RESEARCH COMMUNICATION

Stable correction of maple syrup urine disease in cells from a Mennonite patient by retroviral-mediated gene transfer

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We have successfully used retroviral gene transfer to correct the deficiency of the branched-chain α -oxo acid dehydrogenase complex in lymphoblasts from a homozygous Mennonite maple syrup urine disease (MSUD) patient. The mutation in Mennonites is a Tyr-393 to Asn substitution in the branched-chain α -oxo acid decarboxylase (E1) α subunit of the enzyme complex. This promotes improper assembly of mutant E1 α with E1 β subunits, leading to degradation of both polypeptides. For transduction studies, a full-length human E1 α cDNA was inserted into the retroviral vector LXSIN to produce the recombinant LSN-E1 α . High-titre [6×10^6 colony-forming units/ml] amphotropic retroviral preparations free of helper viruses were obtained by co-cultivation of infected GP+E86 with PA317 cells. Transduction of MSUD lymphoblasts from the Mennonite patient

with LSN-E1 α viruses restored the decarboxylation of α -oxo[1- 14 C]isovalerate to the normal level. The normal decarboxylation activity in transduced MSUD cells remained stable without G418 selection during the 14 weeks studied. Southern-blot analysis indicated that the recombinant E1 α cDNA was integrated into the host genome. Northern and Western blotting showed that both the normal E1 α mRNA and the subunit were properly expressed in transduced MSUD cells. However, the level of E1 β subunits is lower than that of normal cells, suggesting competition of the recombinant E1 α with the mutant form for assembly with E1 β . The results provide a paradigm for the development of somatic gene therapy for disorders involving mitochondrial multienzyme complexes.

INTRODUCTION

Maple syrup urine disease (MSUD) or branched-chain oxoaciduria is an autosomal recessive disorder in the degradation of branched-chain α -oxo acids derived from leucine, isoleucine and valine [1]. The classical form of the disease is characterized by the rapid onset of severe oxoacidosis within the first week of life. This is accompanied by urine with a maple syrup odour and clinically by seizures, coma and death if untreated [1]. There is a high incidence of mental retardation in survivors. Several milder forms of MSUD have also been described, which include intermediate, intermittent and thiamin-responsive conditions [1].

The enzyme deficient in MSUD patients, the mitochondrial branched-chain α -oxo acid dehydrogenase (BCOAD) complex, consists of three catalytic components [2,3]. The E1 component is a decarboxylase comprised of two α (M_r 47 000) and two β (M_r 37 000) subunits. E2, a transacylase, is the core of the enzyme complex consisting of 24 identical lipoate-bearing subunits (monomer M_r 46 500) that form a cubic structure with octahedral symmetry, and E3, a dehydrogenase, exists as a homodimer (monomer M_r 52 000), which is common to the pyruvate and α -oxoglutarate dehydrogenase complexes [4]. The mammalian BCOAD complex also contains two regulatory enzymes, a specific kinase and a specific phosphatase, which regulate the activity of the enzyme complex through a phosphorylation (inactivation)/dephosphorylation (activation) cycle [5]. Genetic heterogeneity in MSUD has been demonstrated by mutations in each of the three E1 α , E1 β and E2 catalytic subunits [1].

The incidence of the classical form of MSUD in Mennonites is high (1 in 176 newborns) because of consanguinity [6]. The

mutation in these patients was identified to be a homozygous T to A transversion in exon 9 of the E1 α gene [7,8], which causes a Tyr-393 to Asn substitution (Y393N) in the E1 α subunit of the BCOAD complex. We subsequently showed that the Y393N mutation in the E1 α subunit impedes proper assembly with E1 β , resulting in degradation of both subunits [9,10]. The absence of a properly assembled stable E1 component accounts for the dysfunction of the BCOAD complex and the resultant MSUD phenotype.

Current treatment of MSUD relies primarily upon dietary restriction of branched-chain amino acids [1]. Dietary treatment is not completely satisfactory as the regimen requires strict adherence. During episodes of catabolic decompensation triggered by infection or stress, neurological damage may occur [1]. One in five classical MSUD patients dies while on dietary therapy [1]. Somatic gene therapy has shown promise in animal models [11–13], and a human protocol for adenosine deaminase deficiency has been described [14]. However, these studies are confined to single-locus diseases. To explore the possibility of gene therapy for disorders involving multiple loci such as MSUD, our laboratory has recently constructed retroviral vectors that carry a normal E1 α or E2 cDNA. We have selected for initial studies a lymphoblast cell line from a Mennonite MSUD patient. We report here stable restoration of BCOAD complex activity in transduced Mennonite MSUD cells.

MATERIALS AND METHODS

Cell cultures

Lymphoblast cultures from normal subjects and a Mennonite

Abbreviations used: BCOAD, branched-chain α -oxo acid dehydrogenase; E1, branched-chain α -oxo acid decarboxylase; E2, dihydrolipoyl transacylase; E3, dihydrolipoyl dehydrogenase; MSUD, maple syrup urine disease; Y393N, tyrosine-393 $\rightarrow$ asparagine substitution; LTR, long terminal repeat.

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Figure 1 Retroviral vectors carrying full-length human E1 α and E2 cDNAs

The LXSN vector contains a retrovirus packaging signal (ψ^+), a neomycin-resistance gene (*NEO*) and 5' and 3' long terminal repeats (LTR) [12]. LSN-E1 α harbours an E1 α cDNA insert (2098 bp) with its coding region in the small hatched bar. The mitochondrial-targeting sequence between initiation Met and the N-terminal Ser residues comprises 45 amino acids. The 5' open bar represents the 320 bp E1 α gene promoter and the 5' untranslated region. The 3' open bar is the 3'-untranslated region of the E1 α cDNA. The transcription initiation site (bent arrow) and the poly(A) signal (pA) for the recombinant E1 α mRNA in the LSN-E1 α vector are shown. LSN-E2 harbours the E2 cDNA insert (1863 bp) shown by a small open and stippled bar. The mitochondrial-targeting sequence in E2 between the initiation Met and the N-terminal Gly residue contains 61 amino-acid residues. The 5' and 3' open bars represent the 5' and 3' untranslated regions of the E2 cDNA respectively. Restriction sites utilized in the construction of the vectors (see the Materials and methods section) and Southern blotting (Figure 4) are indicated.

MSUD patient (LMK), homozygous for the Y393N mutation in E1 α [7,8], were established from peripheral blood lymphocytes by infection with Epstein-Barr virus [9,15]. GP+E86, an ecotropic packaging cell line, was generously provided by Dr. Arthur Bank (Columbia University, College of Physicians and Surgeons, New York, NY, U.S.A.). PA317 cells, an amphotropic packaging cell line, was purchased from American Type Culture Collection (ATCC) (Rockville, MD, U.S.A.). NIH/3T3, and MiCl1 (S⁺L⁻) cells were also obtained from ATCC.

Construction of retroviral vectors

The retroviral vector, LXSN [16], was kindly provided by Dr. A. Dusty Miller (Fred Hutchinson Cancer Research Center, University of Washington, Seattle, WA, U.S.A.). The insertion of a full-length human E1 α cDNA into the LXSN vector was carried out as follows. The 320 bp promoter and 5' mitochondrial-targeting sequences were amplified by PCR using the human E1 α genomic clone (L1) as the template [17]. The sense primer (P2) used was 5'-CAGTCCCACCTCTAACTC-3' (bases -320 to -302). The antisense primer was 5'-AGATCTAGCCAGTCCCCGAGCCC-3' (bases +135 to +113). PCR products were treated with T4 polynucleotide kinase after *Pst*I digestion. The 5' mitochondrial-targeting and the mature human E1 α sequences were derived, respectively, from hE1 α -4 and hE1 α -2 cDNA clones [18] in Bluescript SK⁻ (Stratagene, La Jolla, CA, U.S.A.). A *Pst*I/*Eco*RI fragment (180 bp) was isolated from the hE1 α -4 clone. Two contiguous *Eco*RI/*Acc*I (710 bp) and *Acc*I/*Bam*HI (790 bp) fragments were obtained from hE1 α -2. All four fragments (Blunt/*Pst*I, *Pst*I/*Eco*RI, *Eco*RI/*Acc*I and *Acc*I/*Bam*HI) were ligated into

the Bluescript SK⁻ vector previously digested with *Eco*RV/*Bam*HI. The resultant full-length E1 α cDNA (2098 bp) was subcloned by digesting the Bluescript vector with *Xho*I/*Bam*HI. The insert was ligated into corresponding sites of the LXSN retroviral vector to produce LSN-E1 α (Figure 1). The full-length human E2 cDNA insert was obtained by digestion of an EBO-E2 vector [19] with *Sal*I/*Not*I. The protruding ends were filled in with deoxynucleotides using the Klenow fragment. The insert was cloned into the *Hpa*I-digested LXSN vector by blunt-end ligation. The orientation of the insert was analysed by restriction mapping. The full-length E2 cDNA insert (1863 bp) spans bases +4 to +1866 of the complete E2 mRNA [20], and contains the initiation methionine codon and the translation-termination codon. The recombinant vector harbouring the full-length E2 cDNA is referred to as LSN-E2 (Figure 1).

Virus-producing cell lines

GP+E86 ecotropic packaging cells [21] were transfected with the retroviral vectors by calcium phosphate precipitation [22,23], followed by G418 (GIBCO, Gaithersburg, MD, U.S.A.) selection. PA317 amphotropic packaging cells [24] were infected in the presence of 8 μ g/ml of hexadimethrine bromide (polybrene) (Sigma, St. Louis, MO, U.S.A.) with media from the GP+E86 cells. Virus titres were determined using NIH/3T3 cells as the recipients [23]. To increase the titres of amphotropic virions, transduced PA317 and GP+E86 cells at a ratio of 1:1 were co-cultivated for 2 weeks [25]. Helper virus production was determined by the XC cell plaque assay for ecotropic viruses [26], or by the S⁺L⁻ MiCl1 cell assay for amphotropic viruses [27].

Viral transduction

MSUD lymphoblasts were infected with retroviruses by co-cultivation as described previously [28]. GP+E86 and PA317 cells were seeded at 1×10^6 cells/100-mm-diam. dish the day before transduction of lymphoblasts. The following day, medium from the packaging cells was replaced with RPMI-1640 medium supplemented with 10% (v/v) fetal-calf serum containing 1×10^7 lymphoblasts and 2 μ g/ml hexadimethrine bromide. Both packaging cells and lymphoblasts were co-cultivated for 48 h. The unattached lymphoblasts were resuspended in fresh medium and transferred to a 60-mm-diam. dish. After 3 days, lymphoblasts were cultured in flasks in the selection medium containing 1 mg/ml of G418 for 10 days.

Decarboxylation and enzyme assays with cultured cells

The rates of decarboxylation of α -oxo[1-¹⁴C]isovalerate and [1-¹⁴C]pyruvate were measured with viable intact lymphoblasts [29]. The enzyme activity of the BCOAD complex was determined using the disrupted-cell assay [29]. Protein concentrations were determined by the method of Lowry et al. [30].

RESULTS

The retroviral vectors used in the present study are shown in Figure 1. The parental vector LXSN contains the packaging signal ψ^+ and a selection marker (*NEO*) for G418 resistance. LSN-E1 α harbours a full-length E1 α cDNA insert. The latter consists of (5' to 3') a 320 bp E1 α gene promoter sequence and the sequence coding for a complete mitochondrial-targeting prepeptide (45 residues) and the mature peptide (400 residues) [17]. LSN-E2 contains a full-length E2 cDNA that encodes a complete mitochondrial-targeting prepeptide (61 residues) and

Table 1 Stable restoration of BCOAD activity in transduced MSUD cells

Cultured lymphoblasts from a Mennonite MSUD patient (LMK) were transduced with the retroviral vector LSN-E1 α as described in the Materials and methods section, and analysed for decarboxylation of α -oxo[1- 14 C]isovalerate by the intact cell assay. The number of normal cell lines used for the intact cell assay is shown in parentheses. Variations in specific activity of normal cells at different times appear to result from the use of different batches of substrate.

Weeks after transduction	Specific activity (nmol of CO ₂ /min per mg of protein)		Activity†‡ (% of normal activity)
	Normal cells	MSUD cells transduced with LSN-E1 α	
0	0.059 $\pm$ 0.010 (1)*	< 0.001	0
6	0.067 $\pm$ 0.004 (1)*	0.065 $\pm$ 0.003	96
14	0.119 $\pm$ 0.031 (2)*	0.137 $\pm$ 0.011	115

* The decarboxylation activity in LMK cells transduced with LXSN vector without insert showed undetectable activity. LMK cells transduced with the LSN-E2 vector exhibited a specific activity of 0.003 nmol of CO₂/min per mg of protein (4% of normal).

† Activities of transduced LMK and normal cells assayed simultaneously using the same batch of substrate are compared.

‡ Assays carried out with LMK cells transduced with LSN-E1 α at 7, 10, 11 and 12 weeks after transductions showed restored activities (as % of normal activity) of 120, 84, 88 and 104 respectively.

the mature peptide (421 residues) [20]. Co-cultivation of transduced PA317 and GP+E86 cells produced titres for LXSN, LSN-E1 α and LSN-E2 of 2.7×10^5 , 6.2×10^5 and 3.4×10^5 colony-forming units/ml respectively. Infection of the E1 α -deficient MSUD lymphoblasts (LMK) with retroviruses was performed by co-cultivation with the packaging cell lines. After 48 h of co-cultivation with the GP+E86 and PA317 monolayers, the efficiency of retroviral infection of LMK lymphoblasts ranged from 80% to 92%, as determined by their resistance to G418. Helper viruses were not detected in the co-cultivation media (results not shown).

Decarboxylation of α -oxo[1- 14 C]isovalerate in untransduced and transduced intact lymphoblasts was measured (Table 1). Untransduced LMK cells and LXSN or LSN-E2 transduced cells exhibited only a background level of decarboxylation. Transduction of LMK lymphoblasts with the LXSN vector containing a normal human E1 α cDNA (LSN-E1 α) restored decarboxylation, after 6 weeks, to 96% of that observed in normal cells (Table 1). The decarboxylation activity (84–120% of normal) is stable for at least the 14 weeks studied without G418 selection (Table 1). The activity of the pyruvate dehydrogenase complex, as assayed using [1- 14 C]pyruvate as substrate, is normal in both untransduced and transduced cells (results not shown). The enzyme activity of the BCOAD complex in extracts of normal and LMK cells transduced with LSN-E1 α was 0.03 and 0.04 nmol of CO₂/min per mg of protein respectively.

Figure 2 shows the results of Southern-blot analysis using genomic DNA obtained from untransduced and transduced MSUD lymphoblasts. Both normal and untransduced LMK lanes showed four *Eco*RI fragments when probed with the human E1 α cDNA. The size of these fragments agreed with the restriction map of the human E1 α gene [33]. Genomic DNAs from transduced LMK cells showed four identical fragments. Additional 1.5 kb and 0.6 kb fragments were detected in the LMK + LSN-E1 α lane. These two *Eco*RI fragments were of the expected size for the retroviral E1 α cDNA insert (see Figure 1). This result indicated that the human E1 α cDNA sequence was

Figure 2 Southern-blot analysis of genomic DNA from untransduced and transduced MSUD lymphoblasts

Genomic DNAs were prepared as described [31] from normal and untransduced MSUD (LMK) lymphoblasts, or transduced LMK lymphoblasts that had been cultured for 8 weeks without G418 selection. The DNA (20 μ g/lane) was separated on a 0.7% agarose gel after *Eco*RI digestion, and immobilized on GeneScreen Plus (duPont/NEN, Boston, MA, U.S.A.). A 2.1 kb full-length human E1 α cDNA comprising two contiguous *Eco*RI fragments (0.6 kb and 1.5 kb) (5' to 3') was obtained by digestion of the LSN-E1 α vector (Figure 1) with *Eco*RI. Both fragments were radiolabelled with [α - 32 P]dATP by the random-priming method [32] and used as probes.

correctly integrated into the LMK genome. Densitometric measurements showed that the combined signal intensities of the integrated 1.5 kb and 0.6 kb cDNA fragments were 1.16 times the sum of the four genomic fragments (results not shown). This corresponded to approx. one copy of the retroviral vector (LSN-E1 α) incorporated per LMK cell. The genomic and the retroviral cDNA fragments contained the same 320 bp promoter and the entire exonic sequences that were recognized by the full-length E1 α cDNA probe used in Southern blotting. The integrated E2 cDNA sequence in the LMK + LSN-E2 lane was not detected by the E1 α cDNA probe (see Figure 4), but hybridized with a labelled E2 cDNA (results not shown).

Figure 3 shows the results of Northern-blot analysis using the total RNA isolated from untransduced and transduced MSUD lymphoblasts. Both normal and untransduced LMK cells expressed a single species of the natural E1 α mRNA (2 kb) with similar intensities. LMK cells infected with LSN-E1 α showed an additional signal 5 kb in size. The latter represented the single recombinant E1 α mRNA. Shorter exposure of the filter did not increase resolution of the 5 kb band. The transcription-initiation and polyadenylation sites of the recombinant E1 α message were located in the 5' and 3' long terminal repeat (LTR) of the LSN-E1 α vector respectively (Figure 1).

To determine the protein content of E1 α and E1 β subunits, lysates were prepared from untransduced and transduced MSUD lymphoblasts. Western blotting was carried out using the E1 α or the E1 β antibody as a probe. The E1 α subunit consists of a slower-migrating phosphorylated (inactive) species (E1 α -P) and

Figure 3 Northern-blot analysis of total RNA from untransduced and transduced MSUD lymphoblasts

Total RNA was isolated from lymphoblasts and Northern-blot analysis was carried out as described [14]. Cell lines studied and the E1 α cDNA probe were as described in Figure 2, except that transduced LMK lymphoblasts were cultured for 9 weeks without G418 selection. Each lane contains 20 μ g of total RNA.

a faster-migrating unphosphorylated (active) form (E1 α) [34]. In the normal control, approx. equal amounts of the E1 α subunit exist as phosphorylated and unphosphorylated species, whereas in untransduced LMK cells the E1 α is predominantly unphosphorylated. Transduction of LMK cells with LXSN vector without the insert or with the vector containing the E2 cDNA (LSN-E2) did not significantly increase the content or ratio of phosphorylated to unphosphorylated E1 α subunits. Transduction of LMK cells with the vector containing the normal E1 α cDNA (LSN-E1 α) increased the level of E1 α to greater than that of normal (Figure 4). The distribution of phosphorylated and unphosphorylated E1 α is approx. equal in normal and LMK + LSN-E1 α cells. The level of immunologically detectable E1 β subunits is very low in LMK, LMK + LXSN and LMK + LSN-E2 cells (Figure 4, lower panel). Transduction of LMK with LSN-E1 α significantly increases the level of the E1 β subunits. However, the level of E1 β is less in LMK + LSN-E1 α cells, compared with normal.

DISCUSSION

The present communication is the first report of the stable restoration of activity of the mitochondrial BCOAD complex in cells from an MSUD patient by retroviral gene transfer. The use of retroviral vector LXSN [16] and the packaging cell line GP + E86 allows the production of recombinant retroviral preparations free of helper viruses. The LSN-E1 α vector contains both the 5' LTR promoter and a 320 bp E1 α gene promoter [17] upstream of the full-length cDNA. However, the expression of the recombinant E1 α subunits in transduced lymphoblasts appeared to be driven predominantly by the 5' LTR promoter, as indicated by the size (5 kb) of recombinant E1 α mRNA (Figures 1 and 3). The smaller mRNA, corresponding to transcription initiation 3' to the E1 α promoter, was not detectable. The data indicate that the intrinsic E1 α promoter is suppressed by the 5' LTR. The latter viral promoter is known to function most efficiently in gene transfer into cell culture [16], but is less effective for long-term expression *in vivo* [35].

Western blotting (Figure 4) confirms that E1 α and E1 β subunits are markedly deficient in Mennonite MSUD (LMK) cells [9,19,36]. The apparently weaker signal of E1 β , when

Figure 4 Western-blot analysis of lysates prepared from untransduced and transduced MSUD lymphoblasts

Transduced LMK lymphoblasts were cultured for 11 weeks without G418 selection before preparation of the cell lysate. After SDS/PAGE, proteins were electrotransferred to poly(vinylidene difluoride) membranes. The filters were probed with either anti-E1 α (upper panel) or -E1 β (lower panel) antibodies radiolabelled by coupling with ¹²⁵I-Protein A [18]. E1 α and E1 α -P depict the unphosphorylated and phosphorylated forms of the E1 α subunit respectively. Each lane contains 200 μ g of protein.

compared with that of E1 α , in both normal and transduced LMK cells, results from a lower titre of antiserum for the E1 β subunit. We interpret the present data to indicate that the Y393N mutation in E1 α produces an improperly assembled E1 ($\alpha_2\beta_2$) that is unstable in the MSUD cell. This hypothesis is supported by an earlier study, in which co-expression in *Escherichia coli* of a mutant E1 α containing the Y393N mutation and a normal E1 β resulted in an enzymically inactive large aggregate of the two polypeptides [10]. Introduction of the recombinant normal E1 α subunit increased the level of E1 β in transduced MSUD cells (Figure 4). The inability to fully restore the level of E1 β subunits to that of normal may result from competition of recombinant E1 α with the mutant form for assembly with E1 β . The recombinant E1 α subunit in transduced MSUD cells is phosphorylated to a similar extent to the normal E1 α (Figure 4). Thus, the lower level of functional E1, as indicated by reduced E1 β in transduced LMK cells, appears inconsistent with the demonstration of full restoration of decarboxylation of α -oxo[1-¹⁴C]isovalerate by these cells compared with normal (Table 1). The enzyme activity of the BCOAD complex in LMK + LSN-E1 α lymphoblasts, as determined in cell lysates, is in the normal range (see the Results section). The discordance between BCOAD activity and E1 level in transduced LMK cells may suggest a possible excess of E1 in normal cells, with a portion not bound to E2. In support of this possibility is the evidence that the 'activator' protein of the BCOAD complex in mitochondria is the dissociated E1 component [37,38].

The results of the present study show unequivocally that the recombinant E1 α subunits are imported into the mitochondrial matrix, where the correctly processed mature peptides fold and assemble with endogenous E1 β subunits to form a functional E1 component. E1 and E3 are bound to the E2 core to produce a catalytically active BCOAD complex. The data establish, at the cellular level, the feasibility of stable correction of a mitochondrial multienzyme deficiency by retroviral-mediated transfer of a normal cDNA for the defective subunit. Although lymphoblasts were studied here initially, our plans are to extend retroviral

gene transfer to non-transformed cells. The BCOAD complex is widely distributed in liver, kidney, heart, muscle [39] and lymphocytes [40]. Thus, introduction of the normal gene into a variety of MSUD cell types such as hepatocytes, T-cells and myoblasts would be effective in degrading the branched-chain α -oxo acids. Information derived from these studies can be applied to animal models, for example, MSUD in Hereford calves [41,42].

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REFERENCES

- Chuang, D. T. and Shih, V. E. (1994) in *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), 7th edn., McGraw Hill, New York, in the press
- Pettit, F. H., Yeaman, S. J. and Reed, L. J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4881–4885
- Heffelfinger, S. C., Sewell, E. T. and Danner, D. J. (1983) *Biochemistry* **22**, 5519–5522
- Reed, L. J., Pettit, F. H., Yeaman, S. J., Teague, W. M. and Bleile, D. M. (1980) in *Enzyme Regulation and Mechanism of Action* (Mildner, P. and Ries, B., eds.), pp. 47–56, Pergamon Press, Oxford
- Randle, P. J., Fatania, H. R. and Lau, K. S. (1984) in *Enzyme Regulation by Reversible Phosphorylation – Further Advances* (Cohen, P., ed.), pp. 1–26, Elsevier, Amsterdam
- Marshall, L. and DiGeorge, A. (1981) *Am. J. Hum. Genet.* **33** (Suppl.), 139A
- Matsuda, I., Nobukuni, Y., Mitsubuchi, H., Indo, Y., Endo, F., Asaka, J. and Harada, A. (1990) *Biochim. Biophys. Res. Commun.* **172**, 451–464
- Fisher, C. R., Fisher, C. W., Chuang, D. T. and Cox, R. P. (1991) *Am. J. Hum. Genet.* **49**, 429–434
- Fisher, C. R., Chuang, J. L., Cox, R. P., Fisher, C. W., Star, R. A. and Chuang, D. T. (1991) *J. Clin. Invest.* **88**, 1034–1037
- Davie, J. R., Wynn, R. M., Cox, R. P. and Chuang, D. T. (1992) *J. Biol. Chem.* **267**, 16601–16606
- Chowdhury, J. R., Grossman, M., Gupta, S., Chowdhury, N. R., Baker, J. R. and Wilson, J. M. (1991) *Science* **254**, 1802–1805
- Osborne, W. R. A., Hock, R. A., Kaleko, M. and Miller, A. D. (1990) *Hum. Gene Ther.* **1**, 31–41
- Kay, M. A., Ponder, K. P. and Woo, S. L. C. (1992) *Breast Cancer Res. Treat.* **21**, 83–93
- Hoogerbrugge, P. M., Vossen, J. M. J., Van Beusechem, V. W. and Valerio, D. (1992) *Hum. Gene Ther.* **3**, 553–558
- Miller, G. and Lipman, M. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 190–194
- Miller, A. D. and Rosman, G. J. (1989) *BioTechniques* **7**, 980–990
- Chuang, J. L., Cox, R. P. and Chuang, D. T. (1993) *J. Biol. Chem.* **268**, 8309–8316
- Fisher, C. W., Chuang, J. L., Griffin, T. A., Lau, K. S., Cox, R. R. and Chuang, D. T. (1989) *J. Biol. Chem.* **264**, 3448–3453
- Fisher, C. W., Fisher, C. R., Chuang, J. L., Lau, K. S., Chuang, D. T. and Cox, R. P. (1993) *Am. J. Hum. Genet.* **52**, 414–424
- Lau, K. S., Chuang, J. L., Herring, W. J., Danner, D. J., Cox, R. P. and Chuang, D. T. (1992) *Biochim. Biophys. Acta* **1132**, 319–321
- Markowitz, D., Goff, S. and Bank, A. (1988) *J. Virol.* **62**, 1120–1124
- Corsaro, C. M. and Pearson, M. L. (1981) *Somat. Cell Genet.* **7**, 603–616
- Miller, A. D., Trauber, D. R. and Buttimore, C. (1986) *Somat. Cell Mol. Genet.* **12**, 175–183
- Miller, A. D. and Buttimore, C. (1986) *Mol. Cell. Biol.* **6**, 2895–2902
- Lynch, C. M. and Miller, A. D. (1991) *J. Virol.* **65**, 3887–3890
- Miller, A. D. and Verma, I. M. (1984) *J. Virol.* **49**, 214–222
- Peebles, P. T. (1975) *Virology* **67**, 288–291
- Cone, R. D., Weber-Benarous, A., Baorto, D. and Mulligan, R. C. (1987) *Mol. Cell. Biol.* **7**, 887–897
- Chuang, D. T. and Cox, R. P. (1988) *Methods Enzymol.* **166**, 135–146
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- DiLella, A. G. and Woo, S. L. C. (1987) *Methods Enzymol.* **152**, 199–212
- Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13
- Dariush, N., Fisher, C. W., Cox, R. P. and Chuang, D. T. (1991) *FEBS Lett.* **284**, 34–38
- Kuntz, M. J., Paxton, R., Shimomura, Y., Goodwin, G. W. and Harris, R. A. (1986) *Biochem. Soc. Trans.* **14**, 1077–1078
- Scharfmann, R., Axelrod, J. H. and Verma, I. M. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4626–4630
- Indo, Y., Kitano, A., Endo, F., Akaboshi, I. and Matsuda, I. (1987) *J. Clin. Invest.* **80**, 63–70
- Fatania, H. R., Lau, K. S. and Randle, P. J. (1982) *FEBS Lett.* **147**, 35–39
- Yeaman, S. J., Cook, K. G., Boyd, R. W. and Lawson, R. (1984) *FEBS Lett.* **172**, 38–42
- Shinnick, F. L. and Harper, A. E. (1976) *Biochim. Biophys. Acta* **437**, 477–486
- Dancis, J., Hutzler, J. and Levitz, M. (1960) *Biochim. Biophys. Acta* **43**, 342–343
- Harper, P. A., Healy, P. J. and Dennis, J. A. (1986) *Vet. Rec.* **119**, 62–65
- Baird, J. D., Wojcinski, Z. W., Wise, A. P. and Godkin, M. A. (1987) *Can. Vet. J.* **28**, 505–511