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Versatile cosmid vectors for the isolation, expression, and rescue of gene sequences: Studies with the human α-globin gene cluster

(human recombinant DNA library/selectable gene marker)

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ABSTRACT **We have developed a series of cosmids that can be used as vectors for genomic recombinant DNA library preparations, as expression vectors in mammalian cells for both transient and stable transformations, and as shuttle vectors between bacteria and mammalian cells. These cosmids were constructed by inserting one of the SV2-derived selectable gene markers—SV2-gpt, SV2-DHFR, and SV2-neo—in cosmid pJB8. High efficiency of genomic cloning was obtained with these cosmids and the size of the inserts was 30–42 kilobases. We isolated recombinant cosmids containing the human α-globin gene cluster from these genomic libraries. The simian virus 40 DNA in these selectable gene markers provides the origin of replication and enhancer sequences necessary for replication in permissive cells such as COS 7 cells and thereby allows transient expression of α-globin genes in these cells. These cosmids and their recombinants could also be stably transformed into mammalian cells by using the respective selection systems. Both of the adult α-globin genes were more actively expressed than the embryonic ζ-globin genes in these transformed cell lines. Because of the presence of the cohesive ends of the Charon 4A phage in the cosmids, the transforming DNA sequences could readily be rescued from these stably transformed cells into bacteria by *in vitro* packaging of total cellular DNA. Thus, these cosmid vectors are potentially useful for direct isolation of structural genes.**

The common cloning vectors used to isolate DNA fragments from total genomic DNA are bacterial plasmids, bacteriophages, and cosmids (1). Cosmids are bacterial plasmids to which the cohesive ends of the bacteriophage have been attached to allow packaging with extracts of lysogenic bacteria (2). Up to 45 kilobases (kb) of DNA can be inserted into cosmid vectors. Cloning such large DNA fragments increases the likelihood of isolating an intact gene or gene family from the genomic DNA and thus of preserving any functional domain present in the genes and their flanking sequences.

An efficient system for assaying the expression of cloned mammalian genes is DNA-mediated gene transfer into tissue culture cells (3–7). Gene expression can be studied by either transient (5–10) or stable (3, 4, 11–13) transformation of these cells. For transient transformation, viral enhancing sequences are often used to increase gene expression (14–18), while selectable gene markers are usually used to isolate stable transformants (11–13, 19–21). However, inserting eukaryotic genes into these expression vectors often necessitates trimming the flanking sequences, some of which may influence the expression of the gene(s) under study. If the selectable gene markers and viral enhancer sequences are incorporated into the cosmid vector prior to cloning, the recombinant cosmid containing the genes of interest can be isolated and used directly to transform mammalian cells without further manipulation (22). A major advantage of these cosmid systems, as we will report, is that they can also serve as shuttle vectors to rescue the transforming DNA fragments from the transformed cell lines and reintroduce them in bacteria. The retrieval of such cloned sequences from total cellular DNA permits further definition of the functional units of eukaryotic genes.

We report the construction of a series of cosmid vectors that can serve as (*i*) cloning vehicles for preparing genomic recombinant DNA libraries and gene isolation, (*ii*) expression vectors for both transient and stable transformation in tissue culture cells, and (*iii*) shuttle vectors between bacteria and mammalian cells. We used these vectors to isolate the human α-globin gene cluster, study α- and ζ-globin gene expression, and rescue the human α-globin genes from the transformed cells.

MATERIALS AND METHODS

Cosmid pJB8 (23) and *Escherichia coli* ED8767 (K. Murray) were obtained from R. A. Flavell. All simian virus 40 (SV40)-derived selectable gene markers were obtained from P. Berg. COS 7 cells (24) were obtained from M. R. Botchan. Hypoxanthine/guanine phosphoribosyltransferase-lacking ($HGPRT^-$) Chinese hamster ovary cells (TGA) were obtained from M. Siciliano, and thymidine kinase-lacking (TK^-) mouse L cells, from M. Bishop. *Mbo* I, *Bgl* II, and T4 DNA ligase were from New England BioLabs; *Bam*HI, S1 nuclease, and calf intestine alkaline phosphatase were from Boehringer Mannheim; *Bam*HI and *Bgl* II linker sequences were from Collaborative Research (Waltham, MA). The antibiotic G418 was a gift from P. J. L. Daniels of Schering. High molecular weight DNA was purified from the peripheral blood of a normal adult as described (25).

Construction of Expression Cosmid Vectors. Cosmid pJB8 was modified by insertion of gene markers derived from SV40 hybrid genes containing selectable markers of *E. coli* HGPRT (SV2-gpt) (19), mouse dihydrofolate reductase (SV2-DHFR) (20), and aminoglycoside 3′-phosphotransferase II or the neomycin-resistance gene (SV2-neo) (21). The segment containing the SV40 origin and the selectable gene markers was excised from plasmid pSV2 by digestion with *Bam*HI (for SV2-gpt and SV2-neo) or *Bam*HI/*Pvu* II (for SV2-DHFR) and isolated by glass bead extraction (26). The Pvu II site of the fragment from SV2-DHFR was converted to *Bam*HI sites with synthetic *Bam*HI linkers, and the segments were inserted into the *Bal* I site of pJB8, which had previously been converted to a *Bgl* II site. In the resultant new cosmids, both *Bam*HI and *Bgl* II recognition sequences were abolished at the insertion sites.

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Abbreviations: kb, kilobase(s); SV40, simian virus 40; HGPRT, hypoxanthine/guanine phosphoribosyltransferase; TK, thymidine kinase.

Construction of Human Cosmid Libraries. The general procedures for cosmid library construction have been described (23, 27). Briefly, human leukocyte DNA was partially digested with *Mbo* I and the 30- to 45-kb fractions were collected after centrifugation in a 5–25% sucrose gradient. Vector DNA was completely digested with *Bam*HI and the 5′ end was dephosphorylated with calf alkaline phosphatase. Dephosphorylation was monitored by self-ligation and bacterial transformation (23). Mixtures of 0.2–0.4 μg of the 30- to 45-kb insert DNA and 0.5–1 μg of linearized cosmid DNA were prepared and the DNAs were ligated in 10 μl with T4 DNA ligase; 1–2 μl of ligated DNA was packaged with extracts prepared according to the procedure of Pirotta, as described (27). The packaged cosmids were diluted to 250 μl with 10 mM Tris·HCl, pH 7.4/100 mM NaCl/10 mM $MgCl_2$/0.2% gelatin. They were used to transduce 250 μl of *E. coli* ED8767, which had been grown overnight in L broth with 0.4% maltose and then suspended at double strength in 10 mM $MgCl_2$. After 15 min at room temperature, 1 ml of L broth was added to each transduction mixture and these mixtures were incubated for 30 min at 37°C. Fifty microliters of transduced bacteria was plated on a 100-mm L plate containing ampicillin at 50 μg/ml (LA plates) to quantitate the number of transformants. The remaining cells were either plated directly onto nitrocellulose filter (HATF, Millipore) in a 150-mm LA plate or pooled and amplified in 10 vol of L broth/ampicillin. They were stored at −70°C in 7% dimethyl sulfoxide or 10% glycerol, or as purified DNA.

Cell Transformation and Analysis of α-Globin Gene Expression. Mammalian cells were transformed with DNA from the cosmids and their recombinants by the calcium phosphate precipitation method (28). Transient expression of globin-containing cosmids in COS 7 cells was determined as described (17) using the transforming DNA at 25 μg/ml. Stable cell transformants were selected by using their respective selection systems (19–21). The transforming DNA at approximately 5 μg/ml without carrier was added to 10^6 TGA or L cells in a 100-mm dish for 16–20 hr. Stably transformed cell clones were isolated and grown up to mass cultures, and the number of copies of transforming sequences was estimated by Southern blot analysis (24). Poly(A)$^+$ RNA was purified from both acutely transfected and stably transformed cells as described (29). The expression of the transformed genes was analyzed by RNA blotting (30) and nuclease S1 mapping (9, 31).

Rescue and Analysis of Transforming DNA. Transforming sequences were recovered into bacteria by extracting total DNA from the transformed cell lines and *in vitro* packaging with extracts from lysogenic bacteria, as described above. α-Globin gene organization in the rescued cosmids was compared with that in the original cosmid by Southern analysis using nicked translated α- or ζ-globin gene probes.

RESULTS

Construction of Cosmid Vectors with Selectable Markers. The general structure of the three cosmid vectors reported here is shown in Fig. 1. These vectors contain selectable markers of the SV40-hybrid genes, SV2-gpt, SV2-DHFR, and SV2-neo, as well as the 72- and 21-base-pair (bp) repeats, and the SV40 origin of replication. The markers allow respective selection in hypoxanthine/aminopterin/thymidine (HAT) medium in HGPRT$^-$ cells or dominant selection in the presence of methotrexate or the antibiotic G418. Those cosmids containing SV2-gpt, SV2-DHFR, and SV2-neo selectable markers were designated cosmid vectors pCV103, pCV107, and pCV108, respectively.

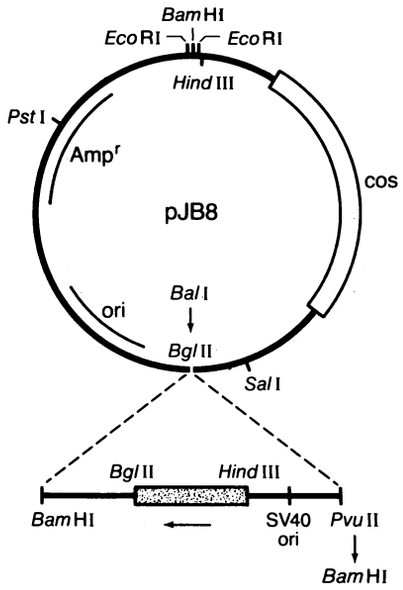

FIG. 1. Structure of the expression cosmid vectors. The stippled bar indicates the location of the selectable gene markers, SV2-gpt, -DHFR, or -neo in the three cosmids, pCV103, pCV107, and pCV108, respectively.

Construction of Human Genomic DNA Libraries by Using Expression Cosmids. We used vectors pCV103, pCV107, and pCV108 to construct human recombinant DNA libraries. An average efficiency of 5.7×10^5 colonies per μg of insert DNA was obtained, as compared with 4×10^8 plaque-forming units per μg of similarly packaged Charon 4A DNA. Restriction enzyme digestion of "minipreps" from randomly picked colonies showed that 98% of all recombinant cosmids contained inserts of 30–42 kb. Each of the cosmid libraries contained $3–7 \times 10^5$ independent colonies.

Isolation of the Globin Gene-Containing Recombinants. About 150,000 colonies from the pCV108 library were screened with a ^{32}P-labeled cDNA probe reverse transcribed from reticulocyte poly(A)$^+$ RNA. Eleven colonies hybridized positively to this probe, and secondary screenings identified five that contained globin genes. After restriction mapping and hybridization with cloned α- and β-globin probes, three were found to contain α-globin sequences and two contained β-globin sequences. One recombinant (pCL9), which had a 42-kb insert and contained the entire α-globin gene cluster, was used for expression and rescue studies. The pCL9 map is shown in Fig. 2.

Expression of the Human α-Globin-Like Genes in Mammalian Cells. We tested the efficiency of the cosmid vectors and the recombinants containing globin genes in stably transforming cultured mammalian cells according to their respective selection systems (Table 1). Similar to previously reported results (19–21), transforming efficiency ranged from 1 per 10^4 to 1 per 10^3 cells.

α-Globin gene expression was studied in COS 7 cells transiently transformed with pCL9 and in four L-cell clones (L9C-1, L9C-2, L9C-3, L9C-4) that had been stably transformed with the same recombinant. Results of Southern blot analysis showed <1, 3, 1, and 25 α-globin gene copies, respectively, in the four stably transformed cell lines (Fig. 3). Poly(A)$^+$ RNA isolated from both acutely transfected COS 7 cells and stably transformed L cells was analyzed by RNA blot analysis (Fig. 4). Hybridization with the α-globin gene probe showed a large amount of α-globin mRNA in the transient transformants. Three out of

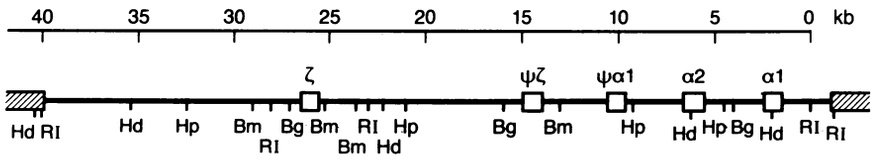

FIG. 2. Partial restriction map of pCL9, a recombinant cosmid containing the entire human α-globin gene cluster in cosmid vector pCV108. RI, *Eco*RI; Hd, *Hin*dIII; Bg, *Bgl* II; Hp, *Hpa* I; Bm, *Bam*HI.

the four L-cell clones showed various amounts of α-globin mRNA expression. The one clone that expressed no α-globin mRNA contained no α-globin DNA sequences by Southern analysis, indicating that these had been deleted from the cosmid sequence. The α-globin transcripts were identical in size to human reticulocyte mRNA, indicating that they were properly processed. Comparison of the intensity of RNA bands in the autoradiograms relative to the amount of poly(A)$^+$ RNA applied to the gel showed 0.5–4% as many α-globin-specific transcripts in the poly(A)$^+$ RNA from these cell lines as in those transcripts from human reticulocytes. Using the calculations of Mellon *et al.* (17), we estimate that the α-globin transcripts in both COS 7 and L cells constitute 0.5–4% of the total cellular poly(A)$^+$ RNA and that 5,000–40,000 copies of α-globin mRNA were present per cell, similar to their results. Since only a fraction of COS 7 cells was transfected in the transient gene expression system, the number of α-globin mRNA molecules per cell probably exceeds this estimate.

When the RNA filter from the pCL9 transformants was washed and hybridization was carried out with a ^{32}P-labeled embryonic ζ-globin gene probe, much less ζ- than α-globin mRNA was detected, and the x-ray film had to be exposed for 10 days for the ζ-globin probe, instead of 20 hr for the α-globin probe. RNA species corresponding to 10S globin mRNA and to higher molecular weight RNA were observed. Comparison of the poly(A)$^+$ RNAs from normal reticulocytes, K562 cells, and the L-cell lines indicated that the upper band at the 10S region was ζ-globin mRNA and that the lower band in this region resulted from cross-hybridization of the ζ-globin probe with α-globin mRNA. This finding is compatible with the known lengths of α- and ζ-globin mRNA sequences (32, 33); excluding the poly(A) region, ζ-globin mRNA is about 50 nucleotides longer than α-globin mRNA. The origin of the high molecular weight transcripts is not known. The ζ-globin genes were not transcribed in acutely transfected COS 7 cells nor in two of the four stably transformed L-cell lines. ζ-Globin mRNA was detected in the other two cell lines, and comparison of the intensity of hybridization in these L-cell lines with the α- and ζ-globin probes showed much less ζ- than α-globin mRNA transcription.

The expression of the selectable gene markers was studied by hybridizing the filters with probes prepared from the SV2-neo marker DNA. RNA from both acutely transfected COS 7 cells and stably transformed L cells hybridized strongly, and RNA band intensity was similar to that of the α-globin transcripts. Transcription of the α- and ζ-globin and the SV2-neo genes was proportional to the number of copies of the transforming cosmid (pCL9) per cell in the stably transformed L-cell lines analyzed.

The duplicated human adult α-globin genes are not transcribed equally in human bone marrow cells and reticulocytes; the α2-globin mRNA is 2–3 times as abundant as the α1-globin mRNA (33–35). We used a 3′ probe starting from the *Bst*EII site in the third exon of the normal α2-globin gene (33) to analyze, by nuclease S1 mapping, the mRNA transcripts from these cells to determine the ratio of these two α-globin mRNAs in the transformed cells. Because of sequence divergence in the 3′ untranslated sequences of the two genes, the probe yielded protected fragments of 218 and 108 nucleotides for α2- and α1-globin transcripts, respectively. The two α-globin genes were transcribed in both transient and stable transformants (Fig. 5). The number of transcripts from the α2-globin gene was equal to or greater than those from the α1-globin gene in the stably transformed mouse cells.

Rescue of the Cosmids from Transformed Cells. The restriction patterns of the human α- and ζ-globin DNA genes in the transformed cells were similar to those of the input cosmids (Fig. 3). As the integration of transforming DNA is often arranged in tandem (36, 37), the cohesive ends in the transforming sequences would be spaced a cosmid-length apart and suitable as substrate in a packaging reaction. We therefore explored the feasibility of recovering the cosmids from the transformed cells by *in vitro* packaging of DNA from cell clones L9C-1, L9C-3, L9C-2, and L9C-4, containing <1, 1, 3, and 25 copies per cell, respectively, of the transformed PCL9 cosmids and from an additional cell line TGA 1B, containing about 250 copies of pCL1 cosmids per cell. (pCL1 is a pCV103 cosmid with a 13-

Table 1. Transformation efficiency of expression cosmids

Transforming DNA	Transformation efficiency*	Selection system	Cell type†
pCV103 (SV2-gpt)	9.6	HAT	TGA
pCV108 (SV2-neo)	3.8	G418	TGA
pCV108 (SV2-neo)	1.2	G418	L
pCL1 (SV2-gpt)	1.6	HAT	TGA
pCL9 (SV2-neo)	3.0	G418	L

*Results represent colonies per 10^4 cells.
†TGA cells are HGRPT$^-$ and L cells are TK$^-$.

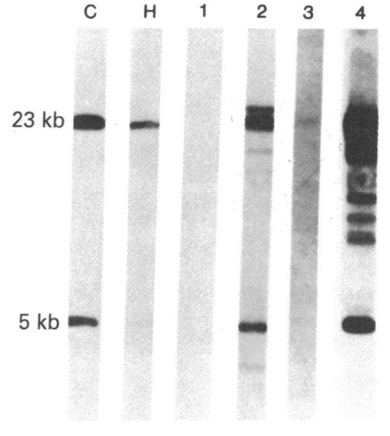

FIG. 3. Southern blot analysis of transforming DNA sequences in stably transformed cell clones. Five micrograms of DNA from each sample was digested with *Eco*RI. Mixed α- and ζ-globin gene probes were used for hybridization. The 23-kb fragment contains the α1-, α2-, and ψζ-globin genes; the 5-kb fragment contains the ζ-globin gene. Lanes: C, 50 ng of pCL9 (2-hr exposure); H, normal human DNA (20-hr exposure); 1–4, DNA from clones L9C-1, L9C-2, and L9C-3 (3-day exposure) and clone L9C-4 (20-hr exposure).

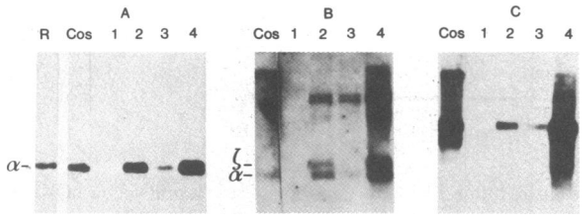

FIG. 4. RNA blot analysis of poly(A)$^+$ RNA isolated from acutely transfected or stably transformed cells with cosmid pCL9. Lanes: R, 10 ng of poly(A)$^+$ RNA from normal human reticulocytes; Cos, 2 μg of poly(A)$^+$ RNA isolated from COS 7 cells 60 hr after transfection with pCL9; 1–4, 2-μg samples of poly(A)$^+$ RNA from stably transformed L-cell clones L9C-1, L9C-2, L9C-3, and L9C-4, respectively. The filters were hybridized with the α-globin gene probe (20-hr exposure time) (*A*); the ζ-globin gene probe (10-day exposure) (*B*); and the SV2-neo gene probe (48-hr exposure) (*C*). With the ζ-globin probe, the upper band at the 10S region was ζ-globin mRNA and the lower band was due to cross-hybridization of the α-globin mRNA with the ζ probe on prolonged exposure. After a 1-wk exposure (data not shown), a small amount of the neo RNA was seen in clone LC9-1.

kb fragment of human DNA containing the $\psi\alpha 1, \alpha 2$-, and $\alpha 1$-globin genes.) Samples (0.5–4 μg) of total DNA of these cell clones were packaged *in vitro*. The number of ampicillin-resistant colonies recovered from the various cell clones is presented in Table 2. No cosmids were rescued from the two cell lines with 1 or <1 copy per cell. In the cell lines containing three or more copies per cell, the efficiency of cosmid rescue increased proportionally with the number of copies of the transforming cosmids per cell. Twenty-two randomly picked colonies from pCL9 rescues were "miniscreened" by Southern hybridization with either human α- or ζ-globin probes (Fig. 6). Four of these rescued cosmids had restriction patterns identical to those of their original transforming cosmids while the remainder showed DNA rearrangements and deletions involving either the ζ- or $\psi\zeta$-globin genes.

DISCUSSION

We have constructed several expression cosmid vectors that can be used as cloning vehicles for preparation of genomic recombinant DNA libraries. These vectors can accommodate up to 45-kb DNA inserts, can be selected for growth in bacteria in the presence of ampicillin, and can stably transform mammalian cells. Insertion of the human DNA into the vectors did not change the efficiency of transformation to tissue culture cells. These cloning vectors therefore provide an easy transition from isolation of DNA sequences to functional analysis of cloned genes.

As shown by the experiments with cosmids containing the human α-globin gene cluster, the cosmid libraries can be used to study transient and stable gene expression in mammalian cells. The transient gene expression in COS 7 cells depended on the ability of introduced circular DNA to replicate and amplify in high copy number (15, 17). The SV2-derived vectors provided the SV40 enhancer sequences with an origin of replication in their selectable gene markers and hence allowed autonomous replication of these recombinant cosmids within the permissive COS 7 cells (19–21). Similar to the stable transformants, these

Table 2. Efficiency of cosmid rescue from cell clones with different copies of transforming DNA

Cell clones	Transforming sequence	Copies, no. per cell	Efficiency of recovery,* colonies: No. per μg	Efficiency of recovery,* colonies: No. per copy
L9C-1	pCL9	<1	0	0
L9C-3	pCL9	1	0	0
L9C-2	pCL9	3	1.3	0.43
L9C-4	pCL9	25	40	1.6
TGA1B	PCL1	250	940	3.8

* Efficiency of packaging of Charon 4A DNA was 3×10^8 plaque-forming units per μg.

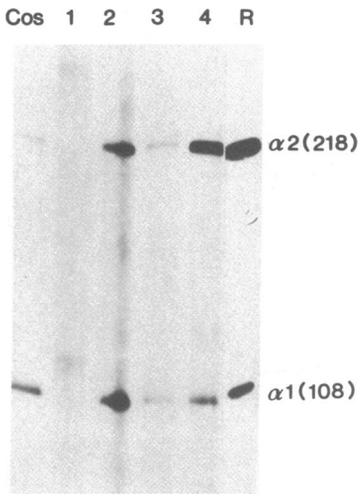

FIG. 5. Nuclease S1 map of α-globin gene transcripts. Because of the sequence divergence between the two α-globin genes, the probes gave protected fragments of 218 and 108 nucleotides, respectively, for $\alpha 2$ and $\alpha 1$ transcripts. Lanes: Cos, RNA from COS 7 cells 60 hr after transfection with pCL9; 1–4, transcripts from the transformed cell lines shown in Fig. 4 (L9C-1, L9C-2, L9C-3, and L9C-4); R, human reticulocyte RNA.

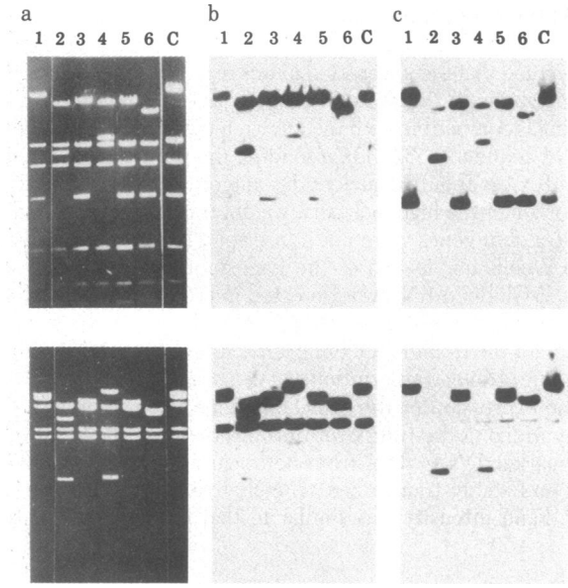

FIG. 6. Southern blot hybridization of rescued cosmids from total DNA of L cells transformed with cosmid pCL9. Identical gels were stained with ethidium bromide to visualize the DNA bands and then denatured and blotted onto nitrocellulose filters (*a*). The filters were hybridized with either α- (*b*) or ζ-globin (*c*) probes. (*Upper*) Digestion was with *Eco*RI. (*Lower*) Digestion was with *Bgl* II. Lanes: 1–6, examples of rescued cosmids; C, the original cosmid, pCL9. Note that all rescued cosmids contained sequences that hybridized with α- and ζ-globin gene probes. Lane 1 shows a rescued cosmid identical to cosmid pCL9, which was originally used to transform the L cells. Lanes 2–6 show examples of rescued cosmids with DNA rearrangements.

α-globin transcripts were properly spliced and polyadenylylated.

Blot analysis of RNA from transformed cells showed more adult α-globin gene transcription than embryonic ζ-globin gene transcription. This difference cannot be explained by the effects of gene dosage, as the average ratio of α- to ζ-globin genes in the rescued cosmids was about 3:1. The marked disparity in transcription rate was much greater and probably reflects the depressed state of the embryonic ζ-globin genes in somatic cells.

Shuttle vectors capable of mediating gene transfer between bacteria and mammalian cells are valuable tools to study the relationship between gene structure and expression. DNA sequences can be introduced into suitable mammalian cells using these vectors to assay their expression and can subsequently be recovered in bacteria to study the structural changes that affect gene function in mammalian cells (7, 17, 18, 38). At present, there are several useful shuttle vectors available; some require extensive DNA cloning and rearrangement before the genes can be introduced into mammalian cells (7, 17, 18, 38) while others require fusion of transformed cells with permissive cells before the transforming sequences can be rescued into bacterial host (39, 40). The cosmid vectors described herein have advantages over currently available shuttle vectors because they can be used as vectors for genomic library preparation to isolate the desired genes, which can then be transferred transiently or stably into mammalian cells. Second, the gene sequences can easily be recovered by *in vitro* packaging reactions of the cellular DNA. In these experiments, as few as three copies per cell of the transforming sequences was sufficient for the cosmid rescue procedure. In addition, we have also successfully rescued α-globin-containing cosmids from packaging total DNA from a mouse that had previously been microinjected with one of these cosmids at the one-cell embryo stage (unpublished data). Furthermore, the cosmid vectors can also be used as rescue vectors to study the role of host DNA sequences in integration, expression, and stability of transforming DNA.

The cosmid vectors described here are potentially useful for direct isolation of functional units of genes (such as *TK* or *HGPRT*) based on their expression in selectable systems. Alternatively, genes encoding surface markers could also be isolated by cell sorting (41).

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