 M Tris-HCl, pH 7.6 buffer containing 0.01 M MgSO₄, and before use it was diluted into 1 ml. of Earle's balanced salt solution without bicarbonate (Earle's BSS) and passed through 'Millipore' filters (0.45 μ m pore size). The filters were rinsed with an additional 1 ml. of Earle's BSS. The cells were infected by the addition of 2 ml. of λ virus (5×10^{10} p.f.u.) in Earle's BSS. The virus was allowed to adsorb for 1 h with gentle rocking at 37° C, followed by the addition of 10 ml. of tissue culture media. Incubation was conducted at 37° C in an atmosphere of 5% CO₂ in air. The cellular RNA was labelled by removing the media and adding 10 ml. of fresh media plus 100 μ Ci of ³H-5-uridine (Lot No. 1745-40 from ICN, 21.7 mCi/mmol). The cells were incubated a further 24 h, rinsed twice with phosphate buffer saline (calcium and magnesium-free), and trypsinized. The RNA was extracted using SDS to break the cells followed by phenol, chloroform-isoamyl alcohol extractions and DNAase digestions using the method of Biswal and Benyesh-Melnick¹⁰. The extracted RNA was precipitated with ethyl alcohol and resuspended in a solution containing 0.003 M EDTA (adjusted to pH 7.4 with HCl). Hybridization was carried out in 6 \times 50 mm test tubes in a total volume of 0.480 ml. containing a final concentration of 2 \times SSC and 50% formamide. Cellulose nitrate filters (Schleicher and Schuell, B-6) containing 50 μ g of denatured DNA each (the DNA is in excess at this concentration) were placed in the tubes and the ³H RNA was added. The tubes were sealed and incubated at 37° C for 24 h (hybridization was complete within 24 h). These filters were then extensively washed with 2 \times SSC, treated with 20 μ g of RNAase A (Worthington) and 10 units of RNAase T₁ (Sankyo) at 37° C for 10 min. They were again washed with 2 \times SSC and incubated in 2 ml. of 1 \times SSC containing 70% formamide for 10 min at 37° C. The filters were again washed with 2 \times SSC, dried, and counted in a Nuclear Chicago refrigerated scintillation counter (model 8914). The scintillation system contained 12 g of BBOT (Packard)/gallon of toluene. This hybridization technique was developed as a modification of the Gillespie and Spiegelman¹¹ method by M. Das and S. Spiegelman (personal communication). All spent medium was tested for bacterial contamination by inoculating 0.5 ml. of medium on to tryptone plates and into 10 ml. of fresh tissue culture media without antibiotics. These were

been assayed through repeated subcultures and enzyme activity has remained relatively constant at the level achieved 4 days after infection. All eight λ gal virus preparations give similar results. Cells were grown in the presence of strepto-

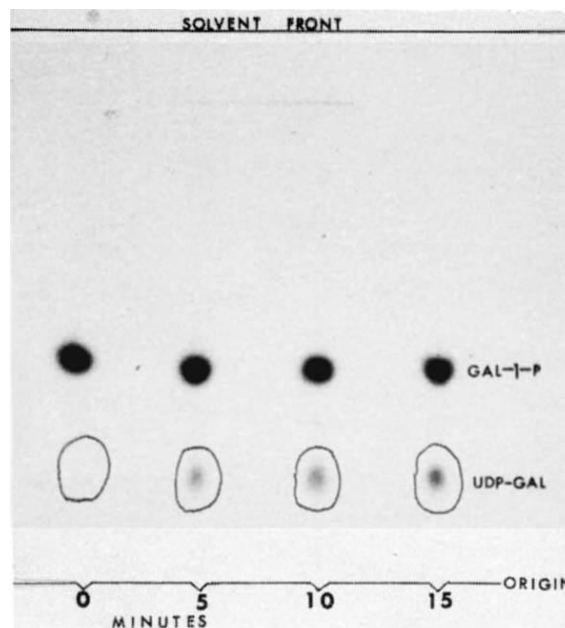


Fig. 2 Chromatography of 5 μ l. aliquots at 5 min intervals from an assay for α -D-galactose-1-phosphate uridyl transferase activity. The ¹⁴C-Gal-1-P reactant is clearly visible in the autoradiogram, while the product UDP-Gal becomes more visible as its concentration increases. The circles represent a UDP-Gal marker (non-radioactive). Cells (5×10^5 , normal rat fibroblasts) were grown as described in Fig. 1, and scraped off the wall, centrifuged (1,400g for 5 min), rinsed in PBS (calcium and magnesium-free) and resuspended in 20 μ l. of glycine buffer (0.08 M, pH 8.7). Sodium dilauryl sulphate (2 μ l. of 0.2% solution) was added and the cells were freeze-thawed three times and centrifuged at 12,000g for 2 min. The supernatant was then assayed for transferase activity and protein concentration was determined by the Lowry method¹⁵. The reaction mix contained 5 μ l. of D-galactose-¹⁴C-1-phosphate (4.5×10^{-4} M, New England Nuclear Corp., 227 mCi/mmol)*, 5 μ l. H₂O, 5 μ l. of glycine buffer (1 M, pH 8.7), 10 μ l. of UDP-glucose (4×10^{-4} M) and 10 μ l. of crude cell lysate. The reaction was performed at 37° C and 5 μ l. aliquots were removed and applied to the thin-layer chromatography plates PEI (Brinkman). Unlabelled UDP-Gal (2 μ l. 10 μ mol/ml.) was spotted at the origin as a marker. The chromatogram was run for 4 cm in 1 N formic acid and 17 cm in 2 N sodium formate, pH 3.6. The position of the UDP-Gal could be determined by the ultraviolet absorption of the unlabelled marker. Similar results were obtained by using DEAE paper ('Whatman DE81') with 0.015 M Na₂HPO₄ \cdot 7H₂O and 0.15 M citric acid, pH 3.8; however, this system gave a higher background. Autoradiography of the chromatogram was performed with X-ray film ('Kodak NS-2T') and the UDP-Gal spots were cut out and placed in a toluene-TLA (Beckman) scintillation system for quantitative counting. A Beckman LS250 scintillation counter was used.

* Commercial Gal-1-P gives a relatively high background in this system (1,000 c.p.m.); however, it is possible to purify this material by utilizing the PEI thin layer chromatography system described above. The Gal-1-P is located by autoradiography and the spots cut out. These are washed twice with 1 litre of distilled H₂O and then placed in 100 ml. of 0.5 M LiCl for 20 min. The LiCl solution is then decanted off and passed through a 'Dowex 50' column, 5 mm \times 20 mm (which was previously washed with 0.5 M LiCl). The column effluent was diluted fifty times and pumped through a 'Dowex 1' column (which was previously washed with 2 M ammonium formate and then with distilled H₂O). The column was eluted with 1 M formic acid and the Gal-1-P collected. The sample was freeze dried and brought to the desired concentration with distilled water. This purified Gal-1-P gives a background of about 100 c.p.m.

cultured for 5 days at 37° C and at room temperature. Cells were also periodically monitored for mycoplasma contamination. All such tests were negative.

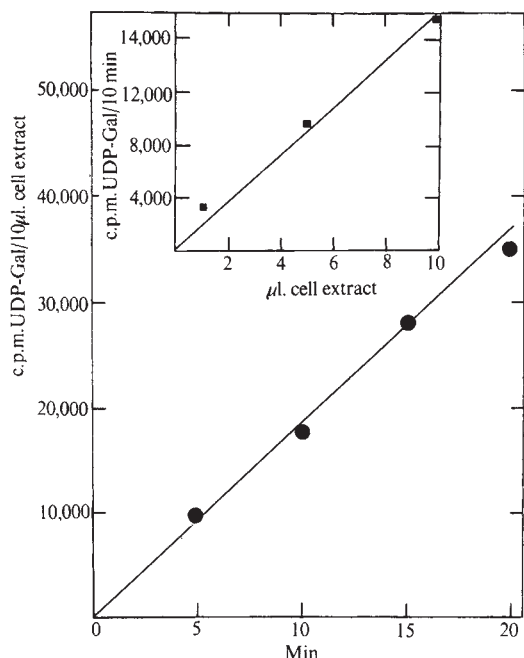


Fig. 3 Linearity of the α -D-galactose-1-phosphate uridyl transferase assay as a function of time. Inset demonstrates the linearity of the assay as a function of protein concentration. The cells used in this assay were galactosemic human fibroblasts infected with a multiplicity of infection of 6×10^4 phage plaque forming unit equivalents of λ pgal T⁺ DNA*. The DNA was added to three flasks, each containing 5×10^5 cells, in 2 ml. of Earle's BSS with 75 μ g/ml. of DEAE-D (MW=10⁶, Pharmacia)¹⁶. This DNA was allowed to adsorb for 1 h with gentle rocking at 37° C, followed by the addition of 10 ml. of tissue culture medium and incubated for an additional 95 h at 37° C in an atmosphere of 5% CO₂ in air. The cells were harvested and the assay performed as described in Fig. 2.

* 1 μ g of λ DNA = 2×10^{10} p.f.u. equivalents, based on a molecular weight of $\lambda = 3 \times 10^7$. Phage DNA was obtained by phenol extraction of 3 ml. of phage with an equal volume of freshly distilled phenol equilibrated with 2 \times SSC followed by an extraction with isoamyl alcohol and chloroform (1 : 100), and a second phenol extraction. The DNA was treated with 1 mg of pronase in 1 ml. of Tris-HCl (0.1 M, pH 7.5) buffer containing EDTA (0.003 M) for 5 min followed by three more phenol extractions and two ethyl ether extractions. It was then exhaustively dialysed against 2 \times SSC at 4° C.

mycin (50 μ g/ml.) and penicillin (100 U/ml.). Mycostatin (50 U/ml.) was tested in cultures with no effect on enzyme production or λ -specific RNA levels. Cycloheximide (1 mg/ml.) which blocks protein synthesis in mammalian cells¹⁹ but not in bacteria²⁰ prevented enzyme production in cultures infected with λ pgal T⁺. These cultures were monitored for bacterial and mycoplasma contamination in the same manner as in the hybridization study.

We determined whether λ pgal T⁺ enhances intracellular galactose metabolism. Morphological evidence of metabolic stress (granular cytoplasm, rounding up and detachment from the surface of the flask) could be detected in galactosemic cells after 72 h incubation with galactose as sole hexose source. Cells infected with λ pgal, however, have survived from 1 to 3 weeks longer than control cells when both were grown in media containing 1 mg/ml. galactose and dialysed foetal calf serum. Normal human fibroblasts have survived in this medium for at least 1 week longer than any galactosemic cells infected with λ pgal T⁺ virus. Furthermore, galactosemic fibroblasts exposed to λ pgal T⁺ produce more ¹⁴CO₂ from galactose-1-¹⁴C than uninfected fibroblasts (our unpublished observations).

The possibility of contaminating organisms giving rise to the data is remote as subcultures of the spent medium in thioglycollate and trypticase soy broths showed no growth, nor did cultures on tryptone plates and tissue culture media

without antibiotics, and the cells were periodically shown to be free of mycoplasma. The presence of organisms in numbers sufficient to yield the transferase data (5×10^5 *E. coli*, N205) should have been detected easily and, furthermore, no *E. coli* specific RNA was detected in the hybridization experiments. As the cells were cultured in penicillin and streptomycin, the contaminant would be insensitive to these antibiotics as well as mycostatin, but inhibited by cycloheximide. As a further control 10⁶ λ lysogens (*E. coli* M5107), were added to a culture of galactosemic fibroblasts and labelled for 24 h with ³H-uridine. These cells were then washed in the normal manner, leaving only the fibroblasts bound to the flask surface. No λ -specific RNA could be found by hybridization, indicating that the λ -specific RNA is not produced by an *E. coli*-like organism. The possibility that the λ gal T⁺ virus is merely transporting enzyme into the cells can be discounted as the enzyme is produced by cells infected with pronase-treated λ gal T⁺ DNA.

The detection of λ -specific RNA supports the occurrence of transcription, while the lack of enzyme production in the presence of cycloheximide indicates that translation is necessary for transferase activity.

The experimental conditions used by us do not permit a differentiation between the various models of phage gene preservation within mammalian cells. The detection of both λ -specific RNA and transferase enzyme activity 41 days after infection with no decrease in levels indicates that the genome is not being segregated out in these conditions of growth. The phage DNA may be preserved by any of the following mechanisms: integration into the host genome, plasmid-like existence in the cytoplasm, interaction with the mitochondria (known to have some bacteria-like properties), or perhaps in some totally unknown manner.

Our results suggest that it might be possible to introduce a selected bacterial gene into human cells *in vivo*, using phage as a vehicle, and significantly to alter a specific metabolic pathway. Our results also agree with recent evidence^{21,22} that there are much greater biochemical similarities between far ranging living species than had been appreciated previously²³.

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