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Genetics

Replication and expression of thymidine kinase and human globin genes microinjected into mouse fibroblasts

(gene transfer/restriction endonuclease/Southern blot/plasmid/herpes simplex virus)

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ABSTRACT **A mixture of two recombinant plasmids was microinjected into mouse thymidine kinase-negative fibroblasts (L cells). One plasmid contained the thymidine kinase gene of herpes simplex virus type I and the other contained the human β globin gene. Approximately 1 or 20 copies of each plasmid were injected. Seven fibroblast colonies arising from injected cells incubated in hypoxanthine/aminopterin/thymidine medium were analyzed. These microinjected cells were shown to: (*i*) produce functionally active herpes simplex type I thymidine kinase enzyme, (*ii*) replicate the human β globin gene, and (*iii*) produce human β globin mRNA sequences at low levels. Thus, the genetic defect (lack of thymidine kinase activity) was corrected by the microinjected thymidine kinase gene, and a coinjected human β globin gene was replicated and weakly expressed.**

Several techniques have been used to transfer genes into tissue culture cells, including somatic cell hybridization (1, 2), chromosome-mediated gene transfer (3, 4), DNA-mediated gene transfer (5–7), and simian virus 40 (SV40) hybrid transfection (8, 9). We wished to develop a procedure whereby a single copy of a gene could be inserted into the nucleus of a single cell under conditions whereby the injected cell could be grown in culture into a cloned population with or without selective pressure. The physical microinjection procedure developed by Diacumakos (10, 11) is ideal for this purpose. A minute volume [10^{-11} ml (= 10 fl)] of solution can be injected into any region of a cell with no apparent interference with cell function.

We have used this procedure to microinject mouse thymidine kinase-negative (TK^-) L cells with a mixture of two plasmids: one containing a cloned fragment of the TK gene from herpes simplex virus (HS) type I and the other a cloned fragment of the β globin gene from humans. The TK^- cells were functionally corrected by the injected TK gene and the globin gene was replicated and expressed at a low level.

MATERIALS AND METHODS

Cell Culture. Mouse TK^- L cells were obtained from Richard Axel of Columbia University (5). Cells were grown in modified minimal essential medium with 10% fetal bovine serum and incubated in 10% CO_2/90% air at 100% relative humidity and 37°C.

Origin and Preparation of Plasmid DNA Molecules. The plasmid χ1, which is a recombinant plasmid that contains a 3.5-kilobase (kb) *Bam*HI restriction endonuclease fragment (coding for TK of HS type I) inserted into pBR322 (12), and pRK1, a recombinant plasmid that contains a 4.4-kb *Pst* I fragment (coding for the human β globin gene) inserted into pBR322 (13), have been described previously. Methods for growth of bacterial cells and extraction of plasmid DNA after chloramphenicol amplification were as described (14) and were in accordance with the National Institutes of Health guidelines on recombinant DNA research.

Physical Microinjection. The microinjection procedure was as described by Diacumakos (11). TK^- L cells (1×10^6) were transferred onto each of several coverslips 12–24 hr prior to microinjection. Cells were injected with approximately 10 fl of solution. After microinjection the cells were incubated in modified minimal essential medium for 24 hr, followed by incubation for 24 hr in HAT medium diluted with 2 parts regular modified minimal essential medium and then by incubation in full-strength HAT medium (100 μM hypoxanthine/0.4 μM aminopterin/16 μM thymidine) (15). Colonies of cells surviving in HAT medium were isolated when they reached 150–200 cells. Porcelain cloning cylinders with vacuum grease to form a seal were placed over the cell colony. Colonies were treated with 0.2 ml of 0.05% trypsin for 3 min and the suspended cells were transferred to a culture dish. Cultures were split after reaching 70% confluency.

DNA and RNA Extraction. Cells were grown to 1×10^9, scraped from 100-mm tissue culture dishes, pelleted, and washed in phosphate-buffered saline. The cell pellet was lysed in 5.4 ml of 6 M guanidine·HCl, adjusted to 0.2 M KOAc, and stirred on a Vortex mixer for 10 min. Twice the volume of 4% Sarkosyl in 0.1 M Tris·HCl, pH 7.5, was added, along with 0.4 g of CsCl per ml (which is 7.2 g for the standard 18-ml lysate). The cell lysate was layered over a 1.6-ml cushion of 5.6 M CsCl in 0.1 M EDTA, pH 8, and centrifuged for 60–72 hr at 34,000 rpm in an SW 41 Beckman rotor (16, 17). After centrifugation, DNA, which appears as a 2- to 5-mm band on the CsCl cushion, was withdrawn and dialyzed against 0.1 M NaCl. Boiled RNase (100 μg/ml) was added and the DNA solution was incubated at 37°C for 1 hr. The DNA was extracted twice with neutralized phenol, precipitated with ethanol, resuspended in deionized water, dialyzed against 10 mM Tris·HCl, pH 7.2/0.5 mM EDTA, and stored at 4°C.

The RNA pellet that resulted from the CsCl centrifugation was resuspended in 5 ml of buffer (10 mM Tris·HCl, pH 7.5/0.5% sodium dodecyl sulfate/0.1 M NaCl/1 mM EDTA), extracted with phenol, precipitated with ethanol, resuspended in water, and stored in liquid nitrogen.

DNA Filter Hybridization. Analysis of recombinants was by restriction endonuclease digestion and Southern blotting (18), using modifications of the hybridization and washing conditions described by Jeffreys and Flavell (19). The ^{32}P-labeled nick-translated double-stranded human β globin DNA probe was prepared from the *Hha* I fragment of the recombinant cDNA

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Abbreviations: SV40, simian virus 40; TK, thymidine kinase; HS, herpes simplex virus; kb, kilobase(s); HAT, hypoxanthine/aminopterin/thymidine; MEL, mouse erythroleukemia.

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plasmid JW-102 (20), or the 4.4-kb *Pst* I fragment containing the β globin gene isolated from human genomic DNA (21) as described (22, 23).

Solution Hybridization. mRNA·cDNA hybridization and synthesis of the anti-mRNA single-stranded ^{32}P-labeled human β globin cDNA probe from JW-102 were carried out as described (14, 23) except that the titration reactions were incubated at 58°C.

Assay for HS Type I TK Enzyme Activity. The initial ^{125}I-labeled deoxycytidine (^{125}I-dCyd) assay (see *Results*) was kindly carried out by William and Wilma Summers at Yale University according to their published procedure (24). Further assays were done with their substrates.

RESULTS

Characterization of Microinjected Colonies. Mouse TK$^-$ L cells were microinjected with the plasmids pRK1 and χ1 as described in *Materials and Methods*. A colony was isolated from each of three coverslips and the colonies were named C1, C2, and C3. Continued culture of the coverslips resulted in the appearance of four additional colonies on the coverslip from which C2 was isolated. Because these might be colonies derived from metaphase cells that migrated from the original C2 colony, these four are named C2A, C2B, C2C, and C2D. Ten to 50 L cells were injected on each coverslip (requiring 5–15 min total injection time). C1 and C3 were obtained from cells injected with plasmids (at a concentration of 1 molecule each of pRK1 and χ1 per injection volume of 10 fl) linearized 80% by digestion with the restriction endonuclease *Sal* I. The extent of linearization was confirmed by electron microscopy (data not shown). C2 and C2A–D were obtained from cells injected at a concentration of 20 copies of each nonlinearized covalently closed circular plasmid per 10 fl. Under the conditions used here, it is possible that more than one injected cell could compose each colony. As for efficiency of gene transfer, we estimate that, under our present conditions, approximately 1 cell in 20 is transformed.

Expression of the HS TK Enzyme in Injected Colonies. The colonies were expanded in HAT medium, which kills all TK$^-$ cells. No revertants were detected when a number of cultures containing 10^6 noninjected TK$^-$ L cells were grown in HAT medium. Growth of injected TK$^-$ cells must, therefore, be due either to expression of the injected HS TK gene or to activation of the mouse TK gene as a result of the microinjection process. A definitive technique for detection of the HS TK enzyme activity has been developed by William and Wilma Summers at Yale University (24). Colonies C1 and C2 were kindly analyzed by the Summers, with the results shown in Table 1. Our parental TK$^-$ L cells (probably identical to the Summers' LMTK$^-$) are negative for TK activity. Both colonies C1 and C2 however, have HS TK enzyme activity (25). Colonies C2B, C2D, and C3 have also been shown to possess HS TK activity (data not shown). Because the assays were done on clones of 5×10^4 cells grown from, presumably, single cells that were microinjected with approximately one copy (or 20 copies) of the TK gene, the injected HS TK gene is clearly replicating and functioning in these cloned cells.

Demonstration of the Human β Globin Gene in Injected Colonies. The TK gene was maintained with a positive selective system. The human β globin gene, however, was simply coinjected with the TK gene. We asked if the human globin gene was still present in these colonies. Each colony was expanded to 10^9 cells, DNA and RNA were extracted, and the DNA was subjected to restriction endonuclease digestion and Southern blot analysis, using the enzymes *Eco*RI, *Pst* I, *Bgl* II, *Bam*HI, *Pvu* II, *Sal* I, and *Kpn* I, either singly or in combination. The fragments hybridizing with the human β globin cDNA probe were compared with the restriction map of pRK1 shown in Fig. 1. The restriction fragments obtained from human genomic DNA, pRK1, and the seven microinjected colonies that hybridize with the human β globin plasmid cDNA probe are shown (in part) in Fig. 2 and a complete list of the data is presented in Table 2.

Table 1. Measurement of HS type I TK in microinjected TK$^-$ L cells*

Cell line	Incorporation of ^{125}I-dCyd, cpm: No H_4Urd	H_4Urd
Controls		
LMTK$^-$	46	54
A9 (TK$^+$)	2576	107
TFC3 (HS TK$^+$)	3477	2917
Clones		
Parental TK$^-$ L	48	53
C1	1541	1227
C2	1979	2000

* Assay of Summers and Summers (24). The HS TK enzyme can phosphorylate ^{125}I-dCyd (which is then incorporated into trichloroacetic acid-insoluble DNA), whereas mouse TK enzyme cannot. However, mouse cells possess an enzyme, deoxycytidine deaminase, that can convert ^{125}I-dCyd into a usable substrate. The inhibitor tetrahydrouridine (H_4Urd) blocks deoxycytidine deaminase activity. Therefore, incorporation of ^{125}I-dCyd into DNA in the presence of H_4Urd is specific evidence for HS TK enzyme activity.

Several conclusions can be drawn. First, human β globin gene sequences are present in every colony, even though there is no selective pressure to maintain globin genes. Because the nick-translated DNA fragment used as the human β globin plasmid cDNA probe contains some sequences of pBR322, Southern blots were also carried out, using as a probe the gel-purified ^{32}P-labeled 4.4-kb *Pst* I human β globin genomic fragment isolated from λ (Fig. 3). Results confirmed the existence of human β globin gene sequences in the microinjected cell DNA. Second, the restriction fragments hybridizing with the human β globin cDNA probe from the DNA of the seven microinjected colonies show distinct differences. Third, the restriction patterns cannot be simply interpreted. However, a considerable number of the bands are consistent with the presence of the entire

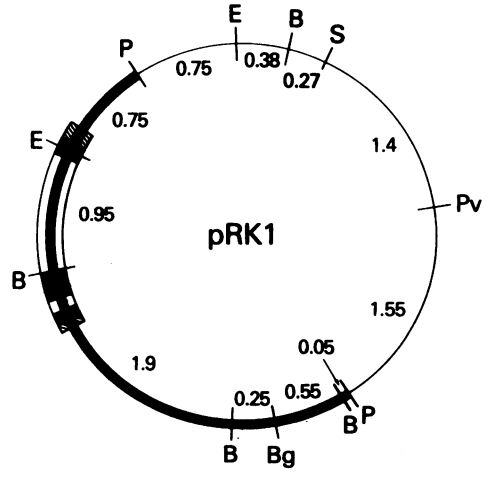

FIG. 1. Restriction map of pRK1. The radial lines are sites of the restriction endonucleases *Eco*RI (E), *Pst* I (P), *Bgl* II (Bg), *Bam*HI (B), *Pvu* II (Pv), and *Sal* I (S). The numbers are in kb. The thin portion of the circle represents pBR322; the thick portion of the circle is the 4.4-kb *Pst* I fragment containing the human β globin gene. The globin gene itself is represented by the boxed area, from bottom to top: hatched, 5′ nontranslated region; solid, 5′ coding region; clear, first intron; solid, middle coding region; clear, second intron; solid, 3′ coding region; hatched, 3′ nontranslated region.

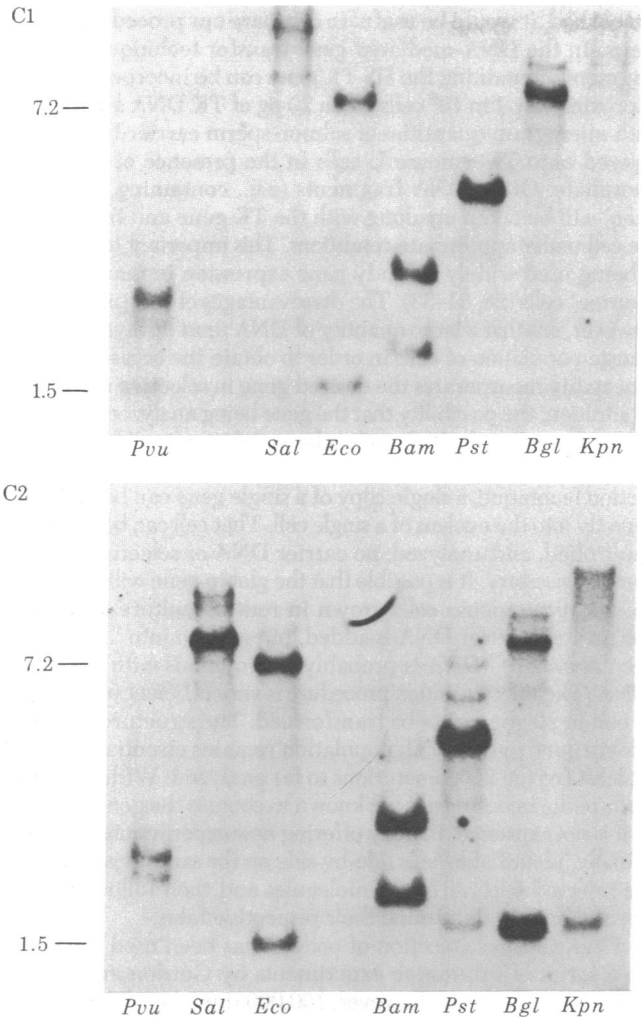

FIG. 2. Southern blot analysis of the DNA isolated from microinjected mouse L cell colonies, using a ^{32}P-labeled human β globin cDNA as probe. High molecular weight DNA was isolated from 10^9 cells (60–90 generations), digested with the specified restriction endonuclease, electrophoresed on 1% agarose slab gels, transferred to nitrocellulose filters, and then hybridized with the ^{32}P-labeled nick-translated *Hha* I fragment isolated from the human β globin cDNA plasmid clone JW-102 (20, 23). Thirty to 40 μg was loaded into each lane. Restriction endonucleases used: *Pvu* II, *Sal* I, *Eco*RI, *Bam*HI, *Pst* I, *Bgl* II, and *Kpn* I. The unlabeled lane contained λ phage DNA marker fragments for use in size determinations (kb scale on the left). All significant bands regardless of intensity are listed in Table 2. (*Upper*) Data for C1; (*Lower*) data for C2.

human β globin gene in the original covalently closed circular pRK1 plasmid (Table 2). We have isolated pRK1 from the undigested DNA of the injected cells and, in addition, have identified (and determined the restriction maps of) a number of unique plasmids representing different recombination (and deletion) events involving pRK1.[§] One of these unique plasmids is a recombinant between partial or complete copies of pRK1 and χ1.[§] All these recombination events appear to have taken place within the mouse cell. The presence of other strong (and light) bands in the digests of the seven colonies might suggest integration of the human β globin gene into high molecular weight DNA. However, these bands might also arise from the unique plasmids, some of which are very large (e.g., 20.7 kb).[§]

[§] P. J. Kretschmer, L. Sanders-Haigh, L. Killos, and W. F. Anderson, unpublished data.

Table 2. Restriction fragments of DNA from microinjected colonies

DNA source	Fragment lengths, kb						
	*Eco*RI	*Pst* I	*Bgl* II	*Bam*HI	*Pvu* II	*Sal* I	*Kpn* I
C1	7.2	4.4	11.0	2.8	2.1	≈20	8.7
	1.5		8.7	1.9	2.0	8.7	
C2	7.2	7.0	11.0	2.8	2.1	≈20	≈20
	1.5	6.0	8.7	1.9	2.0	15.0	1.5
		4.4	1.5			8.7	
		1.5					
C2A	7.2	4.4	—	2.8	8.7	≈20	—
	1.5	3.2		1.9		15.0	
						8.7	
C2B	9.0	4.4	≈20	2.8	8.7	8.7	≈20
	7.2			1.9	6.0		
	4.5				4.3		
	1.5						
C2C	14.0	4.4	—	3.2	15.0	—	≈20
	7.2			2.8	8.7		
	1.5			1.9			
C2D	7.2	4.4	8.7	3.2	8.7	≈20	≈20
	4.5		6.0	2.8	3.1	8.7	
	1.5		5.0	1.9	2.1		
C3	7.2	4.4	10.0	4.2	8.7	8.7	≈20
	4.5	4.2	8.7	2.8	3.1		8.7
	1.5		6.0	1.9	2.1		
			4.8				
pRK1	7.2	4.4	8.7	2.8	8.7	8.7	—
	1.5			1.9			

The probe for these data was the ^{32}P-labeled *Hha* I fragment isolated from the human β globin cDNA plasmid clone JW-102 (20, 23). The value ≈20 means approximately 20 kb and above because the gel does not resolve above 20 kb.

Stability of the Injected Genes. In order to test the stability of the injected genes, we have continued to expand several of the colonies over a period of 9 months. After every 30 generations (10^9 cells), the cells were harvested, DNA was extracted, and Southern blot analysis was repeated. After seven expansions (approximately 200 generations) the cells still survived in HAT (indicating that the TK gene was still functioning) and the restriction fragments that hybridized with the β globin cDNA probe remained essentially unaltered (data not shown).

Expression of the Human β Globin Gene in Injected Colonies. RNA was extracted from the expanded colonies and hybridized against the single-stranded ^{32}P-labeled human β globin cDNA to measure the number of human β globin mRNA sequences. From the data in Fig. 4, it can be calculated that colonies C2B, C3, and C2C contain, respectively, approximately 7, 5, and 2 human globin mRNA molecules per cell. It has not been determined if the mRNA molecules are intact (26, 27). These numbers can be compared with the values of approximately 5 (26) and 2000 (28) rabbit globin mRNA molecules found in mouse L cells cotransformed with the HS TK and rabbit β globin genes.

DISCUSSION

A mixture of the genomic HS type I TK gene and a human β globin gene has been microinjected into TK$^-$ L cells. The injected L cells were grown and shown to produce functional HS TK enzyme from the HS TK gene and low levels of human β

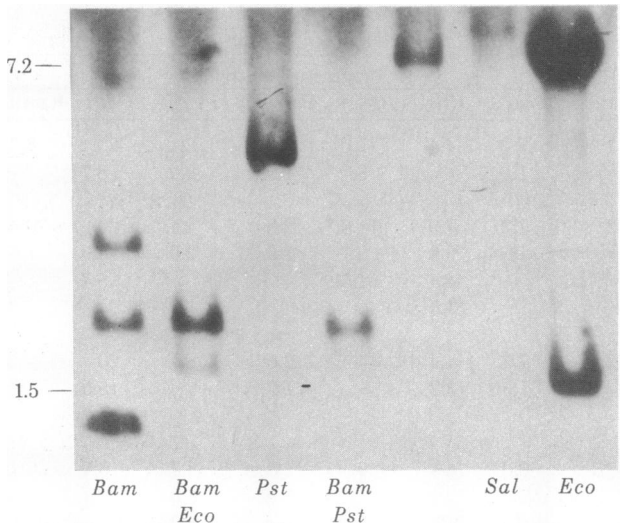

FIG. 3. Southern blot analysis of the DNA isolated from microinjected colony C2B, using the ^{32}P-labeled 4.4-kb *Pst* I fragment from HβG1 (21) (i.e., pure human DNA) as probe. These data demonstrate the presence of human DNA in the microinjected L cell DNA and correlate well with the data in Table 2, which were obtained with a human cDNA probe that contained a small amount of pBR322 sequences (thus producing additional bands on the Southern blots). If the human DNA were present as pRK1 in the microinjected DNA, then the following fragment sizes should be seen: *Bam*HI, 2.8, 1.9, and 0.8 kb; *Pst* I, 4.4 kb; *Sal* I, 8.7 kb; *Eco*RI, 7.2 and 1.5 kb (see Fig. 1). These are the fragments seen. However, double digests of *Bam*HI + *Eco*RI should give fragment sizes 1.9, 1.5, 0.95, and 0.8 kb, but only the larger fragments are seen (1.9 and 1.5 kb). Likewise, the *Bam*HI + *Pst* I digestion should give fragment sizes 1.9, 1.7, and 0.85 kb, but only the 1.9 (and ? 1.7) kb bands are observed. The unlabeled lane contains a low level of *Eco*RI-digested DNA, while the *Eco*-labeled lane contains a high level of DNA.

globin mRNA sequences from the human β globin gene. Thus an intact genomic gene has been successfully microinjected into a mutant mammalian cell with functional correction of the mutation.

Because the DNA-mediated gene transfer procedure developed by Axel, Wigler, and their colleagues (5, 29, 30) is well established, it would be useful to compare our procedure with theirs. In the DNA-mediated gene transfer technique, DNA fragments containing the HS TK gene can be incorporated by approximately 1 in 10^6 cells when 20 pg of TK DNA are mixed with microgram quantities of salmon sperm carrier DNA and layered onto TK$^-$ mouse L cells in the presence of calcium phosphate. Other DNA fragments (e.g., containing a globin gene) can be taken up along with the TK gene and transform the cell under appropriate conditions. This important technique is being used widely to study gene expression in mammalian (murine) cells (28, 31–33). The disadvantages of this procedure, however, are that a large quantity of DNA must be layered onto a large population of cells in order to obtain the occasional cell that stably incorporates the desired gene in selective medium. In addition, the possibility that the gene being analyzed is linked within the cell to the high molecular weight carrier DNA makes analysis of the restriction band data difficult. In the microinjection technique, a single copy of a single gene can be injected directly into the nucleus of a single cell. That cell can be isolated, multiplied, and analyzed; no carrier DNA or selective procedure is necessary. It is possible that the globin gene will replicate in wild-type mouse cells grown in routine culture medium. Because no carrier DNA is added, integration into "high molecular weight" DNA is probably synonymous with genomic DNA. The microinjection procedure is very efficient in that one L cell in 20 appears to be transformed. The structure of the β globin gene in the L cell population remains essentially stable at least for the 200 generations so far analyzed. With this procedure the injected cells are known to contain the gene(s), even if it is not expressed, thereby offering new experimental options. Finally, identifiable cells side-by-side on the same coverslip can be injected with different molecules and then followed over several days to determine their respective fates.

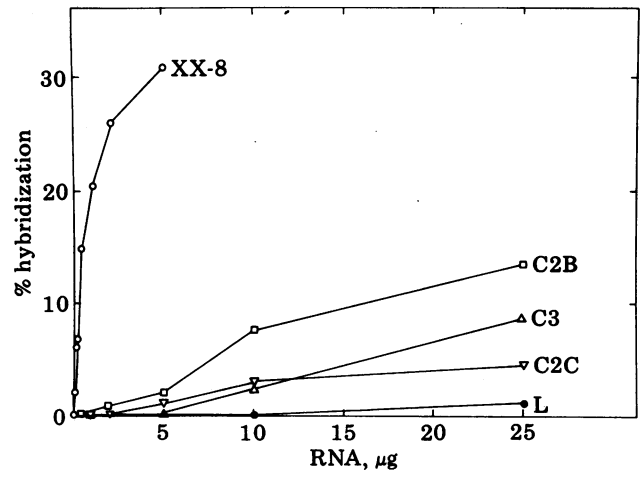

FIG. 4. Hybridization with ^{32}P-labeled human β globin cDNA of RNA extracted from microinjected mouse L cells (30th–60th generations). RNA from the somatic cell hybrid XX-8 [2*s* (*s*, stem line chromosome number) mouse erythroleukemia cell hybridized with human fibroblast (23)] was used as a standard because it has been determined (unpublished observations) that induced XX-8 cells contain approximately 500 human β globin mRNA molecules per cell. On the basis of estimated slopes, the cell lines contain the following numbers of copies of human β globin mRNA per cell: C2B, 7; C3, 5; C2C, 2; and parental L cells, 0.

Physical microinjection of oocytes has been used for years in a series of impressive experiments by Gurdon and others (34–36). Oocytes are, however, 100,000 times larger in volume than mammalian tissue culture cells. Following the report of the injection of DNA into the nucleus of HeLa cells by Diacumakos *et al.* (10), a further application of the microinjection technique for inserting nucleic acid molecules into mammalian cells was reported by Stacey and Allfrey (37). They showed that rabbit globin mRNA injected into HeLa cells can be translated into rabbit globin. Ruddle and his colleagues have used the procedure to inject a number of different mRNA molecules into mouse L cells (38). Diacumakos and Gershey (39, 40) demonstrated that a normal yield of infectious SV40 was produced by CV-1 cell cultures after inoculation of a few cells that had received (on the average) one particle per nucleus. Graessmann and Graessmann, using a relatively similar injection system, have carried out a related study (41) and, in addition, have mapped a number of SV40 proteins to SV40 DNA fragments by injecting the purified DNA fragment and testing for the protein synthesized in susceptible cells (42). Previously, we injected rabbit globin cDNA molecules into HeLa cells and showed that a very low level of transcription and translation of the injected cDNA took place (43). The present work, however, takes the further step of demonstrating expression of an injected genomic gene resulting in phenotypic correction of the mutant cell.

The microinjection technique allows several important questions to be addressed. First, are the expressing injected genes integrated into genomic DNA or free as self-replicating plasmids? The blot data in Fig. 2 and Table 2 show complex patterns. Bands corresponding to the covalently closed circular plasmids pRK1 or χ1 are present. We have recovered not only pRK1 and χ1, but also other plasmids (formed by recombination and deletion events involving pRK1 and χ1 within the

mouse L cell) from the undigested DNA of the expanded microinjected colonies.[§] These data suggest that prokaryotic plasmid DNA, once it is inserted into the mouse L cell nucleus, can replicate and recombine within the eukaryotic cell. Whether it does so while integrated into the genomic DNA or as a self-replicating extrachromosomal element has not yet been determined. However, because covalently closed circular pRK1 plasmids have been rescued directly from the undigested DNA extracted from descendents of microinjected cells, prokaryotic plasmids must exist, at least for part of the cell cycle, in the extrachromosomal state. In addition, it is unknown whether expression of plasmid genes is dependent on an integrated or nonintegrated state. The bands on Southern blots that cannot be accounted for by either pRK1 or χ1 might be due totally or in part to the unique plasmids arising from pRK1 and χ1. Thus, at the present time, there is no definitive data that the globin gene (or the TK gene) is ever integrated into genomic DNA.

Second, will the globin gene be expressed normally if placed into an erythroid environment such as an induced mouse erythroleukemia (MEL) cell? Only a few human globin mRNA sequences were detected in the microinjected L cells (Fig. 4). It is not surprising that the level of expression of the globin gene in L cells is low, because we have previously presented evidence that fibroblasts contain a negative regulatory factor that inhibits globin gene expression (44). Induced MEL cells, however, contain a positive regulatory factor that specifically turns on the expression of globin genes (23, 45). Thus, if a DNA fragment that contains a globin gene along with the appropriate regulatory signals is injected into an uninduced MEL cell, it should be expressed at a very low level (like the mouse L cell) but might be expressed at a very high level when the MEL cell is induced.

Third, the success in correcting a mutant mouse L cell in culture by injecting a normal gene immediately suggests the possibility of correcting human genetic diseases by microinjecting a normal version of the defective gene.

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