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Restoration of Mitochondrial Gene Expression Using a Cloned Human Gene in Chinese Hamster Lung Cell Mutant

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Abstract

Background: Gal$^-$32 is a Chinese hamster lung cell nuclear mutant that is unable to grow in galactose due to a defect in mitochondrial protein synthesis. Since the product of the Gal$^-$32 gene was unknown, it was imperative to use phenotypic complementation to clone a human gene that corrected the Gal$^-$32 mutation.

Results: Recessive Gal$^-$32 cells were co-transformed with pSV2-*neo* plasmid DNA and recombinant DNA from a human genomic library containing the dominant human Gal$^+$ gene and a chloramphenicol-resistance (*cam*^{r}) gene present in the pSV13 vector. Primary transformants were selected by growth in galactose and the neomycin analog G418. In order to rescue the human Gal$^+$ gene, a genomic library was constructed with primary transformant DNA and the pCV108 cosmid vector. The *cam*^{r} gene was used to identify clones with the nearby human sequences. DNA from two *cam*^{r}, *Alu*-hybridizing clones was able to transform the recessive Gal$^-$32 cells to the Gal$^+$ phenotype and to restore mitochondrial protein synthesis.

Conclusion: These data demonstrate the isolation of two pCV108-transformant recombinant clones containing a human gene that complements the Chinese hamster Gal$^-$32 mutation and restores galactose metabolism.

Keywords: Chinese hamster; Gal 32; Cosmid vector; HeLa cell

Abbreviations: Cam^r – Chloramphenicol resistance; CHL – Chinese Hamster Lung; CO – Cytochrome Oxidase; CR – Recombinant Clone; CsCl – Cesium Chloride; GAL – Galactose; Gal$^+$ gene – confers ability to metabolize galactose; Gal$^-$ 32 – Galactose-deficient (inability to metabolize galactose); LB-Luria Broth; ND – NADH dehydrogenase; MXHAT - complete medium containing 78 mM mycophenolic acid (M), 1.6 mM xanthine (X), 0.1 mM hypoxanthine, 4.5 μM aminopterin(A), 40 μM thymidine; Mt – Mitochondrial; pCV108 – cosmid vector containing the *amp*^{r} and SV-*neo* genes; pSV2-*neo* – plasmid containing the gene that confers resistance to the neomycin analog G418; pSV13 – cosmid vector containing genes for chloramphenicol resistance (*cam*^{r}) and xanthine-guanine phosphoribosyl-transferase (SV2-*gpt*); TR – Transformant

Introduction

Gal$^-$32 is an unusual Chinese hamster lung (CHL) cell mutant that lacks the ability to utilize exogenous galactose or fructose in place of glucose due to a single, recessive mutation that causes deficiencies in the mitochondrial (mt) respiratory chain [1,2]. Cells in culture growing in galactose or fructose obtain nearly all of their energy from glutamine via the Krebs cycle, the mt electron transport chain, and oxidative phosphorylation [3]. Therefore, cells that are defective in oxidative energy production by the mt electron transport chain fail to grow in galactose or fructose.

In order to grow or proliferate, cells must comply with the energy demand imposed by vital processes that include macromolecule biosynthesis, DNA replication, ion gradient generation and cell structure maintenance. Mitochondria, in general, play an important role in energy metabolism as they synthesize most of the cellular ATP through oxidative phosphorylation [4].

The mitochondrial genome encodes only 13 polypeptides, all of which are mitochondrially synthesized respiratory complex units [5,6]. However, most mitochondrial proteins, including most respiratory complex subunits, are nuclearly encoded and cytoplasmically synthesized. In Gal$^-$32, the levels of mitochondrially synthesized proteins are all decreased to different extents unrelated to protein size; furthermore, there is a direct correlation between the activities of the respiratory chain and their corresponding mt translation products [7]. For example, NADH dehydrogenase (ND) activity and levels of mt encoded subunits ND 3, 4, 4L, and 5 are greatly reduced. Similarly, cytochrome *c* oxidase (CO) activity and mt synthesized subunits CO I, II, and III are drastically decreased. On the other hand, ATPase activity and mt encoded subunits 6 and 8 are only slightly altered. Likewise, succinate-cytochrome *c* activity, which is dependent on cytochrome *b*, and mt encoded apocytochrome *b* are marginally reduced. This differential reduction in mitochondrially synthesized proteins is a unique property of Gal$^-$32 compared to other mammalian cell and yeast mutants, which usually exhibit no mt translation products [8-10].

Experiments investigating protein degradation as well as steady state levels and sizes of mt transcripts have revealed that the primary defect in Gal$^-$32 is not caused by increased differential degradation of mitochondrial translation products, increased differential degradation of mtRNAs, or less efficient processing of the polycystronic precursor mtRNAs. Surprisingly, the steady state levels of both heavy and light strand mtDNA transcripts were elevated in Gal$^-$32. Therefore, the differential reduction in mitochondrially encoded protein in Gal$^-$32 seems to be the result of a decreased translation of specific mRNAs [11].

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The differential decrease in mitochondrially synthesized proteins in Gal$^-$32 is due to a single mutation as evidenced by the restoration to near normal levels of all mitochondrially encoded proteins and the respiratory complex activities in a spontaneous revertant that has regained the ability to grow on galactose. Using somatic cell hybrids, we have conclusively demonstrated the nuclear origin of the Gal$^-$32 mutation. Fusion of rhodamine-6G-treated Gal$^+$ cells with Gal$^-$32 cells resulted in tetraploid hybrids that grew in galactose; this is expected for a nuclearly encoded gene since rhodamine-6G specifically inactivated mtDNA.

Since the product of the Gal$^-$32 gene is unknown, it is necessary to use phenotypic complementation to clone the gene that corrects the Gal$^-$32 mutation. The dominant wild type Gal$^+$ 32 gene was transferred into recessive Gal$^-$32 cells and phenotypic expression of the wild-type gene was selected for by growth of the transformants in galactose. In this paper, we report the successful isolation of two genomic cosmid clones containing a human gene that complements the Chinese hamster Gal$^-$32 mutation. A preliminary report of this data was presented at a FASEB meeting [12].

Materials and Methods

The Howard University IRB (Internal Review Board) approved the use of the cell lines described herein and determined the protocol to be exempt based on *45 CFR 46.101(b)(4)* and involves minimal risk. The human gene used in this study was derived from the HeLa cell line, which is available commercially and consent was not necessary.

Cell lines and plasmids

The Gal$^-$32 cell line was generously provided by Dr. EH Chu (1). Antibiotics were supplied by BRL (Bethesda, MD). All restriction enzymes were provided by New England Biolabs, Ipswich, MA. Cells were cultured at 37°C in the presence of 5% CO_2. Complete growth medium is GIBCO α-minimal essential medium (GIBCO, now Life Technologies, Bethesda, MD) containing 292 mg/L of glutamine, 17.9 mM NaHCO3, 8.4 mM NaCl, 3-5% heat-inactivated fetal calf serum, 37 μM hypoxanthine (H), 21 μM thymidine (T), and 22 mM D-glucose. Galactose medium is complete medium with 22 Mm galactose in place of glucose and 3-5% dialysed fetal calf serum (BRL, now Life Technologies, Bethesda, MD) (unless otherwise noted) in place of undialysed serum. MXHAT (BRL, now Life Technologies, Bethesda, MD) medium is complete medium containing 78 mM mycophenolic acid (M), 1.6 mM xanthine (X), 0.1 mM hypoxanthine, 4.5 μM aminopterin (A), 40 μM thymidine and no uridine[13]. G418 (BRL, now Life Technologies, Bethesda, MD) medium is complete medium plus 400 or 800 μg/ml G418 (GIBCO, ~50% pure) [14].

The human cosmid library and the pSV13 cosmid vector in *Eschrechia coli* Hb101 were generously donated by Dr. Mary McCormick [15]. pSV13 contains genes for chloramphenicol resistance (*cam*^{r}) and xanthine-guanine phosphoribosyl-transferase (SV2-*gpt*); the human (HeLa) genomic DNA (average size 40 kb) was inserted into the single *Pst*I site of pSV13. The pSV2-*neo* plasmid containing the gene that confers resistance to the neomycin analog G418 was constructed by Drs. P. J. Southern and P. Berg. The cosmid vector pCV108, containing the *amp*^{r} and SV-*neo* genes, was generously supplied by Dr, Y.-F. Lau [16]. The Blur-8 clone, constructed by Deininger et al. [17], was kindly donated by Dr. Antonio Fojo. *E. coli* strain Sure-1 {*mcr*A, Δ[*mcr*CB-hsdSMR-*mrr*] 171, *sbc*C, *rec*B, *rec*J, *umu*C::Tn5 (*kanr*), *uvr*C, *sup*E44, *lac*, *gyr*A96, *rel*A1, *thi-1*, *end*A1 [F' *pro*AB, laclqZΔM15, Tn10, (tetr]} was obtained from Stratagene (La Jolla, CA).

Gene transfer

Gene transfer was performed using slight modifications of the $CaPO_4$-DNA (Sigma, St. Louis, MO) precipitation methods of Abraham et al. and Chen and Okayama [18-20]. Gal$^-$32 cells, at density of 5 x 10^5 / 100 mm dish, were incubated overnight in 10 ml of complete medium, then exposed to a mixture of $CaPO_4$-DNA for 6.5 h (Table 1) or 15 h (Table 3) with 5% CO_2(Table 1, experiments 1 and 2)or 3% CO_2(Table 1, experiment 3; Table 3). The $CaPO_4$-DNA precipitate was prepared with 10 μg/100 mm dish (Table 1) or 20 μg/100 mm dish (Table 3) of each purified DNA and 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, BRL, Bethesda, MD) pH 7.12 (17) (Table 1) (experiments 1 and 2), Hepes, pH 6.95 [19, 20](Table 1, experiment 3), or N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), pH 7.0 (Table 3). After washing the cells with complete medium (Table 1) or phosphate-buffered saline (Table 3), the cells were incubated in complete medium for 40 h (Table 1, experiments 1 and 2), 72 h (Table 1, experiment 3), or 24 h (Table 3) and then re-plated.

DNA purification

For large plasmid purifications, bacteria containing plasmids were grown in Super Broth for 24 – 48 h or in Luria-Bertani medium (LB) plus antibiotic and amplified with chloramphenicol [21]. Plasmids

Table 1: Transformation of Gal$^-$ 32 cells with human genomic library DNA.

Transforming DNA	Transformation frequency(colonies per 10^6 original cells)[a]			
	G418	MXHAT	GAL	GAL+G418
Experiment 1				
Human library + pSV2-*neo*	71	126	2.3	-[b]
pSV2-neo		-	0.33	-
Experiment 2 (yielding Primary Transformants)				
Human library + pSV2-*neo*	135	408	-	2.7
No DNA + $CaPO_4$	-	-	<0.5	-
Experiment 3 (yielding Secondary Transformants)				
Primary Transformant-4 + pSV2-*neo*	102	-	-	1.2
No DNA + $CaPO_4$	-	-	140	-

[a]Selection in medium with 5% fetal calf serum as follows: G418:5x10^5 cells/100 mm plate in 800 μg/ml G418 medium. MXHAT: 5x10^5 cells/100 mm plate in MXHAT medium. GAL: 10^6cells/100 mm plate In galactose medium with undialysed serum. GAL +G418; 10^6 cells/100 mm plate in galactose medium with undialysed serum plus 800 μg/ml G418.
[b] _ Not determined.

Table 2: Plating efficiency of Gal$^+$ 32 transformants in different media.

Cell Line[a]	Media[b]		
	G418	MXHAT	GAL
Relative plating efficacy(%)[c]			
Wild Type	<0.0001	<0.0001	100
Gal$^-$32	<0.0001	<0.0001	<0.0001
Primary Transformant-1	91	6.7	108
Primary Transformant-4	77	8.5	100
Secondary Transformant-1	87	<0.001	92
Secondary Transformant-6	79	<0.001	105

[a] Transformants from GAL +G418 plates were picked into G418 (800 μg/ml) medium
[b]media as described in Table 1 except that 5% dialysed Serum was used tor galactose medium.
The number of colonies in selective medium divided by the number of colonies in the same media without selective agent x 100: galactose medium is compared to the same medium with glucose; 250 to 10^6 cells were seeded per 60 mm plate. Colonies were stained with 0.5% methylene blue in 50% ethanol.

Table 3: Transformation of Gal⁺32 cells with DNA from pCV108-transformant recombinant clones.

Transforming DNA[a]	Total no. of original cells (millions of cells)[b]			Transformation frequency (colonies per 10^6 original cells)			
	G418	GAL+G418	GAL	G418	GAL+G418	GAL	GAL/G418
No DNA	_c	_	2	_	_	<0.5	_
NO DNA							
+ $CaPO_4$	1	1	2	<1.0	<1.0	<1.0	_
4CR-2	3	3	1	19.3	2.0	4.0	0.21
10CR-1	1	1	1	378	6.0	8.0	0.02
12CR-2	2	2	1	217	<0.5	<1.0	_
13CR-2	2.5	2	1	289.6	<0.5	<1.0	_
14CR-2	2	2	1	382.5	<0.5	<1.0	_
16CR-1	2	2	1	79.5	<0.5	<1.0	_
16CR-2	1	2	1	107	<0.5	<1.0	_
20CR-3	2	2	1	191	<0.5	<1.0	_
pSV2-*neo*	2	1	1	166.5	<1.0	<1.0	_

[a]plasmid DNA from individual Alu-hybridizing, chloramphenicol resistant pCV 108- transformant clones.
[b] cells were plated at $5x10^5$/100 mm dish for 24 hours in complete medium then changed to selective medium. Selective media was: G418: 400μg/ml G418 medium: GAL+G418: galactose medium with 3% dialysed fetal calf serum plus 400μg/ml G418:GAL: galactose medium with 3% dialysed fetal calf serum. Selective media were changed twice and stained or picked after 10 days of incubation. Plating efficacy in glucose was 50-100%.
[c] _ not determined.

purified with Qiagen (Germany) columns were grown in Terrific Broth. Plasmid DNA used for gene transfer, for probes, and for construction of the primary transformant library were purified either by double banding in a CsCl-ethidium bromide density gradient or with Qiagen 500 columns. The human genomic cosmid library in host *E. coli* HB101 was grown in Super Broth plus cholrampheicol at 30 μg/ml for 24 h and purified by double banding in a CaCl-ethidium bromide density gradient [22].

For plasmids mini preperations, the alkaline lysis method was used to purify pCV108-transformant recombinant cosmid DNA from *cam*ʳSure-1 bacteria, grown in LB plus chloramphenicol at 30 μg/ml [21].

Primary transformant genomic DNA that was used for gene transfer to yield secondary transformants was purified according to the method of Abraham et al.

Probe preparation

The human *Alu* probe was isolated by digestion of the purified Blur-8 plasmid DNA with *Bam*HI, agarose gel electrophoresis, and electroelution of the 300 bp insert from the gel [21]. Similarly, the 560 bp*cam*ʳ probe was isolated following digestion of the purified pSV14 plasmid DNA with *Eco*RI and *Sal*I [21]. The probes were labeled with [α-^{32}P]dCTP (DuPont) using a nick translation kit or random primer kit (Bethesda Research Laboratories (BRL), Bethesda, MD).

Southern blotting and hybridization

DNA digestion with restriction endonucleases was separated by 0.8% agarose gel electrophoresis in 89 mMTris borate-2 mM EDTA buffer and blotted to GeneScreen membranes (New England Nuclear Corp., Boston, MA) [23]. The Gene Screen blot was hybridized in 50% formamide at 42°C with the ^{32}P-labeled probes as previously described [23].

To eliminate background with the genomic DNA blots, the heat-denatured (95°C, 5 min) radioactive probe was prehybridized to a sheet of blank GeneScreen for 2-3 h in the hybridization solution. The prehybridized probe was denatured at 80°C for 10 min immediately before addition to the DNA blot. This step was omitted for hybridization to the pCV108-transformant recombinant plasmid DNA.

After hybridization for 16-24 h, the membrane was washed with constant agitation as follows: 1) three times with 330 ml of 2X SSPE (0.36 M NaCl, 20 mMNaPi, pH 7.4, 2 mM EDTA) and 0.1% SDS (Sigma, St. Louis, MO) at room temperature for 10 min; 2) twice with 250 ml of the 2X SSPE and 1% SDS at 60°C for 20 min; and 3) three times with 330 ml solution 0.1X SSPE and 0.1% SDS for 20 min at 52°C for the *Alu* probe and at 65°C for the *cam*ʳ probe. The blots were exposed to Kodak XRP-5 film (Kodak, NY) at -70°C until the bands were visible. The probe was removed by incubating the GeneScreen membrane in a final solution of 0.02 M Tris-HCl, (Sigma) pH 7.0, 0.01 M EDTA and 96% (v/v) formamide (Fisher Scientific, Hanover Park, IL) for 1 h at 80°C.

Construction of a Gal⁺ transformant genomic DNA library

A genomic library was constructed from primary Gal⁺transformant-3 (1-TR-3) in the cosmid cloning vector, pCV108. The library was screened by replica-plating on plates containing chloramphenicol.

Preparation and partial digestion of high molecular weight genomic DNA

For each genomic DNA preparation, seven T-150 cm^2 tissue culture flasks were employed to grow 7.0 x 10^7 1-TR-3 cells in galactose and MXHAT media. High molecular weight genomic DNA was purified by CsCl-ethidium bromide (density gradient centrifugation following the protocol of Fleischmann et al.) The size of the DNA was analyzed by electrophoresis in a 0.3% (v/w) agarose gel using oligomers of bacteriophage lambda DNA as markers (BRL, Bethesda, MD). The purified high molecular weight genomic DNA (average size, 150 kb) was partially digested with the restriction enzyme *Mbo*I that recognizes a 4-bp sequence. This partial digestion yields overlapping fragments and cohesive ends which anneal with the *Bam*HI digested pCV108 vector. Analytical pilot experiments were performed varying the time of *Mbo*I digestion. The time of digestion that gave the brightest staining band of DNA between 23 and 50 kb was determined and half of this time was then used in the preparative digestion procedure as described by Seed et al. [24]. Therefore, 40 μg of genomic DNA was digested with20U of *Mbo*I for 1.0 min at 37°C. The resulting fragments were phenol-extracted, ethanol-precipitated, and size-fractioned by 10- 40% sucrose density gradient centrifugation. Following analytical electrophoresis in a 0.3% agarose gel, 35-45 kb gradient fractions were pooled and used for construction of the primary transformant cosmid library.

Digestion and dephosphorylation of vector DNA

The pCV-108 cosmid vector containing the SV2-*neo* and *amp*^{r} genes was used to construct the primary transformant genomic DNA library. Purified pCV108 DNA was digested to completion with *Bam*HI, phenol-extracted, and ethanol-precipitated. The linearized vector DNA was dephosphorylated with calf intestinal alkaline phosphatase[16,21].

DNA ligation

In order to determine an optimal set of conditions for ligation between vector and genomic DNA, a series of test ligation reactions at different concentrations of DNA were performed. The optimal results were obtained with a molar ratio of 9:1 (weight ratio of 2:1) of vector DNA (8.9 kb, *Bam*HI/phosophatase-treated pCV108) to genomic DNA (35-45 kb, *Mbo*I/sucrose-fractioned 1-TR-3). The ligation of the DNA fragments possessing cohesive ends was accomplished using the procedure of DiLella and Woo except that polyethene glycol was omitted from the ligation buffer. The reactions were incubated at 14°C for 14-16 h and the products were analyzed by electrophoresis in a 0.2% agarose gel [25].

In vitro packaging

In vitro packaging of the ligated recombinant DNA or the vector DNA (control reaction) was performed with bacteriophage λ packaging kits from Stratagene or Bethesda Research Laboratories according to the specifications of the manufacturers with a slight but critical modification: The DNA was added to a mixture of the two packaging extracts by thawing the freeze-thaw-lysate (10µl), immediately adding it to the frozen sonicated extract (15 µl), within 5-7 s pipetting in the ligated DNA (vector + genomic, 595 ng in 2.66 µl), and quickly stirring the packaging reaction with the tip of the pipette. The packaging mixture was immediately centrifuged for 5 s and incubated at 22°C for 2 h. The immediate addition of the recombinant DNA to the extracts and the temperature of incubation are very crucial to the success of the packaging reaction.

Transduction of packaged DNA and screening the library

After tittering, the library was plated at high density on LB plates containing ampicillin and screened by replica plating on LB plates containing chloramphenicol.

For transduction, Sure-1 bacteria (Stratagene, La Jolla, CA) were grown in LB medium containing 0.4% maltose and 10mM $MgCl_2$ at an OD_{600} of 0.5 (Stratagene procedure). The cosmid library was tittered by mixing an aliquot of the packaging reaction with the Sure-1 bacteria and spreading on LB agar plus ampicillin (50 µg/ml) plates (150 mm) that were overlaid with detergent-free nitrocellulose filters (Millipore, HATF) [21]. For the large scale transduction, a mixture of the packaging reaction and Sure-1 bacteria were spread at high density (50,000 colonies/150 mm dish) on twenty LB plus ampicillin plates overlaid with nitrocellulose filters. The plates were incubated at 37°C until tiny (0.1-0.2 mm) colonies appeared in 8 to 12 h. The colonies on the LB plates containing ampicillin were replicated onto LB plus chloramphenicol (30 µg/ml) or ampicillin plates that were overlaid with nitrocellulose filters [21,26].

The master filter was stored on LB agar plates containing ampicillin and 25% glycerol at -70°C. The remainder of the packaging reaction containing the cosmid DNA library was stored in 7% (v/v) dimethyl sulfoxide at -70°C. Before further analysis, the *cam*^{r} recombinant colonies were purified by spreading on LB plates containing chloramphenicol and picking single colonies.

Results

Transfer of the human wild-type Gal$^+$32 gene into Gal$^-$32 cells

The objective of this study was to clone the wild type human gene that complements the Gal$^-$32 mutation. Gal$^-$32 cells were co-transformed with pSV2-*neo* plasmid DNA [13] and recombinant DNA from a human genomic library [15] containing the dominant human Gal$^+$ gene. The pSV13 cosmid vector used in construction of the human library carries the SV2-*gpt* gene, which permits mammalian cells to grow in MXHAT [13], as well as *cam*^{r} gene, which enables bacterial cells to grow in chloramphenicol. The dominant SV2-*neo* gene allows mammalian cells to grow in the neomycin analog G418. The frequency of transfer of the SV2-*neo* gene (7.1-13.5 in 105 cells in G418) and the SV2-*gpt* gene (1.26-4.08 in 104 cells in MXHAT) was high, as expected for introduction of a cloned gene (Table 1). In experiment 1, Table 1, Gal$^-$32 yielded spontaneous CHL Gal$^+$ revertants with pSV2-*neo* at the usual frequency of 0.33 in 10^6 cells. However, Gal$^+$ transformants with the human genomic library appeared at a 7-fold higher frequency than spontaneous revertants (2.3 compared to 0.33 in 10^6 cells in galactose, Table 1, experiment 1).

Primary Gal$^+$ transformants were isolated by transferring human genomic library DNA and pSV2-*neo* DNA into Gal$^-$32 cells and selecting for growth in galactose plus G418. The frequency of isolation of primary transformants (2.7×10^{-6}, Table 1, experiment 2) was 10^5 times higher than that (0.5×10^{-6} multiplied by 135×10^{-6}) expected for a CHL Gal$^+$ revertant that incorporated the SV2-*neo* gene.

Secondary transformants were isolated by transferring primary transformant-4 DNA and pSV2-*neo*DNA into Gal$^-$32 cells and selecting for growth in galactose plus G418. Although some CHL Gal$^+$ revertants may have also been present, the frequency (1.2×10^{-6}, Table 1, experiment 3) of isolation of secondary Gal$^+$ transformants in galactose and G418 was 100 times higher than that (102×10^{-6} multiplied by 140×10^{-6}) expected for a CHL Gal$^+$ revertant that incorporated the SV2-*neo* gene.

Plating efficiency of transformants

Isolated colonies of primary transformants exhibited high relative plating efficiency (77-100%) in galactose or G418 and low relative plating efficiency (7-9%) in MXHAT (Table 2). Survival in MXHAT indicates that the primary transformants contain the unselected SV2-*gpt* gene, which is located in the cosmid vector used to construct the human genomic library. Isolated colonies of secondary transformants also demonstrated high relative plating efficiency (79-100%) in galactose or G418. About half of the secondary transformants initially grew in MXHAT, but did not grow in MXHAT after expansion and further testing (Table 2). The human Gal$^+$ was based on (1, growth in MXHAT, which was unselected, and (2, the frequency of isolation of primary and secondary transformants growing in galactose + G418 compared to that expected for CHL Gal$^+$ revertants growing in these media.

Detection of human *Alu* and bacterial *cam*^{r} DNA sequences in the primary and secondary transformants

In order to confirm that the Gal$^-$32 mutation was corrected by the Gal$^+$ gene derived from the human cosmid library, the presence of human and cosmid vector DNA sequences in the transformant here analyzed. Genomic DNA from the putative primary and secondary transformants was digested with restriction enzymes, electrophoresed, and transferred to membranes to test for hybridization to the bacterial

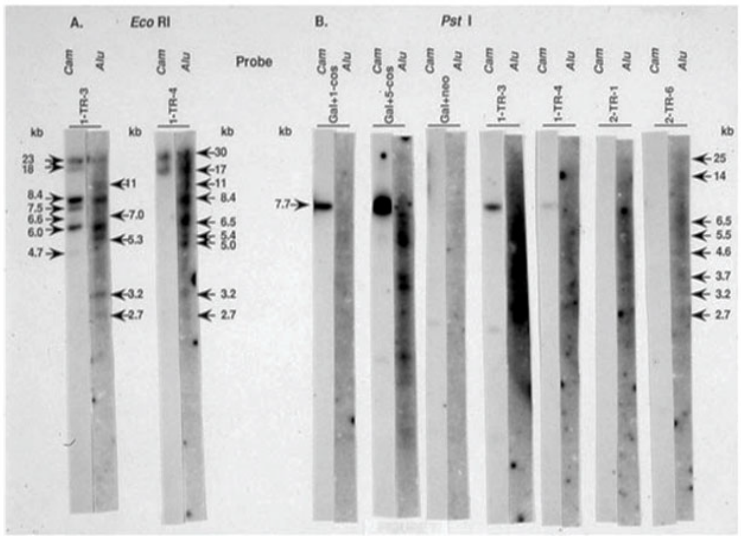

Figure 1: Southern blot of digested primary transformant (1-TR), secondary transformant (2-TR), Gal⁻ 32 (Gal), human genomic cosmid library (cos), and pSV2-*neo* (neo) DNA hybridized with human *Alu* and bacterial *cam*^{r} probes.
*Eco*RI (A) or *Pst*I (B) -digested DNA, 10 µg, was electrophoresed in 0.8% agarose gel, blotted onto GeneScreen, hybridized to the ^{32}P-labeled human *Alu* probe (Blur-8) and autoradiographed. After removal of the *Alu* probe, the same blot was hybridized to the ^{32}P-labeled *cam*^{r} probe and autoradiographed. Gal + 1-cos: 10 µg Gal⁻ 32 DNA (3 x 10^6 kb) and 160 pg of human cosmid library DNA (48 kb; one copy per Gal⁻ 32 genome). Gal + 5-cos: 10 µg Gal⁻ 32 DNA and 800 pg of human cosmid library DNA (five copies per Gal⁻ 32 genome). Gal + neo: 10 µg Gal⁻ 32 DNA and 93 pg of pSV2-*neo* DNA (5.6 kb; five copies per Gal⁻ 32 genome).

cam^r and human *Alu* probes (Figure 1). The *cam*^{r} gene is present in the pSV13 vector used to construct the human genomic library [15]and may still be linked to the human Gal$^+$ gene in the transformants. The highly repeated human *Alu* sequences, which occur every 2-3 kb in the human genome [27], were also expected to be linked to the human Gal$^+$ gene.

The Gal$^+$ transformants hybridized to both the human *Alu* probe and the bacterial *cam*^{r} probe (Figure 1). As expected, both probes hybridized to the human cosmid library (Figure 1B, Gal + 1-cos, Gal + 5-cos) and did not hybridize to the CHL DNA nor the pSV2-*neo* DNA (figure 1B, Gal + neo). The primary transformants (1-TR-3 and 1-TR-4) and the secondary transformants (2-TR-1 and 2-TR-6) hybridized to the human *Alu* probe, indicating the presence of human DNA. It is of interest that the primary transformants, 1-TR-3 and 1-TR-4, have the following *Alu*-hybridizing, *Eco*RI fragments in common: 11 kb, 8.4 kb, 5.3 kb, 3.2 kb, and 2.7 kb.

The primary transformants also hybridized to the *cam*^{r} probe, demonstrating the presence of the pSV13 vector. During gene transfer, a large concatemer of unlinked DNA is formed and the integrated into the chromosomal DNA [18]. Since the *cam*^{r} probe is an *Eco*RI-*Sal*I fragment derived from pSV13, each integrated cosmid copy was expected to produce a single band after hybridization of this probe with *Eco*RI-digested genomic DNA from the transformants. With the *cam*^{r} probe seven bands were observed with *Eco*RI-digested 1-TR-4 (Figure 1A). Furthermore, in the *Eco*RI-digested primary transformants, multiple *Alu*-hybridizing bands which have the same mobility as *cam*^{r}-hybridizing bands (23 kb, 8.4 kb, 6.0 kb, in 1-TR-3; 30 kb, 17 kb in 1-TR-4) were observed.

*Pst*I digestion of the human cosmid library or the primary transformants yielded a 7.7 kb fragment that hybridized to the *cam*^{r} probe (Figure 1B, Gal + cos, 1-TR-3, 1-TR-4). This fragment corresponds to the linear, intact pSV13 vector since the human genomic DNA was inserted into the unique *Pst*I site of the pSV13 vector. The intensities of

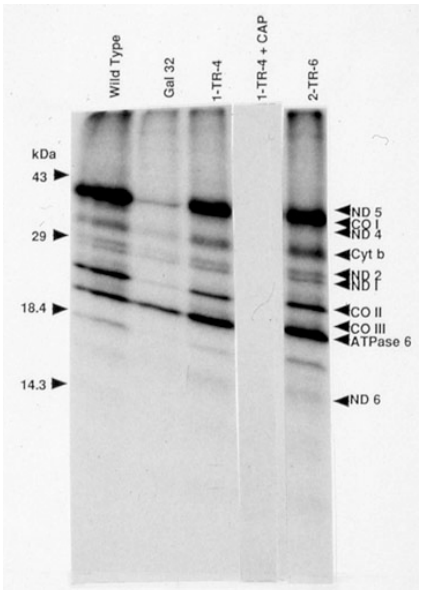

Figure 2: Mitochondrial translation products after SDS-urea-10-18% polyacrylamide gel electrophoresis and fluorography.
Mitochondrially synthesized proteins were labeled for 1 h with ^{35}S-methionine in the presence of cycloheximide, then chased with cold methionine for 0.5 h (lanes 1-3 and 5); chloramphenicol (CAP) was added 6 h before and during labeling in the presence of cycloheximide (lane 4). Mitochondria were isolated, solubilized, electrophoresed and flourographed [7]. Fifty µg of mt proteins were loaded in all lanes except in lane 1 where 26 µg were loaded. Gels were standardized using the BRL ^{14}C-labeled low molecular weight standards. Proteins corresponding to cytochrome oxidase (CO) I, II, III, ATPase 6, and apocytochrome b were identified as previously published [7]. NADH dehydrogenase (ND) subunits are tentatively assigned based on size in these gels compared to the work of Chomyn *et al.*,[5] and on mobility in a nonequilibrium pH gradient [7], compared to the pI of the mouse proteins [36]. ND 5 and CO I are difficult to separate and migrated as single bands as previously observed [7], Figs 1*H* and 2).

the 7.7 kb *cam*^{r} bands suggests that there is approximately one copy of the pSV13 vector (Gal +cos) per genome in 1-TR-3 and much less than one copy in 1-TR-4. Even though the *cam*^{r} probe did not detect any bands in *Pst*I-digested secondary transformants (Figure 1B, 2-TR-1, 2-TR-6), in another experiment not shown, a *cam*^{r} band was observed in *Eco*RI-digested secondary transformant-1.

Mitochondrial protein synthesis in transformants

To verify that the defect in mitochondrial protein synthesis was corrected in the putative Gal$^+$transformants, cells were labeled with [^{35}S]-methionine in the presence of a cytoplasmic protein synthesis inhibitor (cycloheximide), followed by the isolation of mitochondria and analysis of the resulting labeled proteins on SDS polyacrylamide gels (Figure 2). The absence of all labeled proteins (Figure 2, 1-TR-4 + CAP) in the presence of cycloheximide and chloramphenicol, a specific inhibitor of mt protein synthesis, indicates that the proteins labeled in the presence of cycloheximide are indeed mitochondrially synthesized. The intensity of the bands and the mobility pattern of the proteins in the primary and secondary transformants were similar to those of the wild-type, whereas a significant reduction in the level of the same proteins (except ATPase 6) was observed in Gal$^-$32 (Figure 2). These results demonstrate that all the mitochondrially synthesized proteins that were reduced in the mutant are restored to normal wild-type levels in both primary and secondary transformants. Considering the gene transfer, hybridization, and mt protein synthesis data, we conclude that a human gene is present in both the primary and secondary transformants and is capable of correcting the Chinese hamster Gal- 32 mutation.

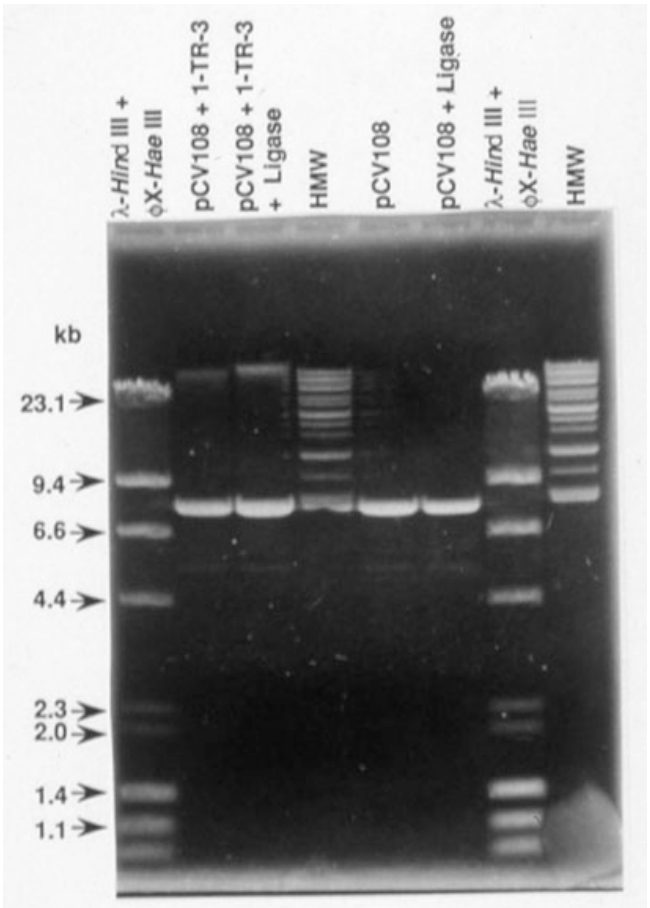

Figure 3: Preparative ligation between cosmid vector (pCV108) and primary transformant genomic DNA (1-TR-3) at a molar ratio of 9:1.
The *Mbo*I-digested/sucrose gradient-fractionated 1-TR-3 DNA (35-50 kb, 1.5 µg = 0.055 pmol) was ligated to the *Bam*HI/phosphatase-treated vector DNA (8.9 kb, 3µg = 0.5 pmol) in the presence of T4 DNA ligase (16 Weiss units). Aliquots of the ligation products were analyzed by 0.2% agarose gel electrophoresis and stained with ethidium bromide. As size markers, high molecular weight DNA standards and *Hind*III-digested λ and *Hae*III-digested ΦX DNAs were used.

Construction of a primary transformant cosmid library

Since primary transformant-3 exhibits multiple *Eco*RI restriction fragments that hybridize to both the human *Alu* probe and to the *cam*^{r} probe (Figure 1), it is likely that the human DNA is still linked to the *cam*^{r} gene as in the pSV13-human cosmid DNA library used to obtain the primary transformant. Therefore, a genomic DNA library was constructed from primary Gal$^+$ transformant-3 and the cosmid cloning vector, pCV108. High molecular weight DNA (>150 kb) was purified from primary transformant-3 and partially digested with *Mbo*I. *Mbo*I genomic DNA fragmetns of 35–50 kb were isolated from a sucrose density gradient. The pCV108 cosmid vector was completely digested with *Bam*HI to linearize it and to produce ends complementary to the *Mbo*I-digested genomic DNA. The digested vector was treated with alkaline phosphatase to remove the 5'-terminal phosphate and thereby suppress self-ligation. None of the phosphatase-treated, *Bam*HI-digested pCV108 changed mobility after incubation with T4 DNA ligase, indicating the absence of self-ligation (Figure 3, pCV108 + ligase). When the *Mbo*I-digested, size fractioned, primary transformant-3 DNA was ligated to the *Bam*HI-digested, dephosphorylated pCV108 vector, all of the transformant DNA was converted to a larger size DNA (Figure 3, pCV108 + 1-TR-3 ± ligase). Since there was a nine to one molar excess of vector DNA to genomic DNA, most of the vector DNA did not yield a larger size DNA. The recombinant DNA was packaged into bacteriophage λ particles, and transduced into *E. coli*.

Cam^r gene identifies primary transformant clones that contain human DNA

The primary transformant cosmid library was plated on ampicillin to select for the presence of the pCV108 vector, which contains the *amp*^{r} gene. The library was replica-plated onto chloramphenicol because the *cam*^{r} gene is present in the primary transformant and is likely to remain linked to the human DNA. The total library obtained was 1.9 x 10^6*amp*^{r} colonies. Only half of these colonies were replica-plated on chloramphenicol since this number would be more than enough for a 99% probability of isolating a single copy gene [14]. There was an average of 7 *cam*^{r} clones per 50,000 *amp*^{r} colonies isolated. The total number of *cam*^{r} clones recovered was 144 from 9.5 x 10^5*amp*^{r} colonies. Based on the average size of the insert (3 x 10^4 base pairs) and the mammalian genome size (3 x 10^{9}base pairs), this corresponds to approximately 10 copies of the *cam*^{r} gene per genome. The average size of the DNA inserts from 32 *cam*^{r} recombinant clones was 35 kb.

These *cam*^{r} colonies were digested with *Eco*RI, electrophoresed, transferred to GeneScreen, and hybridized to a ^{32}P-labeled *Alu* probe. Of the 32 pCV108-transformant recombinant plasmids digested with *Eco*RI, all exhibited some DNA fragments of approximately the same size that were not vector fragments. After Southern blotting, 28 of these 32 plasmids hybridized to a human *Alu* probe at a location or intensity different from that of the vector; 10 of these exhibited very distinct *Alu*-hybridizing signals. Figure 4 shows eight of these *cam*^{r} recombinant clones. The *Alu* probe also hybridized very weakly with the *Eco*RI-released cosmid vector (~8.9 kb). Since the vector was released intact

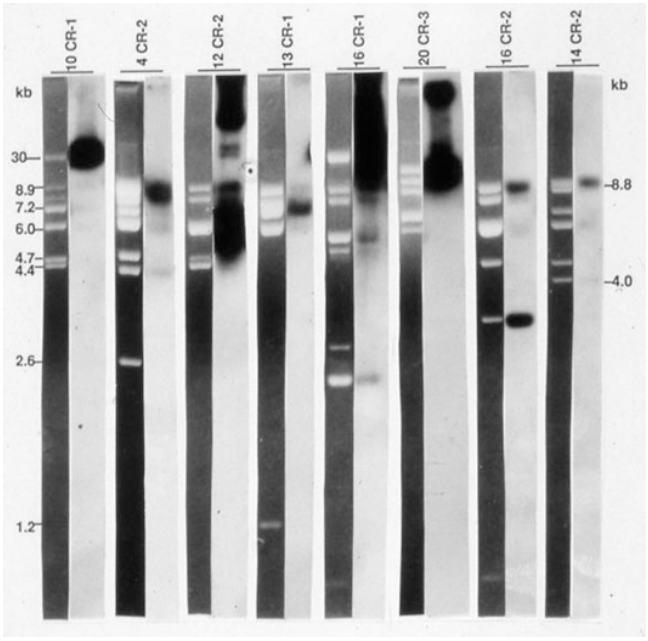

Figure 4: Southern blot of DNA from individual *cam*^{r} pCV108-transformant recombinant clones hybridized with a human *Alu* probe. pCV108-transformant plasmid DNA was purified by the alkaline lysis method for minipreparations. *Eco*RI-digested plasmid DNA was electrophoresed in 0.8% agarose gel, stained with ethidium bromide (left lane), blotted onto GeneScreen, hybridized to ^{32}P-labeled human *Alu* DNA, and autoradiographed (right lane). The intact, linear pCV108 vector is present at 8.9 kb. λ-HindIII, ΦX 174-*Hae*III, Sp6-*Kpn*I/*Hind*III DNAs were used as size markers (not shown).

(as evidenced by its size) by *Eco*RI digestion, its weak hybridization with the *Alu*probe may be attributed either to the presence of short *Alu*-like sequences within its DNA structure or contamination of the probe with pBR vector sequences.

Gal$^-$32 mutation is complemented with DNA from pCV108-transformant recombinant clones

The identification of the recombinant clone that corrects the Gal$^-$ 32 mutation ultimately depends on gene transfer since the product of the gene is not known. To identify which one of the *Alu*-hybridizing, *cam*^{r} recombinant cosmid clones contains the Gal$^+$ 32 gene, purified DNA from each recombinant clone was used to transform Gal$^-$32 mutant cells selecting for growth in GAL or GAL + G418. Even though the efficiency of transformation was high with all eight clones for the SV2-*neo* gene (G418 selection), only two of the recombinant clones, 10CR-1 and 4CR-2, enabled the mutant to grow in GAL or GAL + G418 (Table 3). Eight and four colonies per 10^6 cells were recovered in GAL with 10CR-1 and 4CR-2, respectively. Similarly, in the double selection (GAL + G418) three times more colonies were retrieved with 10CR-1 than with 4CR-2.

In case that expression of the Gal$^+$ gene was extremely low, the G418-resistant transformant colonies for each cosmid clone were also pooled, grown in complete medium, and plated at different cell densities in GAL or GAL and G418 media. As a negative control for the presence of human DNA inserts, the pSV2-*neo*-transformants were cultured as well. The same results were obtained as in Table 3; only 4CR-2 and 10CR-1 produced transformants in GAL and GAL + G418 (data not shown).

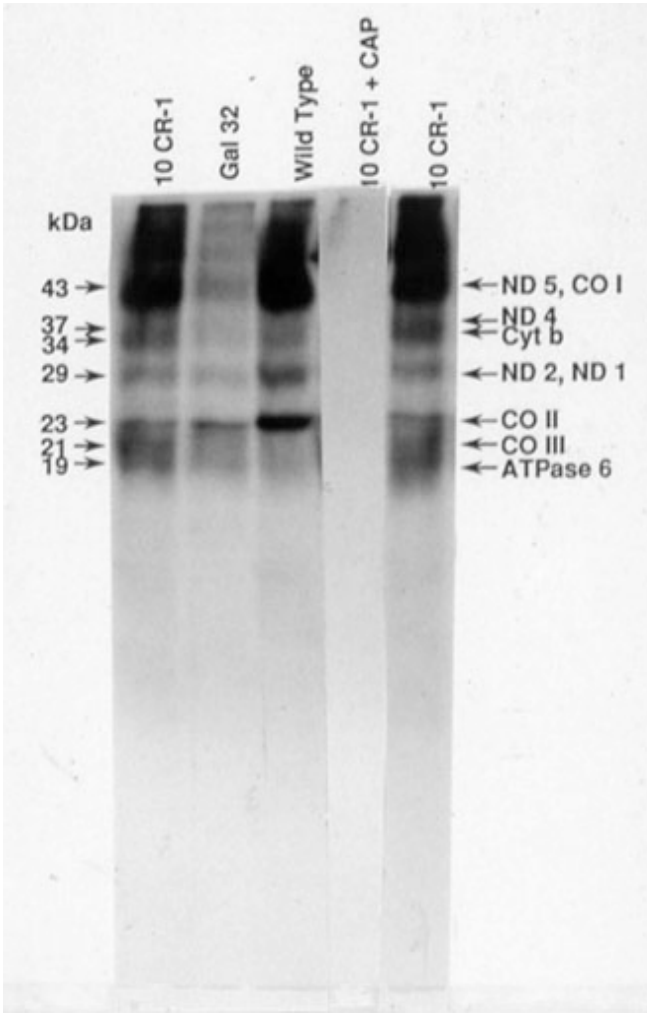

Figure 5: Mitochondrial translation products of 10 CR-1 after SDS-urea-10-18% polyacrylamide gel electrophoresis and fluorography. Mitochondrially synthesized proteins were labeled as described in Figure 2. Thirty-three µg of mt proteins were loaded in all lanes.

Mitochondrial protein synthesis is restored in Gal$^-$32 complementing recombinant clones

To determine if the putative Gal$^+$ transformants restored mitochondrial protein synthesis, growing cells were labeled with [^{35}S] methionine in the presence of cycloheximide and proteins from isolated mitochondria were analyzed on SDS-urea polyacrylamide gels. In Gal$^-$ 32 cells transformed with 10CR-1, mitochondrial protein synthesis is restored to the wild-type level; while mitochondrial protein synthesis is greatly reduced in the mutant (Figure 5). When expanded to test for mitochondrial protein synthesis, the Gal$^+$ phenotype was not stable in Gal$^-$32 cells transformed with 4CR-2; therefore, mitochondrial protein synthesis could not be accurately measured in this case.

Restriction digestion of 10CR-1 and 4CR-2

Since both 10CR-1 and 4CR-2 complemented the Gal$^-$32 mutation, purified DNA from these clones was digested with different restriction enzymes (Figure 6). When 10CR-1 was digested with *EcoRI,* four fragments were observed: 7 kb (vector), 5.7 kb, 4.7 kb, and 4.6 kb; therefore, the vector size (7 kb) and total insert size (15 kb) are smaller

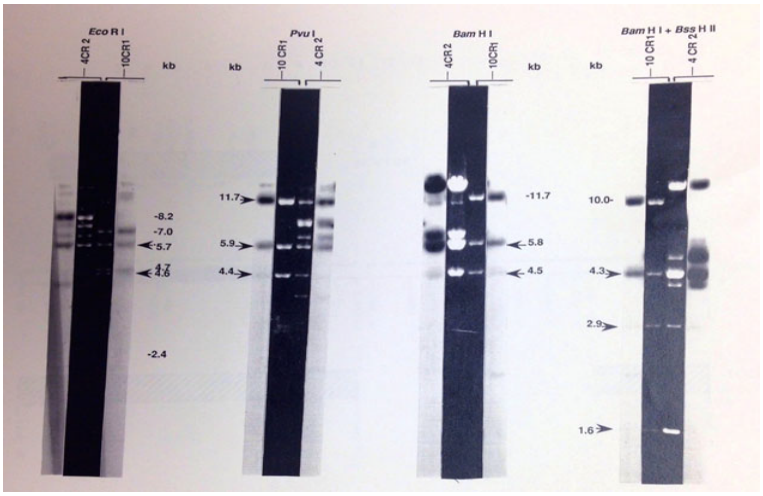

Figure 6: Southern blot of digested DNA from pCV108-transformants, 4CR-2 and 10CR-1, hybridized with a human *Alu* probe.
Plasmid DNA (used for the experiment in Table 3) was purified by CsCl centrifugation or Qiagen columns and digested with several restriction enzymes. The digested DNA, 60 ng, was electrophoresed in an 0.6% agarose gel, stained with ethidium bromide (central lanes in each panel), blotted onto GeneScreen, hybridized to ^{32}P-labeled human *Alu* DNA, and autoradiographed (lanes with the black bands). The intact linear vector is at 8.2 kb for 4CR-1/*Eco*RI and 7.0 kb for 10CR-1/*Eco*RI. λ-*Hind*III and ΦX 174-*Hae*III DNAs were used as size markers.

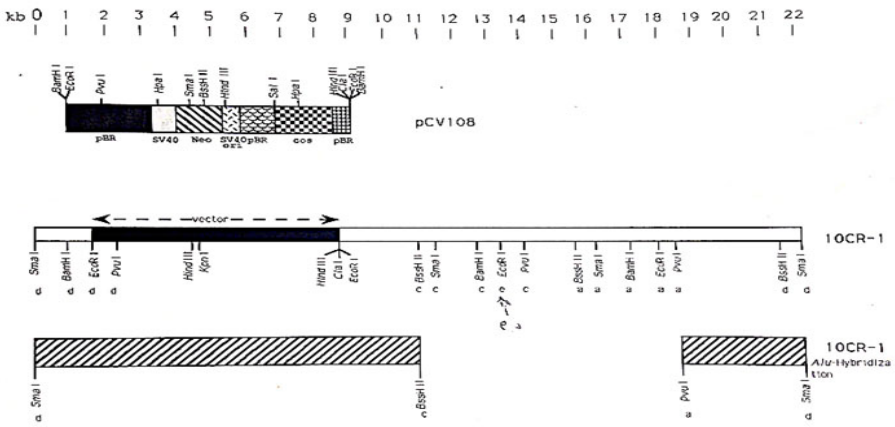

Figure 7: Restriction map and *Alu* hybridization of pCV108-recombinant clone 10CR-1 and restriction map of pCV108.
Clone 10CR-1 was digested with *Eco*RI, *Bam*HI, *Sma*I, *Bss*HII, *Cla*I, *Pvu*I, *Hind*III, *Kpn*I, *Eco*RI + *Bam*HI, *Cla*I + *Pvu*I , *Bam*HI +*Sma*I, *Bam*HI + *Bss*HII, *Sma*I + *Bss*HII, *Bss*HII + *Cla*I, *Sma*I + *Cla*I, *Sma*I + *Kpn*I, *Bss*HII + *Kpn*I, *Bam*HI + *Kpn*I, *Bam*HI + *Cla*I. The pCV108 vector was digested with *Hpa*I, *Sma*I, *Bss*HII, *Cla*I, *Pvu*I, *Hind*III, *Kpn*I (no digestion), *Sal*I, *Hpa*I + *Cla*I, *Hpa*I + *Sma*I, *Bss*HII + *Hind*III, *Hpa*I + *Bss*HII, *Sma*I + *Hind*III, *Pvu*I +*Hind*III, *Cla*I + *Sal*I. Electrophoresis, blotting, and hybridization with the human *Alu* probe are described in Figure 6. The striped regions of 10CR-1 hybridized to the *Alu* probe. The solid region of 10CR-1 is the altered pCV108 vector. The pCV108 map is based on this data and published maps of the component parts [19].

than expected (8.0 kb and 35 kb, respectively). Cosmid clones are known to rearrange. Interestingly, digestion of 10CR-1 with *Bam*HI, *Bss*HII, *Pvu*I, or *Sma*Iall resulted in three fragments with nearly the same size (11.7 kb, 5.8 kb, 4.5 kb for *Bam*HI, Figure 6). Double digestions revealed that the sequence *Bss*HII, *Sma*I, *Bam*HI, *Eco*RI, *Pvu*Iis repeated three times (Figure 7). Two of these *Bss*HII-*Sma*I-*Bam*HI-*Eco*RI-*Pvu*I sequences (a and c sites) do not hybridize to the *Alu*probe; while the cluster with *Eco*RI-*Pvu*Iin the vector (d sites) does hybridize to the *Alu*probe.

*Eco*RI digestion of 4CR-2 resulted in the expected ~8.2 kb vector and several insert fragments with a total size of ~36 kb. With 4CR-2 and 10CR-1, several restriction fragments with the same size and same *Alu*-hybridizing properties were observed (Figure 6). In 4CR-2 and 10CR-1, there are 5.7 kb *Eco*RI, 5.9 kb *Pvu*I, 5.8 kb *Bam*HI, and 4.3 kb *Bam*HI/*Bss*HII fragments which hybridize to the *Alu* probe. In addition, both 4CR-2 and 10CR-1 contain 4.6 kb *Eco*RI, 4.4 kb *Pvu*I, 4.5 kb *Bam*HI, 2.9kb *Bam*HI/*Bss*HII, and 1.6kb *Bam*HI/*Bss*HII fragments which do not hybridize to the *Alu* probe. The fragments which do not hybridize to the *Alu*probe could represent human Gal$^+$ DNA or CHL DNA. Fragments with the same size and *Alu*-hybridizing properties in 4CR-2 and 10CR-1 are likely to encode the same portion of the human GAL$^+$ gene.

Discussion

Gal$^-$32 is a Chinese hamster lung cell mutant that is unable to grow in galactose or fructose due to a differential decrease in mitochondrially synthesized proteins [7]. In a separately published data, it was demonstrated that the majority of the Rhodamine 6-G-treated hybrids

grew in galactose as expected for a nuclearly encoded gene considering that Rhodmnine 6-G interferes with transmission of mt DNA but not nuclear DNA. Therefore, these results are compelling in their demonstration of the nuclear origin of the Gal$^-$32 mutation [28].

The objective of this research was to isolate a human gene that complements the Gal$^-$32 mutation. Recessive Gal$^-$32 cells were co-transformed with pSV2-neo plasmid DNA and recombinant DNA from a human genomic library containing the dominant human Gal$^+$ gene and a *cam*^{r}gene present in the pSV13 vector (14). Primary transformants were selected by growth in GAL and G418. The double selection in GAL + G418 was utilized to distinguish cells that were Gal$^+$ as a result of incorporating the human gene from cells that were Gal$^+$ because of the spontaneous reversion of the CHL Gal$^-$32 gene. Hybridization of primary transformant DNA with a human *Alu*sequence and a *cam*^{r} probe confirmed the presence of human DNA sequences that may remain linked to the *cam*^{r}gene. In order to rescue the human Gal$^+$ gene, a genomic library was constructed with primary transformant-3 DNA and the pCV108 cosmid vector, which contains *amp*^{r} and SV2-neo genes. The pCV108-transformant library was plated on ampicillin and replica plated onto chloramphenicol. Thus, the *cam*^{r} gene was used to identify clones with the nearby human sequences. In fact, most of the *cam*^{r} clones contained human sequences that hybridized to the human *Alu* probe. DNA from two *cam*^{r}, *Alu*-hybridizing clones (10CRA and 4CR-2) was able to transform recessive Gal$^-$32 cells to the Gal$^+$ phenotype and, in the case of 10CR-1, to restore mitochondrial protein synthesis.

Primary and secondary transformants

During gene transfer, 1- 6 copies of the plasmid are integrated into high molecular weight DNA in tandem or at separate sites [14, 19]. The *cam*^{r} probe hybridized to seven different *Eco*RI-primary transformant-3 (1-TR-3) restriction fragments, suggesting that pSV13 -*cam*^{r}-human cosmids had integrated at seven different sites (Figure 1). Hybridization of the *cam*^{r} probe to the *Pst*I-digested 1-TR-3 DNA indicated that there is approximately one pSV13 cosmid copy per genome. On the other hand, in the pCV108-transformant library, the frequency of *cam*^{r} colonies among the *amp*^{r} colonies indicates that there are 10 copies of the pSV13-*cam*^{r}-human cosmids per genome. The explanation for these contradictory pSV13 cosmid copy numbers in 1-TR-3 is not clear.

The frequency of gene transfer with a single copy gene using genomic DNA is expected to be at least 200 times lower, than the frequency with a cloned gene [18, 29]. In this study (Table 1), the transformation frequency with the cloned SV2-*neo* (G418 selection, 135 or 102 in 10^6 cells) or SV2-*gpt*(MXHAT selection, 126 or 408 in 10^6 cells) genes was at the expected level [13]. Surprisingly, the frequency of isolation of primary transformants (GAL and G418, 2.7 in 10^6 cells) and secondary transformants (GAL and G418, 1.2 in 10^6 cells) was higher than expected: only 50-150 times lower than the frequency for cloned SV2-*neo* or SV2-*gpt*genes. The unexpectedly high frequency of isolation of primary transformants may be due to multiple copies of the Gal$^+$ gene in the human cosmid library, which may have arisen during amplification of the library [30]. Gene amplification is also known to occur during gene transfer, perhaps accounting for the high frequency in isolating secondary transformants.

Human gene complements the Gal$^-$32 mutation

We conclude that a human gene was transferred into Gal$^-$32 cells and corrected the Chinese hamster mutation for the following reasons: 1) The frequency of isolation of primary Gal$^+$ transformants in GAL + G418 media was 10^5 times higher than expected for a CHL Gal$^+$ revertant that incorporated the SV2-*neo* gene; 2) the frequency of isolation of secondary Gal$^+$ transformants in GAL + G418 media was 100 times higher than expected for a CHL Gal$^+$revertant that incorporated the SV2-*neo* gene; 3) primary transformants were able to grow in MXHAT media even though the SV2-*gpt* gene present in the pSV13 vector was unselected; 4) detection of multiple human *Alu*-hybridizing DNA in primary and secondary transformants; 5) detection of multiple *cam*^{r}-hybridizing restriction fragments corresponding in size to the *Alu*-hybridizing fragments indicated the presence of multiple human-pSV13 recombinant clones in the primary transformants; 6) the restoration of mt protein synthesis in primary and secondary transformants. These data also support the conclusion that the Gal$^-$32 gene is nuclearly encoded, since mtDNA has not been reported to yield stable transformants by $CaPO_4$ precipitation. ***It is significant that the human gene corrects the Chinese hamster Gal$^-$32 mutation because many human mt proteins are unable to substitute for Chinese hamster proteins.*** For example, in human-rodent or mouse-rat hybrid cell lines, mtDNA is usually not maintained unless nuclear DNA from the same species is also retained [31, 32].

Isolation of two recombinant human clones that correct the Gal$^-$32 mutation

Two pCV108-transformant clones (10CR-1 and 4CR-2) that complement the Gal$^-$32 mutation were identified by transferring the purified recombinant cosmid DNA into Gal$^-$32 cells and selecting for growth in GAL + G418. If a particular pCV108-recombinant clone contains the Gal$^+$ gene, the transformation frequency in galactose is expected to be about the same as the transformation frequency in G418. However, when plasmid DNA from 4CR-2 was transferred into Gal$^-$32 cells, the transformation frequency selecting in galactose was one twelfth to one fifth that of selecting in G418; with 10CR-1, the frequency was one fiftieth. A very likely explanation for the lower frequency of expression of the Gal$^+$ gene compared to the SV2-*neo* gene is a difference in

promoter strength. Alternatively, the lower transformation frequency in galactose may be the result of DNA rearrangements in the presumably larger Gal$^+$ gene. Furthermore, DNA methylation and changes in chromatin structure may decrease gene expression of transferred DNA [33].

It is concluded that we have isolated two human genomic cosmid clones (10CR-1 and 4CR-2) that correct the CHL Gal$^-$32 mutation because 1) the genomic clones were constructed from primary transformant-3 (1-TR-3), which contains CHL and human DNA; for the reasons given above, the Gal$^+$ phenotype of 1-TR-3 is due to the human DNA; 2) 10CR-1 and 4CR-2 grow in chloramphenicol and hybridize to the human *Alu* probe; thus, they contain human and *cam*^{r} DNA, derived from the pSV13-human library that generated primary transformant-3; 3) 10CR-1 and 4CR-2 transform Gal$^-$32 to the Gal$^+$ phenotype; whereas, six other *cam*^{r}, *Alu*-hybridizing, primary transformant clones did not; 4) 10CR-1 and 4CR-2 exhibit restriction fragments with the same size and with the same *Alu*-hybridizing properties, 5) mitochondrial protein synthesis is restored in Gal$^-$32 cells transformed with clone 10CR-1 and could not be measured accurately with clone 4CR-2 due to instability.

Primary defect in Gal$^-$32

The primary defect in Gal$^-$32 causing the differential decrease in mitochondrial protein synthesis is unknown. It is unlikely that Gal$^-$32 is deficient in a general protein synthesis component such as mt ribosomal

protein or mt aminoacyl synthetase because mutations in these components completely abolish all mt translation products [34,35]. A mutation altering tRNA structure, tRNA base modification, or tRNA charging might differentially affect the synthesis of mitochondrially encoded proteins according to their amino acid composition or nucleotide sequence, or unique context of a particular codon. In fact, the mouse mtDNA sequence reveals that there are more cysteine, aspartate, glutamate, and tyrosine residues in the subunits of NADH dehydrogenase and cytochrome *c* oxidase than in ATPase subunits[36]. Thus, a nuclear mutation affecting $tRNA^{Cys}$, $tRNA^{Asp}$, $tRNA^{Glu}$ or $tRNA^{Tyr}$, might decrease cytochrome oxidase and NADH dehydrogenase peptides more than ATPase peptides, as is observed in Gal$^-$32. Many disorders of the mitochondrion involve defects in the oxidative phosphorylation system, which comprises five multisubunit enzyme complexes encoded by both the nuclear and the mitochondrial genomes [37, 38]. Due to the multitude of proteins and intricacy of the processes required for a properly functioning oxidative phosphorylation system, identifying the genetic defect that underlies an oxidative phosphorylation deficiency is a formidable task [39]. Further investigation of the cloned human gene that corrects the Gal$^-$32 mutation will yield information about its regulation, structure, and gene product and thereby enhancing our understanding of mitochondrial gene expression. This may also open possibilities for therapeutic modalities for those affected by mitochondrial disorders.

Human respiratory-deficient diseases

Deficiencies in human mt respiratory complexes have been associated with cardiac failure, stroke, skeletal muscle movement disorders, renal malfunction, diabetes, liver disease, Alzheimer's disease, Parkinson's disease, blindness, dementia, deafness, and myoclonic epilepsy (reviewed by Wallace [32]. Many of these diseases are maternally inherited due to missense, insertion-deletion, and copy number mutations in mtDNA. Mitochondrial tRNA mutations have been reported for myoclonic epilepsy and ragged-red fiber disease; mitochondrial encephalomyopathy, lactic acidosis, and stroke like symptoms; and maternally inherited myopathy and cardiomyopathy [32]. Like Gal 32, these tRNA mutations preferentially decrease cytochrome *c* oxidase and NADH dehydrogenase, which have the greatest number of mitochondrially encoded subunits.

The gene locus of Gal-32 is not known

Zheng et al. [40] have studied a lethal infantile mitochondrial disease due to deficiencies in the same respiratory complexes as Gal 32. NADH dehydrogenase and cytochrome *c* oxidase were drastically decreased in heart and skeletal muscle, but not brain; whereas, succinate-cytochrome *c* reductase activity was not greatly affected. Both parents were normal, suggesting a recessive, nuclear mutation, as in Gal 32. Gal$^-$32 provides an intriguing animal model system for these human patients with analogous deficiencies in the mitochondrial respiratory complexes.

Authors' Contribution

ZAS carried out all the experiments in this study including the molecular biology and genetic studies: cloned the human Gal$^+$ gene, conceived the theme of the project, designed and drafted the manuscript. CWB conceived the idea, participated in the design of the study and help draft the manuscript. All authors read and approved the final manuscript.

Acknowledgement

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References

1. Chu EH (1974) Induction and analysis of gene mutations in cultured mammalian somatic cells. Genetics 78: 115-132.
2. Malczewski RM, Whitfield CD (1982) Respiration-defective Chinese hamster cell mutants containing low levels of NADH-ubiquinone reductase and cytochrome c oxidase. J Biol Chem 257: 8137-8142.
3. Reitzer LJ, Wice BM, Kennell D (1979) Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. J Biol Chem 254: 2669-2676.
4. Diaz-Ruiz R, Rigoulet M, Devin A (2011) The Warburg and Crabtree effects: On the origin of cancer cell energy metabolism and of yeast glucose repression. Biochim Biophys Acta 1807: 568-576.
5. Chomyn A, Mariottini P, Cleeter MW, Ragan CI, Matsuno-Yagi A, et al. (1985) Six unidentified reading frames of human mitochondrial DNA encode components of the respiratory-chain NADH dehydrogenase. Nature 314: 592-597.
6. Chomyn A, Cleeter MW, Ragan CI, Riley M, Doolittle RF, et al. (1986) URF6, last unidentified reading frame of human mtDNA, codes for an NADH dehydrogenase subunit. Science 234: 614-618.
7. Malczewski RM, Whitfield CD (1984) A novel mutation selectively decreases complex I and cytochrome c oxidase subunits in Chinese hamster mitochondria. J Biol Chem 259: 11103-11113.
8. Ditta G, Soderberg K, Scheffler IE (1977) Chinese hamster cell mutant with defective mitochondrial protein synthesis. Nature 268: 64-67.
9. Michaelis G, Mannhaupt G, Pratje E, Fischer E, Naggert J, et al. (1982) Mitochondrial Translation Products in Nuclear Respiration-deficient pet Mutants of Saccharomyces cerevisiae. In Mitochondrial Genes first edition:311-321.
10. Soderberg K, Mascarello JT, Breen GA, Scheffler IE (1979) Respiration-deficient Chinese hamster cell mutants: genetic characterization. Somatic Cell Genet 5: 225-240.
11. Whitfield CD1, Jefferson LM (1990) Elevated mitochondrial RNA in a Chinese hamster mutant deficient in the mitochondrially encoded subunits of NADH dehydrogenase and cytochrome c oxidase. J Biol Chem 265: 18852-18859.
12. Sherif ZA, Whifield-Broome C (1992) Characterization of Mitochondrial Gene Expression and Protein Synthesis Related to Oxidative Phosphorylation. FASEB 6:454.
13. Mulligan RC, Berg P (1981) Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyltransferase. Proc Natl Acad Sci U S A 78: 2072-2076.
14. Southern PJ, Berg P (1982) Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J Mol Appl Genet 1: 327-341.
15. Fleischmann R, McCormick M, Howard BH (1987) Preparation of a genomic cosmid library. Methods Enzymol 151: 405-416.
16. Lau YF, Kan YW (1983) Versatile cosmid vectors for the isolation, expression, and rescue of gene sequences: studies with the human alpha-globin gene cluster. Proc Natl Acad Sci U S A 80: 5225-5229.
17. Deininger PL, Jolly DJ, Rubin CM, Friedmann T, Schmid CW, et al. (1981) Base sequence studies of 300 nucleotide renatured repeated human DNA clones. J Mol Biol 151: 17-33.
18. Abraham I, Tyagi JS, Gottesman MM (1982) Transfer of genes to Chinese hamster ovary cells by DNA-mediated transformation. Somatic Cell Genet 8: 23-39.
19. Chen C, Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7: 2745-2752.
20. Chen CA, Okayama H (1988) Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. Biotechniques 6: 632-638.
21. Sambrook J, Fritsch E F, Maniatis, T (1989) Molecular Cloning: A laboratory Manual.
22. Gorman CM, Merlino GT, Willingham MC, Pastan I, Howard BH (1982) The

Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc Natl Acad Sci U S A 79: 6777-6781.

23. Whitfield C, Abraham I, Ascherman D, Gottesman MM (1986) Transfer and amplification of a mutant beta-tubulin gene results in colcemid dependence: use of the transformant to demonstrate regulation of beta-tubulin subunit levels by protein degradation. Mol Cell Biol 6: 1422-1429.

24. Seed B, Parker RC, Davidson N (1982) Representation of DNA sequences in recombinant DNA libraries prepared by restriction enzyme partial digestion. Gene 19: 201-209.

25. DiLella AG, Woo SL (1987) Cloning large segments of genomic DNA using cosmid vectors. Methods Enzymol 152: 199-212.

26. Hanahan D, Meselson M (1980) Plasmid screening at high colony density. Gene 10: 63-67.

27. Schmid CW, Deininger PL (1975) Sequence organization of the human genome. Cell 6: 345-358.

28. Sherif ZA, Jefferson LM, Whitfield-Broome CD (1996) Nuclear inheritance of a gene affecting mitochondrial gene expression. Somat Cell Mol Genet 22: 443-451.

29. Pulciani S, Santos E, Lauver AV, Long LK, Robbins KC, et al. (1982) Oncogenes in human tumor cell lines: molecular cloning of a transforming gene from human bladder carcinoma cells. Proc Natl Acad Sci U S A 79: 2845-2849.

30. Frischauf AM (1987) Construction and characterization of a genomic library in lambda. Methods Enzymol 152: 190-199.

31. Hayashi J, Tagashira Y, Yoshida MC, Ajiro K, Sekiguchi T (1983) Two distinct types of mitochondrial DNA segregation in mouse-rat hybrid cells. Stochastic segregation and chromosome-dependent segregation. Exp Cell Res 147: 51-61.

32. Wallace DC1 (1992) Diseases of the mitochondrial DNA. Annu Rev Biochem 61: 1175-1212.

33. Rolando M, Sanulli S, Rusniok C, Gomez-Valero L, Bertholet C, et al. (2013) Legionella pneumophila effector RomA uniquely modifies host chromatin to repress gene expression and promote intracellular bacterial replication. Cell Host Microbe 13:395-405.

34. Myers AM, Crivellone MD, Tzagoloff A (1987) Assembly of the mitochondrial membrane system. MRP1 and MRP2, two yeast nuclear genes coding for mitochondrial ribosomal proteins. J Biol Chem 262: 3388-3397.

35. Koerner TJ, Myers AM, Lee S, Tzagoloff A (1987) Isolation and characterization of the yeast gene coding for the alpha subunit of mitochondrial phenylalanyl-tRNA synthetase. J Biol Chem 262: 3690-3696.

36. Bibb MJ, Van Etten RA, Wright CT, Walberg MW, Clayton DA (1981) Sequence and gene organization of mouse mitochondrial DNA. Cell 26: 167-180.

37. Ugalde C, Morán M, Blázquez A, Arenas J, Martín MA (2009) Mitochondrial disorders due to nuclear OXPHOS gene defects. Adv Exp Med Biol 652: 85-116.

38. Shoubridge EA (2001) Nuclear genetic defects of oxidative phosphorylation. Hum Mol Genet 10: 2277-2284.

39. Smits P, Smeitink J, van den Heuvel L (2010) Mitochondrial translation and beyond: processes implicated in combined oxidative phosphorylation deficiencies. J Biomed Biotechnol 2010: 737385.

40. Zheng X, Shoffner JM, Lott MT, Voljavec AS, Krawiecki NS, et al. (1989) Evidence in a lethal infantile mitochondrial disease for a nuclear mutation affecting respiratory complexes I and IV. Neurology 39: 1203-1209.