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ABOUT THE COVER:
Characteristics of endothelial cells. Endothelial cells stained for Factor VIII-related antigen (upper photograph). Endothelial cells stained red due to uptake of DiI-acetylated LDL (bottom photo). (see article on page 10)

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MONOCLONAL ANTIBODY PRODUCTION IN THE CELLMAX[®] ARTIFICIAL CAPILLARY SYSTEM

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Hybridomas grown in conventional cell culture systems, such as flasks, suspension culture, or roller bottles, are exposed to decreasing concentrations of nutrients and oxygen and increasing concentrations of lactic acid and ammonium ion (1–3). In contrast, hybridomas cultured in artificial capillaries are continuously bathed in a fresh supply of oxygen and nutrients from the circulating growth medium, while at the same time lactate and growth-inhibitory secreted proteins, such as TGF- β_1 , diffuse out of the extracapillary space (ECS) surrounding the cells and are diluted into the circulating medium (3–5).

Artificial capillary cell culture provides an ideal environment for high-level monoclonal antibody (MAb) production. Hybridomas cultured in artificial capillary systems generally secrete an increased amount of MAb in comparison to MAb production in conventional

culture (8,11,12). With some hybridomas, a single Artificial Capillary Module can produce levels of MAb similar to ascites concentrations (1–5 mg/ml) and 15–60 mg of antibody per harvest. Furthermore, 3-dimensional culture emulates *in vivo* pericellular growth conditions and cell-cell interactions (6–9), allowing for a 5- to 10-fold reduction in serum and rapid adaptation to serum-free or protein-free medium formulations.

METHODS

The hybridomas were mouse hybridoma OKT8, secreting IgG_{2a} (ATCC #CRL 8014, P3X63Ag8UI-derived); mouse hybridoma 3G8, secreting anti-CD16 IgG₁; and a human/mouse heterohybridoma (human B cell/HAT-sensitive heterohybridoma-derived) secreting an anti-B cell human IgM.

Hybridoma cell lines were cultured in RPMI-1640 or CO₂-Independent Medium

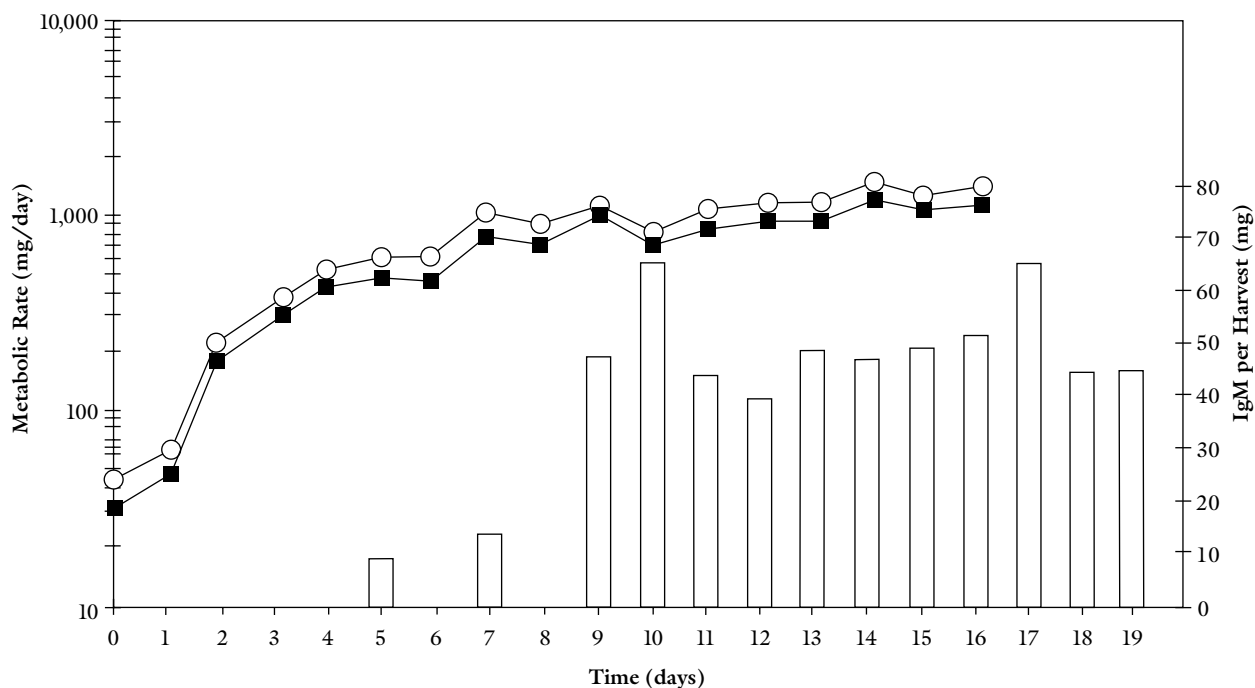


FIGURE 1. Glucose utilization, lactate production, and MAb production. The mouse/human IgM heterohybridoma (1.0×10^8 cells) was cultured in RPMI-1640 with 10% FBS. Samples were removed daily from the reservoir bottle for glucose and lactate determination. Glucose consumed (\circ), lactate produced (\blacksquare), or MAb produced (\square) per 24-h time period was calculated.

supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µM 2-mercaptoethanol, and 1% to 10% fetal bovine serum (FBS); or Hybridoma-SFM or Protein-Free Hybridoma Medium (PFHM-II) supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin. Unless otherwise indicated, all cell culture media and supplements were GIBCO BRL brand.

Cells were cultured in the CELLMAX Artificial Capillary System using an MPS Artificial Capillary Module as recommended by the manufacturer. Hybridoma cells (0.5×10^8 to 1×10^8) were inoculated into the ECS of the artificial capillary cartridge. A 1-ml sample of the growth medium was removed from the reservoir bottle daily and monitored for lactate and glucose. Glucose depletion and lactate production rates were calculated to measure the metabolic activity of the culture. Medium was replaced when the glucose concentration of the medium in the reservoir bottle was depleted to 50% of the initial concentration. Reservoir volume was increased from an initial 125 ml, to 500 ml, and lastly to a 1-L medium bottle at successive medium changes.

At day 7 to 10 following inoculation, or when glucose or lactate rates reached 700 to 1,000 mg/24 h, regular MAb harvests were initiated from the ECS. The MAb supernatant was clarified by centrifugation and stored at 4°C. As the glucose consumption and lactate production rates increased, the CELLMAX system required more frequent harvests and reservoir bottle changes, often several times per week. The ECS was harvested each time the 1-L reservoir bottle was changed.

RESULTS AND DISCUSSION

Following inoculation of hybridoma cells into the ECS of an MPS Artificial Capillary Module, a logarithmic increase in daily consumption of glucose and a concomitant production of lactate were observed as the cells rapidly expanded within the fiber bundle (figure 1). By monitoring these parameters, it was possible to predict the medium usage. Also, the rate of MAb production correlated to glucose consumption. As hybridoma cell lines reach the mature stage of their growth cycle, they display a maximum metabolic activity range characteristic that hybridoma cell line.

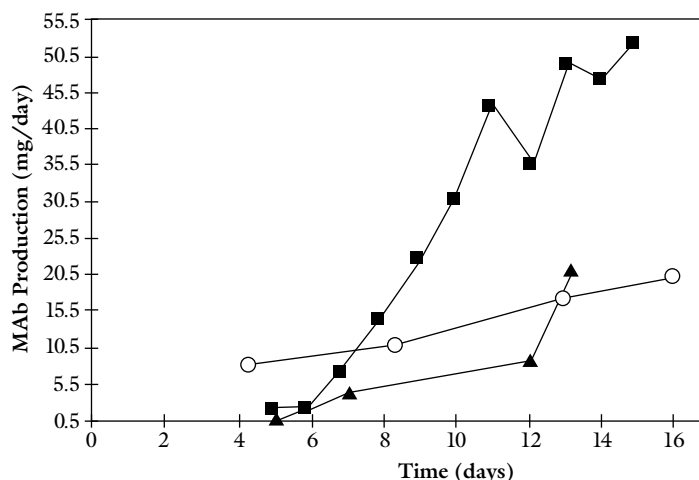


FIGURE 2. MAb production for 3 cell lines. Hybridomas (1×10^8 cells) producing IgG_{2a} (▲), IgG₁ (○), and IgM (■) were cultured in RPMI-1640 with 10% FBS.

The mouse/human IgM heterohybridoma exhibited a consistent glucose consumption rate of 800 to 1,100 mg of glucose consumed/24 h beginning on day 8, even with multiple ECS harvests and medium changes. At this homeostatic growth phase, this IgM-secreting hybridoma consumed a liter of medium every 24 h. Therefore, ECS MAb harvests were performed on a daily basis. After day 9, when the system reached optimal cell density, consistently high levels of MAb (30–60 mg) were harvested on a daily basis. From day 9 through day 19, the average daily production was approximately 50 mg of IgM per harvest.

Three different hybridoma cell lines were grown in the CELLMAX system. There was a marked difference in the production rates for each of these three hybridomas (figure 2). Since each hybridoma is a unique somatic cell fusion product, it can be expected that each hybridoma will exhibit a characteristic level of glucose consumption and MAb secretion (8,11,12). For long-term production of MAbs, using routine aseptic technique and proper daily maintenance, CELLMAX Artificial Capillary Modules can produce consistent amounts of MAb for many months.

Growth of cells in artificial capillaries, in contrast to T-flasks, is characterized by an environment where cell-specific growth factors are concentrated in the ECS in the absence of metabolic waste. Cells are nurtured in a microenvironment containing autoregulated

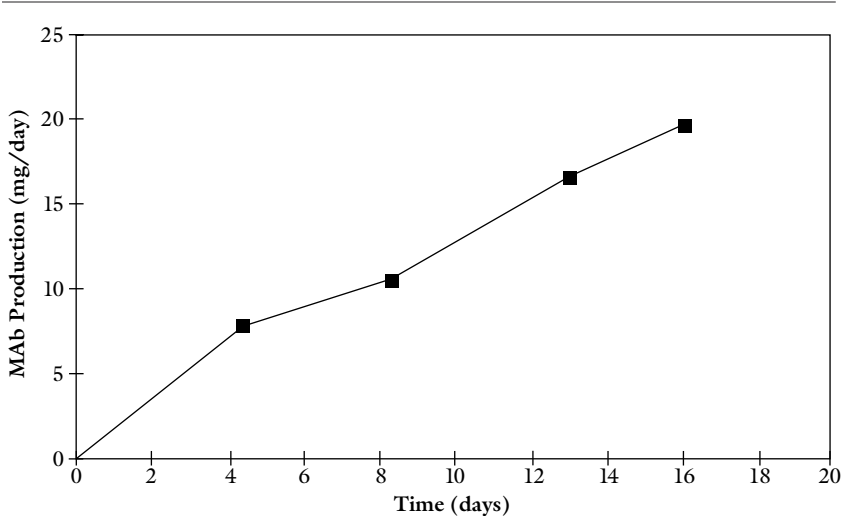


FIGURE 3. MAb production during serum reduction. Hybridoma 3G8 was inoculated into the ECS in RPMI-1640 with 10% FBS. FBS amounts were decreased to 7.5%, 2.5%, and 1.25% on days 5, 7, and 11, respectively.

concentrations of cell-specific growth factors for extended periods of time. Since these culture conditions are closer to the *in vivo* environment, the amount of serum necessary

should be less than in conventional cell culture. Initially, monitoring glucose and lactate production was used to measure the effect of reduced serum as well as adaptation to alternative media formulations on the growth rate of the hybridoma cell line (data not shown). Many cell lines can be adapted to serum concentrations as low as 1% to 2%. For hybridoma 3G8, antibody production was not affected by a reduction of serum from 10% to 1.25% over 11 days (figure 3).

Serum-free and protein-free media may reduce the need for the downstream purification of antibody. Many hybridomas readily adapt to growth in a serum-free medium, while maintaining the same growth rate and levels of MAb secretion. MAb production and glucose consumption of a hybridoma adapted to serum-free medium were examined (figure 4). 7 days after adaptation to serum-free medium (low-protein formulation), the cultured medium was changed to a completely protein-free medium for MAb production. During day

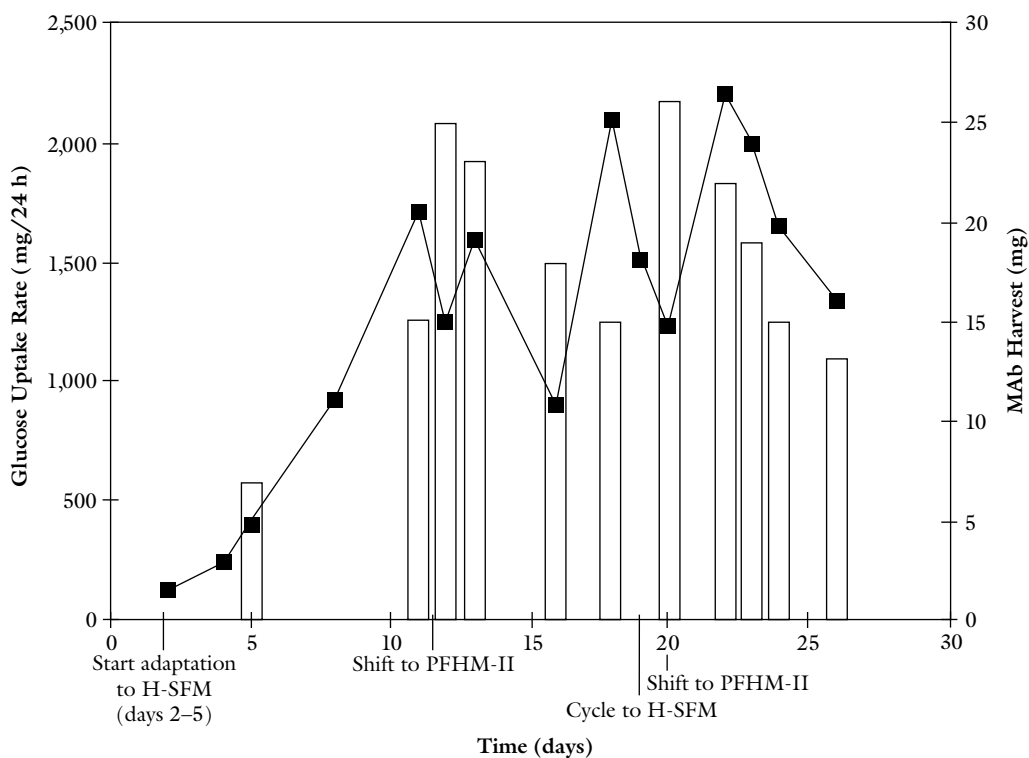


FIGURE 4. MAb production using Hybridoma-SFM and PFHM-II. Hybridoma OKT8 (1×10^7 cells) was adapted in flask culture to RPMI-1640 with 1% FBS. Cells were inoculated into the ECS of a hybridoma module supplemented with RPMI-1640 and 1% FBS. The cells were adapted to Hybridoma-SFM (H-SFM) over 3 days by a stepwise increase in Hybridoma-SFM in the circulating medium. MAb harvests (\square) and glucose (\blacksquare) determinations were performed periodically. On day 12, the medium was changed to PFHM-II. On day 18, the medium was changed to Hybridoma-SFM for 2 days and then back to PFHM-II on day 20.

12 through day 18, MAb production decreased slightly. To boost production, at day 18 the PFHM-II medium was changed back to Hybridoma-SFM for 2 days, and then at day 20 the medium was cycled back to PFHM-II. This serum-free/protein-free medium cycling produced nearly the same MAb production as from serum-free medium alone, with the added benefit of a MAb sample that was collected in a protein-free medium.

CO₂-independent medium may provide an alternative cell culture method to hybridoma culture in conventional CO₂ incubators. For most cell lines, if they grow well in T-flask culture using CO₂-independent medium, then the hybridomas will perform well using artificial capillary cell culture in CO₂-independent medium. The CELLMAX Artificial Capillary System was used successfully to culture hybridoma cells in CO₂-independent medium with FBS (figure 5).

In summary, hybridomas cultured in artificial capillaries yield antibody concentrations of 0.1 to 5 mg/ml depending on the secretion characteristics of the specific hybridoma. These yields are comparable to those seen with mouse ascites fluid and much greater than cultures in T-flasks or roller bottles (5 to 20 µg/ml, ref. 8,11,12). Hybridoma cells adapted to serum-free, protein-free, or CO₂-independent media can also be grown in artificial capillaries with high yields of MAb.

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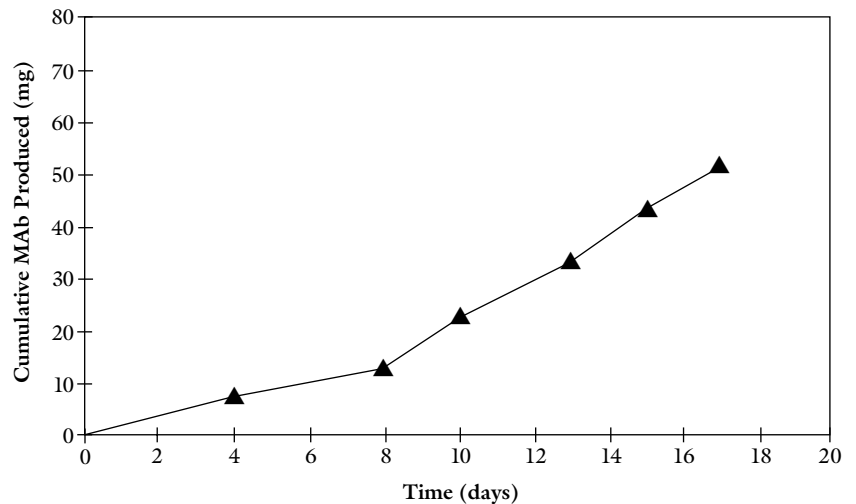


FIGURE 5. MAb production in CO₂-Independent Medium. Hybridoma 368 cells (5×10^7 cells) adapted to CO₂-Independent Medium with 10% FBS in a T-flask were cultured in the Artificial Capillary System.

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ISOLATION AND CULTURE OF HEPATOCYTES

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An important function of the liver is the detoxification and metabolism of xenobiotics. This is accomplished in the hepatocytes (cells that comprise over 70% of the liver cell mass), by a group of microsomal hemoprotein enzymes that have different substrate specificities (1,2). These enzymes are collectively known as the cytochrome P450 (CP450) system. Inducibility by hormones, drugs, and chemicals associated with enhanced microsomal oxidation and reduction (1-3) is a characteristic property of this enzyme system.

Hepatocytes in culture, besides presenting a system for studying xenobiotic metabolism, are also being used extensively for the development of an extracorporeal liver support system for the treatment of acute liver failure, and as an ectopic organ for the correction of numerous inborn errors of metabolism of liver origin.

As with other primary culture systems, there are several requirements for the successful establishment of hepatocyte monolayer cultures. Unlike most primary culture systems, adult hepatocytes are multinucleated and only a small percentage of the population divide. The requirements of this culture system are an optimized isolation protocol (4,5), medium and substratum for the attachment of viable hepatocytes (6), and provision of optimal conditions for the long-term survival and function (7) of the cells.

Adult rodent hepatocytes in culture lose cell viability and function over time (8-10) as measured by decreasing levels of CP450 (11,12) or of CP450-catalyzed reactions (9,12,13), although the CP450 inducibility (12) is still apparent. A number of approaches have been tried to maintain hepatocyte CP450 isozymes. Attachment matrices used include a liver cell biomatrix (14), an extracellular matrix (EHS) (15,16), a collagen matrix (17), two layers of hydrated collagen (18) or a layer of collagen and EHS (19), to generate a sandwich matrix or co-culture with other cell types (20,21). Also, the choice of medium (17,22) and sup-

plementation of media with hormones (13,23) such as dexamethasone are important. DMSO, testosterone, polycyclic hydrocarbons, or barbiturates (24) can stimulate CP450 levels.

In the present study, we describe media for the isolation, purification, and culture of adult rat hepatocytes. HepatoZYME-SFM (Serum-Free Medium) for attached hepatocytes is based on a modification of Chee's medium (25). Chee's medium is able to preserve the protein content of adult rat hepatocytes in long-term culture (26). HepatoZYME-SFM has been optimized for maintaining the long-term viability of primary rat hepatocytes and is useful for the maintenance and induction of selected CP450 isozymes. The present study investigates the effects of HepatoZYME-SFM on CP450 levels and metabolic capacity of cells cultured at high density on collagen and EHS matrices as compared to those found with serum-supplemented Williams' E Medium. The gel electrophoretic profiles of microsomal proteins are also investigated.

METHODS

Preparation of isolated hepatocytes. Hepatocytes were isolated from adult male Sprague-Dawley rats by the two-step method of Seglen (5). The liver was perfused *in situ* with Liver Perfusion Medium (Ca-free buffer, Cat. No. 17701) followed by treatment with Liver Digest Medium (collagenase-dispase L-15, Cat. No. 17703). The liver was aseptically transported to the cell culture laboratory in cold Liver Transport Medium (Hepes-buffered L-15 with BSA, Cat. No. 17702). The hepatocytes were purified by Percoll (27) density gradient separation and washed twice with the Hepatocyte Wash Medium (Williams' Medium E with BSA, Cat. No. 17704) before being resuspended in Hepatocyte Attachment Medium (Modified Williams' Medium E, Cat. No. 17706) supplemented with 2 mM L-glutamine and 5% fetal bovine serum (FBS).

About 10×10^6 cells were plated in 150-cm² tissue culture flasks precoated with

12.5 $\mu\text{g}/\text{cm}^2$ rat tail collagen I (28) matrix or flasks coated with 100- $\mu\text{g}/\text{cm}^2$ MATRIGEL™ EHS matrix (an extract isolated from mouse Engelbreth-Holm-Swarm sarcoma containing laminin, collagen IV, proteoglycans, and growth factors) (Collaborative Research Inc.) and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. After 2 h, unattached cells and medium were removed and replaced with HepatoZYME-SFM (Cat. No. 17705), supplemented with 2 mM L-glutamine or with Williams' E Medium supplemented with 2 mM L-glutamine and 5% FBS. Both media were supplemented with 1.25 $\mu\text{g}/\text{cm}^2$ rat tail collagen to form a sandwich matrix (18,29,30). Cultures were refed with media without collagen at 24 h and every 48 h thereafter. Serum-supplemented Williams' E was chosen as the control, as this combination is widely used and developed specifically for the culture of hepatocytes. To some cultures, 0.5 mM hexobarbital (HB) was added 48 h before harvesting the cells.

Preparation of microsomes. Cells were harvested from the tissue culture dishes by scraping and recovered by centrifugation (800 $\times g$ for 5 min). The pellet was resuspended in 5 ml of ice-cold 100 mM phosphate buffer (pH 7.4) containing 1 mM dithiothreitol (DTT) and cells disrupted with a sonicator. The nuclei and mitochondria were removed by centrifugation at 30,000 $\times g$ for 10 min. The microsomal pellet was obtained by centrifugation of the supernatant fraction at 105,000 $\times g$ for 30 min. The microsomal pellet either was stored frozen at -70°C or was analyzed after being homogenized in 40 mM phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA, 0.1 mM DTT, 0.5 $\mu\text{g}/\text{ml}$ leupeptin, 0.7 $\mu\text{g}/\text{ml}$ pepstatin, and 68 $\mu\text{g}/\text{ml}$ toluenesulfonyl fluoride (TSF).

Analytical procedures. Protein was determined by the method of Lowry (31). CP450 concentrations were determined from the dithionite-reduced CO-difference spectrum, using an extinction coefficient of 91 $\text{mm}^{-1}\text{cm}^{-1}$ (32). The spectra were recorded on a Shimadzu Dual Beam Spectrophotometer equipped with baseline correction and peak wavelength indicator.

Metabolic studies. Catalytic activity of the CP450 isozymes was measured using

7-ethoxycoumarin *O*-deethylase (ECOD) (33,34) activity.

Immunological studies. Antibodies to 3 different isozymes of CP450 (IA1, IIIA1, and IIB1) and to NADPH CP450 reductase were obtained from Oxygene. SDS/polyacrylamide gel electrophoresis was performed according to the method of Laemmli (35). After separation, the proteins were transferred to nitrocellulose membranes (36). Signals were detected with a peroxidase-conjugated second antibody and 4-chloro-1-naphthol.

RESULTS AND DISCUSSION

Several technical advancements in the isolation and culture of hepatocytes have cumulatively led to tissue culture systems for the long-term maintenance of primary hepatocytes expressing differentiated functions. Plating of hepatocytes at high density under conditions that allow cell-cell contact as well as cell-matrix contact helps promote maintenance of the differentiated phenotype *in vitro* (37). With our procedure in more than 30 isolations, the yield of viable hepatocytes (cells that did not stain with trypan blue) was about 1×10^8 to 4×10^8 cells/liver. The hepatocytes attached at efficiencies of 65% to 75% within 2 h independent of the matrix used.

Phase contrast microscopy of the adult non-proliferating hepatocytes cultured for 24 h in either serum-free or serum-supplemented medium revealed a number of flattened, binucleated cells on the collagen matrix. In contrast when cells were cultured on the EHS matrix, they remained round and formed small aggregates that adhered strongly to the matrix. The morphology of cells on either matrix was not related to the ability to induce CP450 by HB

TABLE 1. Effect of culture medium and substratum on CP450 activity. Hepatocytes were cultured for 9 days in Williams' Medium E with 5% FBS (WE + FBS) or HepatoZYME-SFM on a collagen I or an EHS sandwich matrix. Results are the average of 2 determinations.

Medium/Matrix	HB	ECOD (nmol/min/mg protein)
Primary Suspension	-	7.0
WE + FBS/EHS matrix	+	3.2
WE + FBS/collagen I	+	3.8
Hepatocyte-SFM/EHS Matrix	-	4.7
Hepatocyte-SFM/EHS Matrix	+	15.3
Hepatocyte-SFM/collagen I	-	3.5
Hepatocyte-SFM/collagen I	+	11.1

TABLE 2. Metabolic studies of the microsomal fraction of primary adult rat hepatocytes. Cells were cultured in Williams' Medium E with 5% FBS (WE + FBS) or HepatoZYME-SFM on a collagen I sandwich matrix. Results are the average from 3 animals with each condition in triplicate \pm SD.

Day	Medium	HB	Total CP450 (pmol/mg protein)	ECOD Activity (pmol/min/mg protein)
0			189 \pm 14	10.3 \pm 0.1
4	WE + FBS	+	38 \pm 2	5.3 \pm 0.1
	HepatoZYME-SFM	-	146 \pm 17	7.6 \pm 0.1
6		+	160 \pm 16	15.4 \pm 0.2
	WE + FBS	+	not detectable	2.5 \pm 0.1
	HepatoZYME-SFM	-	117 \pm 14	8.8 \pm 0.4
8		+	146 \pm 12	13.1 \pm 0.6
	WE + FBS	+	not detectable	2.3 \pm 0.1
	HepatoZYME-SFM	-	95 \pm 15	6.3 \pm 0.2
		+	132 \pm 10	15.1 \pm 0.7

(table 1). In HepatoZYME-SFM, cell morphology remained unchanged over an 8-day culture period, contrary to the loss of cells in serum-supplemented cultures, starting at about day 3. Cell morphology at 24 h was poorer in collagen IV and V alone compared to collagen I or the EHS matrix (data not shown), suggesting that some individual components of the matrix may not in themselves support hepatocyte growth (16).

Table 2 shows a difference in the level of total P450 and ECOD activity in cells cultured in HepatoZYME-SFM versus that of cells cultured in the Williams' Medium E with serum. There was a large variability in the CP450 content and monooxygenase activity of the isolated

hepatocytes prepared from different rats. The CP450 levels varied from 150 to 250 pmol/mg protein between experiments. This was apparently caused by animal variation, since viability and attachment efficiency were always similar. In uninduced cells with HepatoZYME-SFM, there was a loss of 23% in total CP450 on day 4 and 50% after 8 days in culture. In uninduced cells with serum-supplemented Williams' E, no CP450 signal was obtained after 24 h in culture. Treatment of cultures with HB resulted in an increase in microsomal CP450. This retention of inducibility closely mimics *in vivo* conditions, where the liver maintains very low levels of enzyme until stimulated by a xenobiotic.

Cells cultured in HepatoZYME-SFM showed a decrease in de-ethylase activity (table 2) over the 8 days, although this decrease was much less than for cells in serum-supplemented Williams' E. Following HB induction, there was a significant increase (3-fold of uninduced or 1.5-fold over Day 0) in the de-ethylation activity of cells cultured in HepatoZYME-SFM. De-ethylase activity has been linked to CP450 IA1, IIA1, IIB1, and IIB2, with CP450 IA1 giving the highest activity (2).

Western blots of induced microsomes isolated from hepatocytes were performed to monitor levels of CP450 isozymes. In the absence of HB, the signals for CP450 IA1 and IIIA1 isozymes were absent in cells cultured in Williams' E and were very faint in

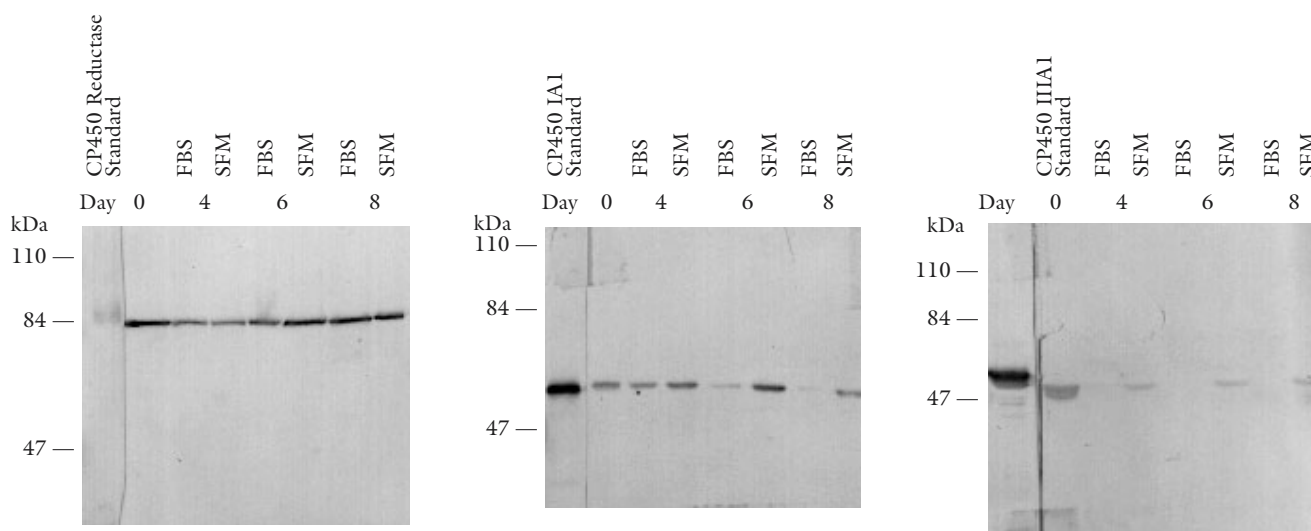


FIGURE 1. Western blot analysis of CP450 reductase and isozymes of CP450. Hepatocytes were cultured in Williams' E + 5% FBS (FBS) or HepatoZYME-SFM (SFM) for 0, 4, 6, and 8 days. Cells were stimulated with HB 48 h prior to harvest. 25 μ g of sample were loaded per lane. The blots were immunostained for CP450 IA1, IIIA1, or for CP450 reductase.

HepatoZYME-SFM (data not shown). With HB treatment (figure 1), the expression of CP450 reductase was constant while the expression of CP450 IA1 and IIIA1 was dependent on the medium used. For serum-supplemented Williams' E, there was a decline in the level of CP450 IA1 antigen, and for CP450 IIIA1 no signal remained by 6 days in culture. In HepatoZYME-SFM, the levels of both these antigens remained unchanged. Although apparent in freshly iso-lated adult hepatocytes, a lower molecular weight isoform of CP450 IIIA1 was lost in culture even in HepatoZYME-SFM. The results in figure 1 correlated to the enzyme activity shown in table 1. CP450 IIB1 was not detected at day 0 or after induction with HB (data not shown).

In summary, HepatoZYME-SFM improved both the culture of primary monolayers of adult rat hepatocytes on collagen I or EHS matrices, and the retention of inducibility of CP450 at near-normal levels for at least 9 days. Also, the cells maintained their ability to carry out metabolism of xenobiotics such as ethoxycoumarin. The long-term maintenance of functional adult hepatocytes *in vitro* without loss of enzymatic activity is a valuable tool for studies on metabolic processing of drugs and carcinogens *in vitro*, and it may allow for the development of an extracorporeal liver support system.

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A SERUM-FREE MEDIUM FOR THE CULTURE OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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The ability to cultivate vascular endothelial cells *in vitro* has led to the development of many useful models for the study of endothelial cell physiology under both normal and pathological conditions. However, most of the studies of endothelial cell function have been *in vitro* experimentation utilizing serum-supplemented media (1). The use of high concentrations of animal sera creates many obstacles, such as lot-to-lot performance variability, presence of adventitious agents, and fluctuations in price and availability (2,3). The presence of serum can mask a desired effect or detection of a mediator present in low quantities (4,5). Additionally, the undefined character of serum makes it undesirable for media supplementation as endothelial cell research moves toward therapeutic applications.

We previously reported on Endothelial Serum-Free Medium (SFM) designed specifically for the culture of nonhuman vascular endothelial cells (6). Endothelial-SFM effectively supports the growth and maintenance of

bovine, porcine, canine, and ovine large vessel endothelial cells while maintaining standard markers of physiologic function. However, this nutrient medium does not support the growth of human endothelial cells without further supplementation with bovine pituitary extract, EGF, and hydrocortisone. Even with additional hormones and growth factors, human endothelial cells could only be maintained for a limited number of passages in Endothelial-SFM. This article describes Human Endothelial-SFM, a low-protein SFM specifically optimized to support the long-term culture of human umbilical vein endothelial cells (HUVEC).

MATERIALS AND METHODS

Isolation and culture of human endothelial cells. Unless otherwise indicated, all media, growth factors, and cell culture reagents were GIBCO BRL brand. HUVEC were isolated from umbilical cords obtained from normal vaginal and caesarean section deliveries using a modification of the procedure described by Jaffe *et al.* (7). Briefly, untraumatized umbilical vein segments were cannulated and flushed with 50 ml of Medium 199 supplemented with 5 µg/ml gentamicin. Endothelial cells were isolated by incubating umbilical veins with 0.1% collagenase, type II, in Medium 199 for 25 min at 22°C. Vessels were then flushed with 50 ml of Medium 199, the cell suspension centrifuged (100 × *g*, 22°C, 5 min), and the cell pellet washed 2 times with Medium 199. The final cell pellet was suspended in 2 ml of Human Endothelial-SFM. Primary cultures were established in 25 cm² tissue culture flasks (Costar) preincubated with 20 µg/ml human plasma fibronectin (HPFN) in a final volume of 5 ml. Fibronectin can be added directly to the culture medium when establishing primary cultures. However, better cell yields were observed with flasks pretreated with fibronectin. Serum-free cultures were supplemented with 20 ng/ml human recombinant basic FGF (hrbFGF),

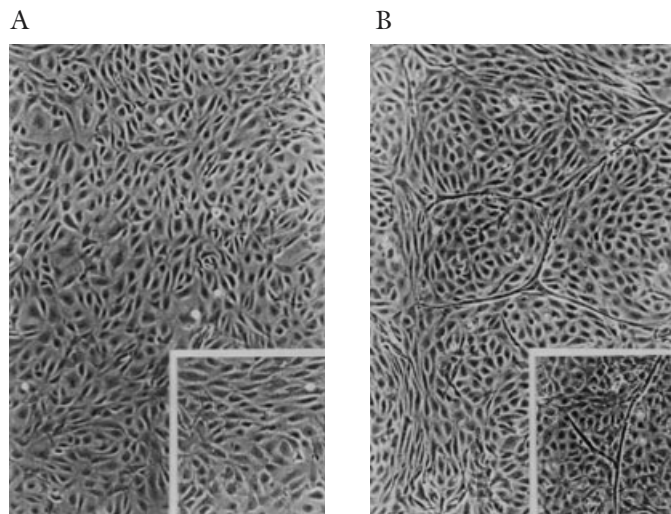


FIGURE 1. Phase contrast microscopy of endothelial cells cultured in Human Endothelial-SFM. Panel A. Normal “cobblestone” morphology (inset: serum-supplemented Medium 199). Panel B. Capillary-like tube structures (inset: serum-supplemented Medium 199).

10 ng/ml human recombinant EGF (hrEGF), and 5 µg/ml gentamicin. Cultures were maintained with a loosened cap at 37°C in humidified air containing 5% CO₂. Serum-supplemented cultures were established as described above and maintained in Medium 199 supplemented with 20% fetal bovine serum (FBS), 20 ng/ml human recombinant acidic FGF (hraFGF), 17.6 U/ml heparin, and 5 µg/ml gentamicin. Primary cultures were fluid changed 24 h postseeding (without gentamicin) and subcultured upon reaching confluence using 0.05% Trypsin-0.53 mM EDTA. Secondary cultures were established in SFM supplemented with 20 ng/ml hrbFGF, 10 ng/ml hrEGF, and HPFN added directly to the SFM at a concentration of 10 µg/ml.

Evaluation of Factor VIII-related antigen. HUVEC previously cultured in Human Endothelial-SFM or serum-supplemented Medium 199 were processed for the visualization of Factor VIII-related antigen using indirect immunofluorescence (8). The primary antibody was rabbit anti-human von Willebrand antigen (1:40 dilution, Dako) and the secondary antibody was GIBCO BRL *Premium Quality* FITC-Conjugated Goat Anti-Rabbit IgG (1:50 dilution).

Evaluation of DiI-acetylated LDL uptake. Endothelial cell uptake of DiI-acetylated LDL (1:20 dilution, Biomedical Technologies, Inc.) was evaluated using fluorescence microscopy (9).

Quantitation of cyclic adenosine monophosphate (cAMP) production. For the evaluation of cAMP production, HUVEC from control and serum-free cultures were seeded at a density of 4×10^5 cells/well (100,000 cells/cm²); following a 24-h culture period, spent culture medium was removed, the cell sheet was washed (3X), and 2 ml of Human Endothelial-SFM or serum-free Medium 199 was added. Cultures were treated with the following agonists/inhibitors: 100 µM 3-isobutyl-1-methylxanthine (IBMX), 1 µM isoproterenol, 50 µM forskolin, and no addition (control). Following a 15-min incubation (37°C), culture media were removed and the cell monolayers washed with D-PBS (4°C). Cellular cAMP was extracted using 70% ethanol (4°C)(10). Ethanol extracts were evaporated using a Savant Automatic SPEEDVAC® (22°C, 6 h) and stored at -20°C prior to being reconstituted in 1 ml

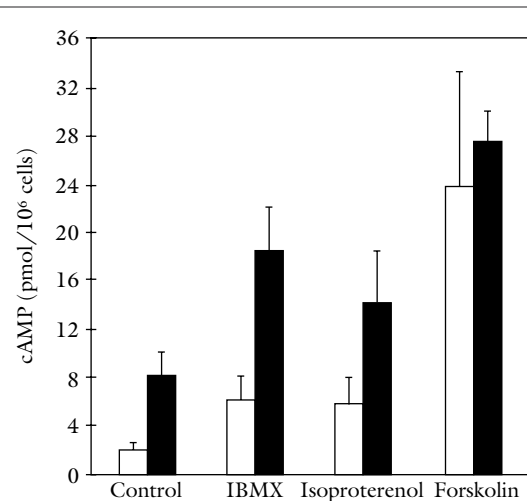


FIGURE 2. cAMP production by endothelial cells cultured in serum-free versus serum-supplemented media. Medium 199 with 20% FBS (□), Human Endothelial-SFM (■). Values are the mean ± SEM, n = 5.

of 0.1 M sodium acetate. cAMP concentrations were determined by the GIBCO BRL Non-Isotopic Immunoassay System for cAMP (11) and are expressed as picomoles of cAMP/ 1×10^6 cells.

Evaluation of HUVEC growth in SFM. For growth kinetic analysis, HUVEC from control and serum-free cultures were seeded at densities of 0.4×10^5 cells/well (10,000 cells/cm²), 0.8×10^5 cells/well (20,000 cells/cm²), 1.2×10^5 cells/well (30,000 cells/cm²), and 1.6×10^5 cells/well (40,000 cells/cm²). The total number of cells/well was determined at 24-h intervals for 96 h without media replacement. Cells cultured in SFM were supplemented with 20 ng/ml hrbFGF, 10 ng/ml hrEGF, and 10 µg/ml HPFN. Serum-containing HUVEC cultures were supplemented with 20% FBS, 20 ng/ml hraFGF, and 17.6 U/ml heparin.

RESULTS

HUVEC cultured in Human Endothelial-SFM exhibited the same histotypic “cobblestone” morphology as observed in serum-supplemented cultures (figure 1). As is sometimes observed in serum-supplemented cultures (12), HUVEC grown in SFM developed capillary-like tube structures upon reaching confluence (figure 1). HUVEC cultured in SFM expressed Factor VIII-related antigen (cover photograph), a standard marker

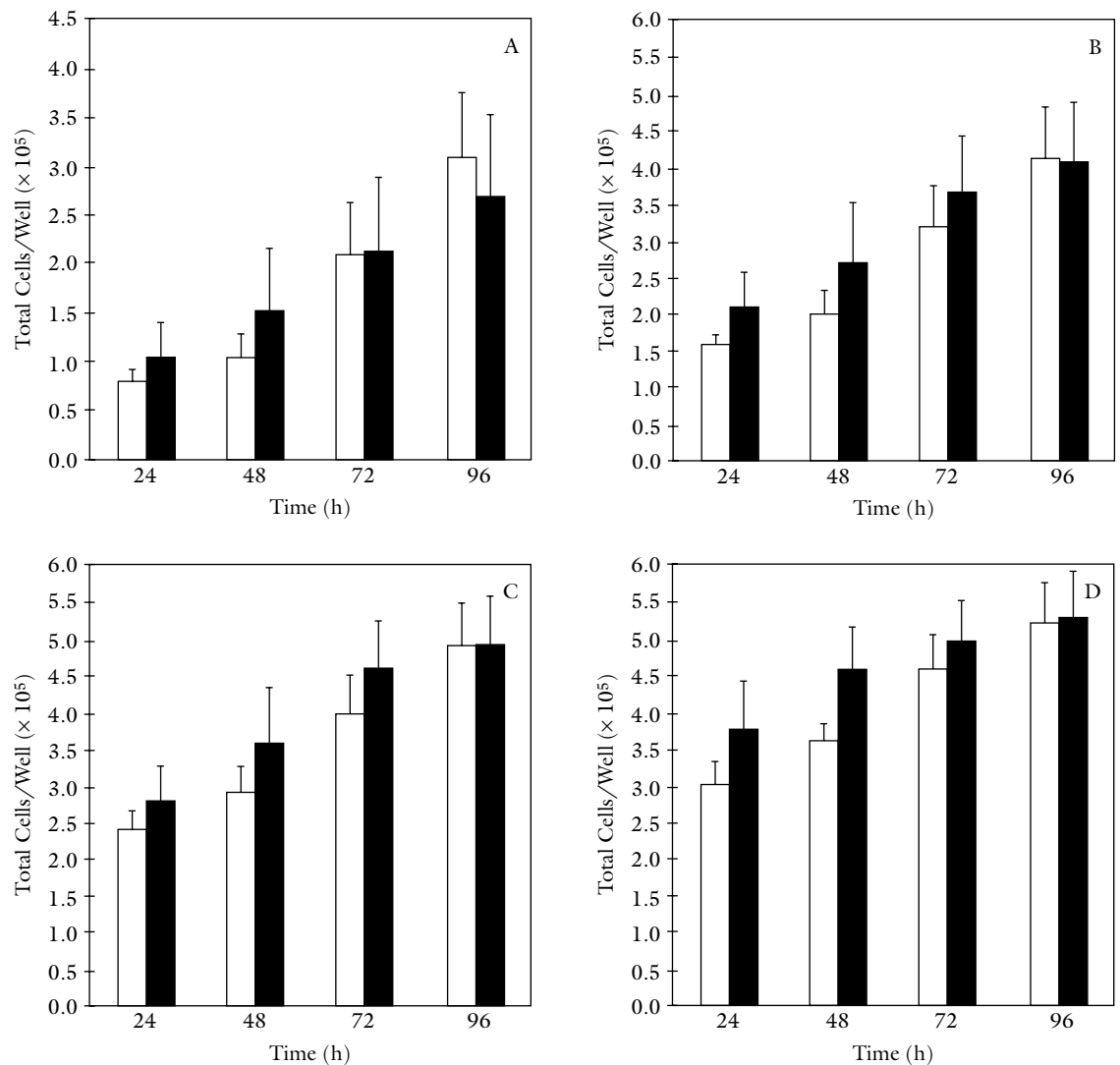


FIGURE 3. Growth kinetic analysis of endothelial cells cultured in serum-free versus serum-supplemented media. Initial seeding densities were: Panel A, 0.4×10^5 ; Panel B, 0.8×10^5 ; Panel C, 1.2×10^5 ; and Panel D, 1.6×10^5 cells/well. Medium 199 with 20% FBS (\square), Human Endothelial-SFM (\blacksquare). Values are the mean \pm SEM, n = 3.

of vascular endothelial cells, and took up DiI-acetylated LDL (cover photograph), indicating the functionality of the scavenger pathway for acetylated-LDL metabolism. HUVEC cultured in Human Endothelial-SFM have also been demonstrated to maintain UEA-1 lectin binding, vinmentin, and IL-1 α -induced ICAM-1 expression (data not shown). Basal and treatment-induced increases in cAMP production were similar between serum-free and serum-supplemented cultures (figure 2). The growth of HUVEC in SFM was comparable to that observed for serum-supplemented cultures at all of the seeding densities tested (figure 3). Human Endothelial-SFM successfully sup-

ported primary isolation and subsequent secondary growth of HUVEC for >10 passages.

DISCUSSION

HUVEC cultured in Human Endothelial-SFM exhibit the histotypic "cobblestone" morphology and retain endothelial-specific markers including: expression of Factor VIII-related antigen, uptake of DiI-acetylated LDL, UEA-1 lectin binding, vinmentin, and IL-1 α -induced ICAM-1 expression. Additionally, HUVEC cultured in SFM have been shown to maintain a functional adenylate cyclase/cAMP signal transduction system. When supplemented with hrbFGF and hrEGF, the growth rate of

HUVEC in SFM is similar to that obtained for cells cultured in Medium 199 supplemented with 20% FBS, hraFGF, and heparin.

Serum-free culture of endothelial cells eliminates many of the problems associated with the use of high concentrations of animal sera and may provide the controlled environment necessary to conduct definitive studies of endothelial cell physiology and pathophysiology. Additionally, the development of therapeutic applications involving cultured endothelial cells will be facilitated by serum-free technology.

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EDITOR'S NOTE:

GIBCO BRL Human Endothelial SFM Medium is available alone (Cat. No. 11111-044) or in a system containing basic FGF, EGF and HPFN (Cat. No. 11111-036).



What do you recommend for trypsinizing my endothelial cells grown in serum-free medium?

Cells in serum-free medium are very sensitive to trypsin. Use 0.05% trypsin for dissociation. Trypsin should be removed prior to cell detachment and cells resuspended in serum-free medium. Wash cells twice by centrifuging at $100 \times g$ for 3 to 4 min. Stopping trypsin activity with soybean trypsin inhibitor is not necessary.

SERUM-FREE CULTURE OF HUMAN ENDOTHELIAL CELLS FOR LEUKOCYTE ADHESION APPLICATIONS

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The endothelium is the interface between the circulatory and tissue compartments. Recent advances in understanding the human endothelial cell at the molecular level have made it clear that the vascular endothelium is an active, potent, and finely tuned physiological monitor and information transduction system (1-7). The vascular endothelial cell is involved in both cell-mediated and humoral immune responses. Species-specific, activation-specific, and site-specific functional subsets of vascular endothelial cells also exist (8-17).

One area of recent advance has been in stimulated expression of cell surface and cell adhesion molecules that are involved in the interaction of human endothelial cells and leukocytes (18). These endothelial adhesion macromolecules (19) and their soluble isoforms (20) are involved in mechanisms of specific information transfer and physiologic monitoring operative at the cellular and organismal levels.

The presence of serum in an experimental system can mask detection of an effect (21,22) or result in detection of effects not observed in the presence of serum in primate and animal systems (23,24). This paper describes a new cell culture tool that combines primary human vascular endothelial cell cultures with Human Endothelial Serum-Free Medium (SFM).

METHODS

Isolation of human venous endothelial cells. Thirty-six umbilical cords obtained from normal deliveries were individually tested for HIV antibody in cord blood using an HIV-1 ELISA test. Endothelial cells were isolated using standard methods (25). Briefly, veins were cannulated, flushed free of blood, and incubated with 50 $\mu\text{g}/\text{ml}$ collagenase to remove the endothelial cells. The primary cell isolates were pooled, washed, and plated in plastic culture flasks coated with a nondefined endothelial cell

attachment factor (ECAAF), CS-AF-1.0 (Cell Systems). Five percent of the pooled primary isolate was cultured in GIBCO BRL M199, a proprietary endothelial cell growth supplement (CS-C supplement, Cell Systems) with 10% FBS. Ninety-five percent of the pooled primary isolate was cultured in GIBCO BRL Human Endothelial-SFM supplemented with 20 ng/ml basic FGF and 10 ng/ml EGF. The cultures were incubated at 37°C in humidified 5% CO₂. When the cultures were first fed at 24 h, cell coverage was >25%. The cultures were fed every other day. At confluence, the cells were removed from the flasks with 0.5 U/ml trypsin, 0.18 mg/ml EDTA; the protease was inactivated with an equal volume of trypsin inhibitor solution; and cells were cryopreserved in 50% conditioned and 50% fresh Human Endothelial-SFM + 7.5% DMSO.

The pooled serum-free venous endothelial cell culture consisted of 500 identical aliquots of $>5.0 \times 10^5$ recoverable cells. The serum-containing venous endothelial cell culture consisted of 25 identical aliquots of $>5.0 \times 10^5$ recoverable cells. Average yield per umbilical vein as assessed at 24 h *in vitro* was 1.0×10^6 cells. At the time of freezing, all pooled venous endothelial cells were at confluence in primary culture [<3 cumulative population doublings (CPD) *in vitro*]. No antibiotics were used at any stage in the process.

Growth assays. An aliquot of the pooled cryopreserved cell culture was recovered and cultured in 25-cm² culture flasks coated with CS-AF-1.0 ECAAF using Human Endothelial-SFM supplemented with basic FGF and EGF. Alternatively, cells were cultured in 25-cm² culture flasks using Human Endothelial-SFM, growth factors, and 10 $\mu\text{g}/\text{ml}$ GIBCO BRL Human Plasma Fibronectin (HPFN). Cell coverage at 24 h was >25%. The cultures reached confluence within 5 days of plating and were subcultured into 75-cm² culture flasks (1:3 split). Subcultures at a 1:3 split ratio have

TABLE 1. Immunofluorescent characterization of human venous endothelial cells.

Marker	Result
Factor VIII-related antigen	>95% Positive
CD 62P (P-selectin)	>99% Positive
Vimentin cytoskeleton	>99% Positive
Smooth muscle actin	<1% Positive
Smooth muscle desmin	<1% Positive
R-PE secondary Ab	<1% Positive
Sham primary Ab (mouse IgG ₁)	<1% Positive

Cells were cultured on chamber slides coated with CS-AF-1.0 ECAF in Human Endothelial-SFM. Cells were fixed with methanol and then incubated with various primary antibodies. Positive cells were detected by fluorescence after incubation with a secondary antibody — R-phycoerythrin (PE) conjugate.

been maintained for 8 passages. For the quantitative determination of growth, 25-cm² flasks coated with CS-AF-1.0 ECAF were seeded with 20 × 10³ cells/cm² in serum-containing or serum-free medium. Cell growth was determined at 24-h intervals.

Adherence assays. For cytokine-stimulated adherence, cells were plated in 24-well plates at a density of 20 × 10³ cells/cm² on CS-AF-1.0 ECAF and grown to confluence in Human Endothelial-SFM. Triplicate wells were stimulated with each cytokine. 1.0 × 10⁶ THP-1 human monocytes (ATCC TIB 202) grown in M199 + 10% FBS and resuspended in Human Endothelial-SFM were added to all wells and incubated for 30 min. Nonadherent leukocytes were removed by gentle washing.

For quantitative analysis of the relative effect of extracellular matrix attachment factors on the *E. coli* LPS-stimulated leukocyte-endothelial cell adhesion interaction, cells were plated in 24-well plates at a density of 20 × 10³ cells/cm². Half of the wells were coated with CS-AF-1.0 ECAF and half were seeded in medium containing HPFN. The cells were grown to confluence in Human Endothelial-SFM. Three wells were not stimulated. Nine wells were stimulated with 100 ng/ml of *E. coli* LPS (O111:B4) for 4 h. Six of the LPS-treated wells were supplemented with 1% FBS at the time of LPS addition (as a source of LPS-binding protein). Three of the LPS + 1% FBS wells were incubated for the final 30 min of LPS treatment with 50 µg/ml CAMFolio MAb to CD 62E (26) (common names: E-selectin, ELAM-1). 1.0 × 10⁶ THP-1 human monocytes (ATCC TIB 202) were

added to all wells and incubated for 30 min. Nonadherent cells were removed by gentle washing.

RESULTS AND DISCUSSION

Characterization of cell cultures. A pool of primary human vascular endothelial cells never exposed to serum or to nondefined mitogens was isolated, cryopreserved, and recovered using the Human Endothelial-SFM System. Recovered cells tested negative for HIV by PCR and tested negative for mycoplasma. Cell cultures were derived from pooled primary isolates to minimize individual functional assay variability due to individual donor heterogeneity. We compared *E. coli* LPS-stimulated adherence of human polymorphonuclear leukocytes to endothelial cells (at >6 CPD *in vitro*) for 50 individual isolates and 50 aliquots of a pool of 121 individuals. More than five times as many experimental data points were needed using individual isolates to achieve the same confidence level as compared to a multi-donor primary pool (data not shown).

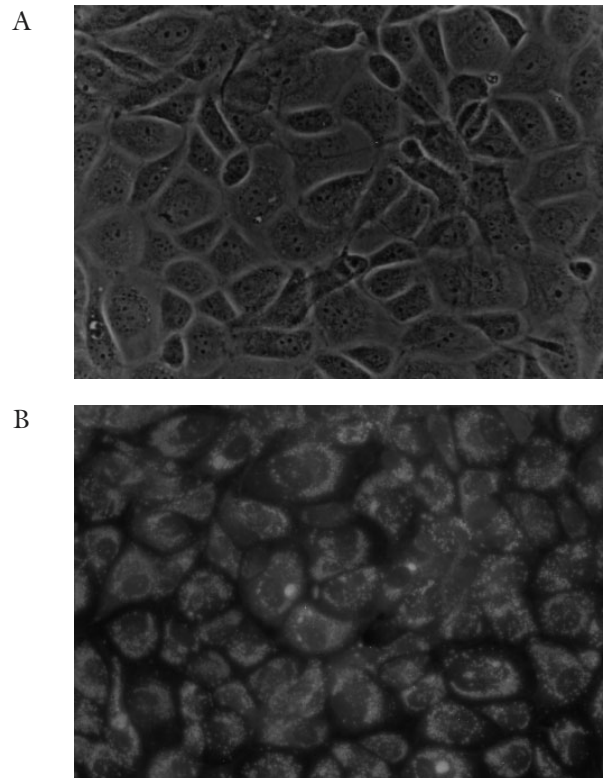


FIGURE 1. Presence of cytoplasmic CD 62P. Cells tested at <6 CPD *in vitro* were cultured and characterized as described in table 1. Cells were photographed by phase contrast (panel A) and by indirect immunofluorescence (panel B) (400 X).

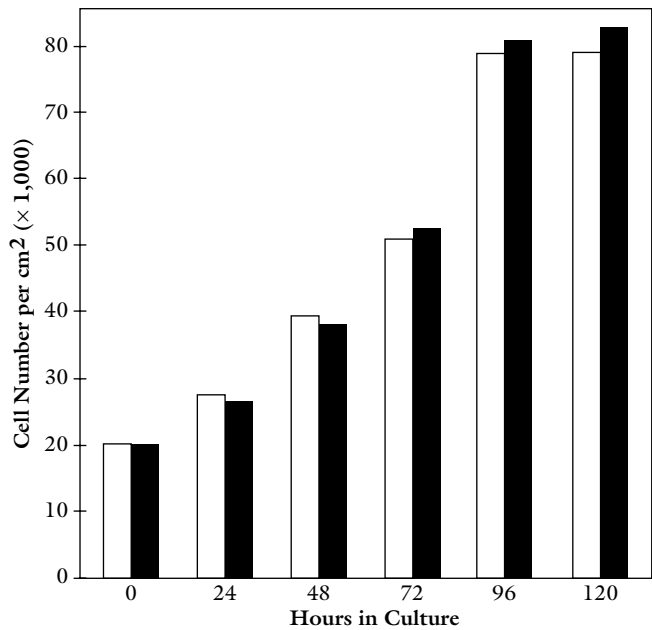


FIGURE 2. Endothelial cell growth. Cultures were plated in either M199 with supplements (□) or Human Endothelial-SFM with growth factors (■). The arithmetic mean of cells counted in six digitized low-power phase-contrast images was determined. Values are the arithmetic mean and the standard error was <10%.

Cells cultured in serum-free medium exhibited the histotypic cobblestone morphology and took up DiI-acetylated LDL (see cover photographs). Cells were also characterized for other physiological markers using standard immunofluorescence procedures (table 1). Serum-free cultures were positive for Factor VIII-related antigen, CD 62P (common names:

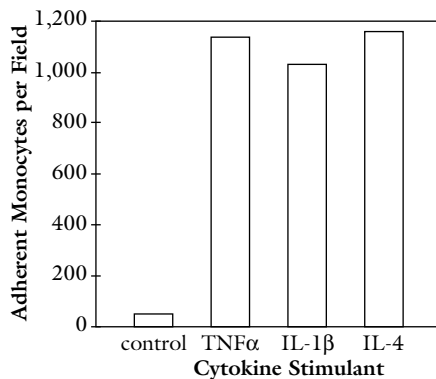


FIGURE 3. Cytokine-stimulated monocyte adherence to endothelial cells. Endothelial cells grown in serum-free medium were stimulated with 20 ng/ml recombinant human TNFα for 6 h; 10 ng/ml recombinant human IL-1β for 24 h; or 10 ng/ml recombinant human IL-4 for 24 h. The number of adherent leukocytes was quantitated by counting and averaging two digitized low-power phase-contrast images from each well. Values are the mean and the standard error was <10%.

P-selectin, GMP-140, PADGEM) (26), and vimentin. Less than 1% of the population stained for smooth muscle actin and desmin. Figure 1 shows the presence of cell adhesion molecule CD 62P. Human vascular endothelial cell growth in Human Endothelial-SFM with growth factors was not different from that in serum-containing medium supplemented with a CS-supplement, whether the cells were plated using HPFN (data not shown) or the less defined CS-AF-1.0 ECAF (figure 2).

Adherence studies. The leukocyte-endothelial cell adherence interaction is the result of cell-surface ligands expressed on the endothelial cells following cytokine or endotoxin exposure. Human vascular endothelial cell cultures isolated and grown in Human Endothelial-SFM medium showed the endothelial-cell specific adherence of human leukocytes after stimulation by the cytokines TNF-α, IL 1-β, and IL-4 (figure 3) or gram-negative bacterial endotoxin (*E. coli* LPS) exposure (figure 4). The adherence interaction can be prevented by incubation of the endothelial cell monolayer with neutralizing monoclonal antibodies to specific adhesion ligands such as CD 62E (figure 4).

This report describes the first stage in developing human vascular endothelial cell applications based on a serum-free *in vitro* operating system. Pooled primary human venous endothelial cell cultures isolated and grown in Human Endothelial-SFM comprised this human endothelial cell culture operating system of known origin and reproducible performance. Functional testing of this operating system has confirmed that these endothelial cells have typical endothelial morphology, display endothelial cell-specific markers, and display the expected cytokine-induced leukocyte adherence.

Access to a serum-free culture operating system for human vascular endothelial cells will enable applications and assays to be established based on common functional standards and system definitions. Application and assay results can be compared over time and between research groups. Serum-free human vascular endothelial culture system applications will also be increasingly important in providing stability and reproducibility as research using human endothelial cells moves closer to therapeutic applications.

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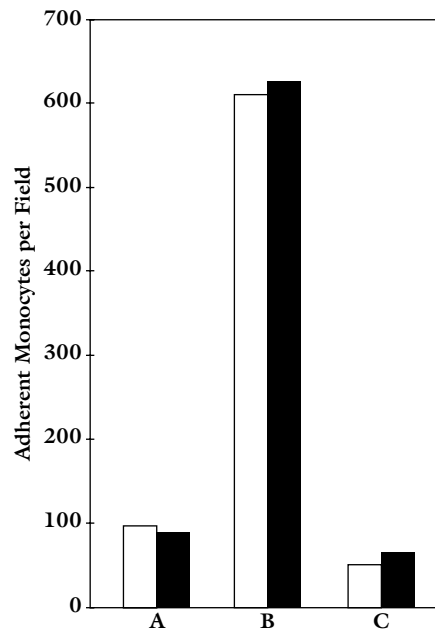


FIGURE 4. The effect of extracellular matrix attachment factors on the *E. coli* LPS-stimulated monocyte adherence. Cells were plated with CS-AF-1.0 ECAF (□) or HPFN (■). Monocyte adhesion was monitored after treatment with LPS (A), treatment with LPS + 1% FBS (B), or treatment with LPS + 1% FBS + CAMfolio MAb to CD 62E (C). The number of adherent cells was quantitated by counting and averaging two digitized low-power phase-contrast images from each well. Values are the mean and the standard error was <10%.

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EDITOR'S NOTE

To purchase the system containing cells, Human Endothelial-SFM, HPFN, EGF, and basic FGF, contact Cell Systems at: phone, 206/823-1010; fax, 206/820-6762. The GIBCO BRL Human Endothelial-SFM System containing medium, basic FGF, EGF, and HPFN (Cat. No. 11111-036) can be purchased from Life Technologies.

SINGLE-STEP EXTRACTION OF VIRAL RNA FROM SEMEN FOR RT-PCR

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The reverse transcription-polymerase chain reaction (RT-PCR) has greatly enhanced the capabilities of a diagnostic virology laboratory by allowing rapid and sensitive detection of virus-specific RNA from biological material. The initial steps involved in RNA extraction, however, can be cumbersome and time consuming when testing many samples by RT-PCR, as each sample must be processed individually. The RT-PCR process also requires the extraction of a sufficient amount of clean and intact RNA. Thus, methods that shorten the time spent in RNA extraction from samples while maintaining the integrity of viral RNA are desirable. For semen, which contains substances inhibitory to PCR (1,2), the method of nucleic acid extraction must also inactivate or remove these substances in order to achieve a high sensitivity for PCR. In this report we describe a one-step method using TRIzol™ Reagent, a monophasic solution of phenol and guanidine isothiocyanate (3,4), for extraction of viral RNA from semen for use in an RT-PCR assay. The method described herein allows the efficient extraction of RNA from 10 semen samples in less than 1 h.

METHODS

Equine semen samples from noninfected stallions or from stallions persistently infected with equine arteritis virus (EAV), a single-stranded RNA virus (5), were microcentrifuged at $14,000 \times g$ for 5 min. The seminal plasma (100 μ l) containing 20 μ g of glycogen was added to 1 ml of TRIzol Reagent and vortexed for 15 s. After the addition of chloroform (220 μ l), the mixture was vortexed for 15 s and subjected to microcentrifugation at $14,000 \times g$ for 5 min. The RNA in the aqueous phase (~650 μ l) was precipitated with 750 μ l of isopropanol for 15 min, microcentrifuged at $14,000 \times g$ for 10 min, washed with 75% ethanol, and air dried. The pellet was then redissolved in 50 μ l of HPLC-grade water. All RNA extraction procedures were performed at room temperature.

Standard procedures used for the RT-PCR and primer design will be described in detail elsewhere (Gilbert *et al.*, in preparation). Briefly, 200 units of GIBCO BRL Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) and 2 μ l of extracted RNA were incubated at 37°C for 60 min in transcription buffer (total volume 20 μ l) containing a downstream oligonucleotide (20-mer) primer complementary to the 3' region of the EAV polymerase sequence, GIBCO BRL dNTPs (1 mM each), and 5 units of GIBCO BRL Human Placental RNase Inhibitor. The mixture was then heated at 95°C for 5 min. First-round PCR reagents including the upstream oligonucleotide (20-mer) primer and 2.5 units of GIBCO BRL *Taq* DNA Polymerase in 80 μ l of PCR buffer were added to the reaction tubes containing the reverse transcriptase mixture. After denaturation at 94°C for 3 min, the reactions were cycled 30 times as follows: 94°C for 20 s, 42°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 15 min. A second round of amplification was performed with 1 μ l of the first-round product and internal nested oligonucleotide (20-mer) primers, using the same reaction conditions as described for the first round of PCR amplification. The final product (15%) was electrophoresed on a 2% agarose gel in 1X TAE with a GIBCO BRL HORIZON® 58 Horizontal Gel Electrophoresis Apparatus using a GIBCO BRL Model 250 Power Supply and stained with ethidium bromide.

RESULTS AND DISCUSSION

The results of RT-PCR after two rounds of amplification for two positive semen (field) samples and a negative semen sample are presented in figure 1. A product consistent in size with that predicted (186 bp) from the EAV sequence was obtained from positive semen samples. These samples contained relatively low titers of 300 and 850 pfu of EAV/ml semen, lanes 2 and 3 respectively, as determined by a plaque infectivity assay (6). Samples with as low

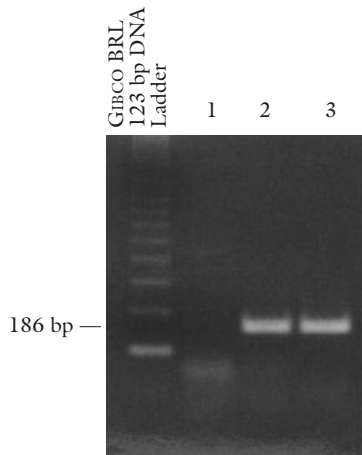


FIGURE 1. RT-PCR of extracted RNA from equine semen samples. Ethidium-bromide-stained agarose gel. Lane 1, EAV-negative equine semen. Lanes 2 and 3, EAV-positive equine semen.

as 2.5 pfu of EAV/ml semen gave equally intense product bands (data not shown). No product bands were observed from the negative semen (lane 1). Furthermore, the number and size of restriction fragments obtained after enzymatic digestion of the RT-PCR product obtained from positive semen samples were consistent with those predicted for virus-specific cDNA amplification (data not shown).

Based on the findings of an initial study, in which tissue culture-derived EAV was used to spike semen, RT-PCR could detect as little as 0.01 pfu/ml, as demonstrated by ethidium bromide staining of the product. This suggests that viral RNA not associated with infectious particles was also being detected. Thus, the results indicate that the TRIZOL Reagent extraction for viral RNA was efficient and that the procedure could be used in an RT-PCR assay for the detection of viral RNA in equine semen samples.

Methods for RT-PCR for the detection of EAV-specific RNA in semen have been reported previously (1,7). In each case, the RNA extraction procedure was more involved and time consuming than that described herein, and it included overnight or double precipitations of the RNA. Previously, cellular RNA isolated

with TRIZOL Reagent was used successfully for RT-PCR (3). In this report, we extend the use of the reagent to the isolation of viral RNA from semen. This involved the simple addition of TRIZOL Reagent directly to seminal plasma containing glycogen.

In addition to the isolation of viral RNA from seminal plasma, we have also used this procedure successfully for the extraction of RNA from EAV and a related RNA virus, porcine reproductive and respiratory syndrome virus (8), directly from infected tissue culture supernatants for RT-PCR assays. It is anticipated that this procedure will be generally useful in the extraction of viral RNA from a variety of biological sources for RT-PCR.

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For small samples, can I add glycogen with TRIzol Reagent to maximize my RNA yields?

Yes. However, glycogen is soluble in TRIzol Reagent. Therefore, we recommend you add 5 to 10 µg glycogen per 0.8 ml TRIzol Reagent to the aqueous phase after chloroform extraction.

RAPID EXTRACTION OF MULTIPLE RNA SAMPLES FROM PLANT SUSPENSION CELLS

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Extraction of nucleic acids from plant cells is a labor-intensive and time-consuming process. The main reason is the cell wall that must be broken, typically by mechanical means, so that extraction buffers can exert their effect. Grinding, mechanical homogenization or repeated pipetting procedures are cumbersome, especially when handling multiple samples, and inherently cause variation in yield and quality of RNA obtained.

We have extended the single-step RNA isolation method based on guanidine isothiocyanate/phenol/chloroform, described by Simms *et al.* (1) for animal cells and tissues, to plant cells. We have found that TRIzol™ Reagent can be used to extract total RNA from alfalfa suspension cells without the need for grinding or any other form of mechanical breaking of the plant cell wall.

EXPERIMENTAL PROCEDURES

Alfalfa (*Medicago varia*) A2 suspension cells (2) in early to late log phase were collected into

a 0.5- to 1.0-ml pellet from 5 to 15 ml culture by centrifugation at $150 \times g$ for 4 min at 20°C in a capped, sterile, 15-ml polypropylene tube. The culture medium was decanted and the tube with cell pellet was placed directly in a -70°C freezer until use. RNA extraction was performed in lots of eight tubes. GIBCO BRL TRIzol Reagent (2.5 ml) was pipetted on top of the frozen pellet and the tubes were placed in a waterbath of 37°C for 3 min with vigorous shaking by hand every min for 10 s. Subsequently, the eight tubes were vortexed together for 2 min at room temperature. After 5 min at room temperature, 0.5 ml chloroform was added to each tube and shaken vigorously by hand for 10 s. Room temperature incubation was continued for 3 min. The tubes were centrifuged at $4,000 \times g$ for 25 min at 4°C. At this time, RNA isolation from the next batch of eight samples was started. The colorless upper phase in each tube was collected and RNA was precipitated with 1.25 ml isopropanol and washed with 4 ml of 75% ethanol according to Simms *et al.* (1). RNA was solubilized in 80 μ l DEPC-treated 0.5% (w/v) SDS in water.

Total RNA was electrophoresed in agarose gels under nondenaturing conditions and under denaturing conditions in formaldehyde (3). Northern analysis of alfalfa histone H3 transcripts was performed with gene-specific, digoxigenin-labeled hybridization probes, which were visualized by chemiluminescence.

RESULTS AND DISCUSSION

Plant cell suspension culture conditions produce small clusters of vacuolized cells, surrounded by cell walls. Freezing followed by rapid thawing at 37°C in excess TRIzol Reagent, a strong chaotropic solvent with guanidine isothiocyanate, produced effective cell lysis with simple mixing. Mechanical breakage of cell walls was not required. Thus, it is possible to prepare intact total RNA from many small samples, in reproducibly high yields (fig-

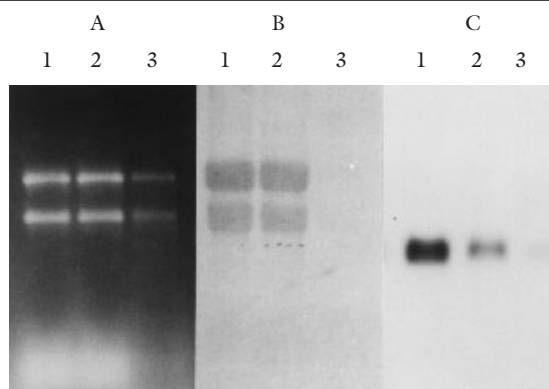


FIGURE 1. Analysis of RNA recovered from alfalfa suspension cells or callus. Panel A. Total RNA (2 μ g), obtained from alfalfa A2 cells at two different times from a partially cell-cycle-synchronized suspension culture (lanes 1 and 2), was electrophoresed in Tris-borate-EDTA buffer and stained with ethidium bromide. Lane 3. Total RNA obtained from an equivalent amount of alfalfa callus, cultured on plates. For Northern analysis, 7.5 times more RNA was separated on an agarose-formaldehyde gel. Panel B. Transferred RNA stained with methylene blue (4). Panel C. The membrane was hybridized with a digoxigenin-substituted alfalfa histone H3 probe that is specific for histone H3.1 (2).

ure 1). Additional homogenization of TRIzol Reagent lysates failed to increase the yield of RNA (data not shown). The volume of TRIzol Reagent that was required for plant cell lysis, a 2.5-fold excess, is less than the 10-fold excess recommended for the preparation of RNA from animal cells (1). The minimal amount of reagent required was established experimentally for our plant cell culture and may vary with factors such as the buffering capacity of cells and growth medium. In general, 0.5 to 1.0 mg total RNA ($A_{260/280} = 2.0-2.1$) was obtained from 1 ml cell pellet, corresponding to 50 mg lyophilized tissue. This yield of RNA from plant is similar to that obtained with more conventional, laborious methods (2).

When callus cultures, grown on agar plates, were used, considerably less RNA was obtained (figure 1, lane 3). The yield of RNA was increased if a mechanical homogenization step was added (data not shown). This suggests that the large cluster of cells in the callus impedes access of the chaotropic buffer to the deeper cell layers.

RNA isolated with TRIzol Reagent was not degraded, as judged by the pattern of ribosomal RNAs in the gel when stained with ethidium bromide, and after transfer of the RNA to a membrane and stained with methylene blue. Also, hybridization with an alfalfa histone H3 gene probe produced a sharp band (figure 1, panel C).

In summary, TRIzol Reagent is fast, inexpensive, and produces high-quality RNA from multiple samples in reproducibly high yields.

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ISOLATION OF FULL-LENGTH cDNA CLONES ENCODING THE COMMON β -SUBUNIT OF GM-CSF, IL-3, AND IL-5 RECEPTORS BY RT-PCR.

Reverse transcription-polymerase chain reaction (RT-PCR) is a sensitive method that can detect rare transcripts. However, a long mRNA, the presence of high G+C content, secondary structure in the target mRNA, and very low levels of expression often hinder RT-PCR.

High-affinity receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3), and interleukin 5 (IL-5) consist of ligand-specific, low-affinity α -subunits (1-3) and a common β -subunit (β_c)(2-4). cDNAs encoding the low-affinity α -subunits have been isolated from a variety of tissues by RT-PCR (5-8). The cDNA clone (pKH97) encoding β_c isolated from a cDNA library of a human hemopoietic cell line, TF-1

(6), has been used in several laboratories (9-11). The cDNA encoding β_c is 2,996 bp, has a high G+C content (63%), and is expressed at very low levels in normal human hemopoietic cells making this cDNA difficult to detect by RT-PCR. The alternative method to detect the β_c subunit by constructing and screening a cDNA library is quite time consuming, labor intensive, and tedious. We need to compare β_c from normal cells to cells from patients with various diseases. Here we present an RT-PCR strategy that resulted in full-length cDNA clones of β_c from total RNA of peripheral blood mononuclear cells.

METHODS

RNA isolation. Total RNA was isolated

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TABLE 1. Oligonucleotides for the β_c Chain.

Fragment A

1. ([GC]₂₇-Not I-Hind III-4-23)
[GC]₂₇GCG GCC GCA AGC TTG CCG TCC AGA GCT GAC CA
2. (2125-2106) AGA GCT CAT GGG TAT AGC CCA
3. (1780-1761) AGC CTG TTT CTC AGG TGT GT

Fragment B

4. ([GC]₂₈-Not I-Xba I-2798-2778)
[GC]₂₈GCG GCC GCT CTA GAA ATG ACT GAG GAA GGT CA
5. (1382-1401) CTC ACC ACT GCT GTG CTC CT
6. (1629-1648) TGT CAC CTC TCA CCA TAG AG

The numbering system used for all oligonucleotides represents the numbering of the originally published KH97 (β_c) cDNA sequence. The GC-clamps at the 5'-end of primers 1 and 4 were used for denaturing gradient gel electrophoresis and were irrelevant to the studies reported here.

from human peripheral blood mononuclear cells (PBMNC), using the single-step acid guanidinium thiocyanate method (12).

Reverse transcription. Total RNA (1–5 μ g) was heated for 10 min at 70°C and cooled on ice for 5 min. The RNA was mixed in a final volume of 20 μ l with 1 μ g of GIBCO BRL Random Hexamers, 500 μ M of each dNTP, and 200 units of GIBCO BRL SUPERSCRIPT™ RNase H⁻ Reverse Transcriptase in 1X reverse transcription buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂]. Alternatively, 250 ng of cDNA-specific antisense primer (CAG GGA GAG AAG TGA TTT GCT) was used to prime cDNA synthesis. The mixture was incubated at 42°C for 1 h, and the

reaction was stopped by adding 4 μ l of 0.5 M EDTA. The cDNA was purified by phenol extraction, precipitated with 7.5 M ammonium acetate and 3 volumes of ethanol, and then dissolved in water.

PCR. Target cDNA derived from the RT reaction was amplified by PCR using two sets of primers. The primers for the first round of PCR were 1+2 for fragment A and 4+5 for fragment B (table 1, figure 1). The primers for the second round of PCR were 1+3 for fragment A and 4+6 for fragment B. The 5' portion (fragment A) and 3' portion (fragment B) of target β_c cDNA overlap the *Bgl* II site at nucleotide 1695. Target cDNA was combined with 15 pmol of each PCR primer, 200 μ M dNTPs, and 2.5 units of *Taq* DNA polymerase in 1X PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 100 μ g/ml BSA] in a total volume of 100 μ l. PCR was 30 cycles of 94°C for 1 min, 58°C for 2 min, and 72°C for 3 min, followed by an extension step at 72°C for 7 min. About 3% (3 μ l) of each first-round PCR product was used as template for second-round PCR at the same conditions as that used for the first-round PCR.

Cloning. PCR products were excised from a low melting point agarose gel, purified with a glass matrix product, and digested with *Hind* III and *Bgl* II (fragment A) or *Xba* I and *Bgl* II (fragment B) at 37°C for \geq 4 h. Both fragments A and B were cloned into the eukaryotic expression vector pCDM8 that had been linearized by digestion with *Hind* III and *Xba* I. The triple-ligated cDNA clones with the correct sizes of insert fragments were sequenced.

RESULTS AND DISCUSSION

The 5' and 3' portions of cDNA were amplified with cDNA-specific primers. For fragment A, the expected 1.78-kb fragment was seen after the second-round PCR (figure 2). The efficiency of PCR was related to the amount of RT reaction used, with 25% of the RT reaction giving optimal amplification of fragment A (figure 2). This may be due to carryover of some components from the RT reaction.

To see if the primer used for the RT reaction affected the ability to obtain PCR product, random hexamer and specific antisense oligonucleotide-primed cDNA were used for fragment B. Random primers resulted in successful ampli-

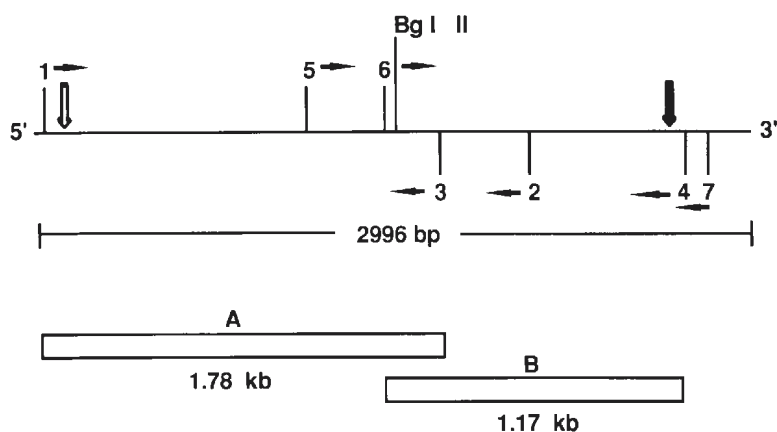


FIGURE 1. Schematic diagram of cDNA (KH97) encoding the β_c -subunit. The location of primers used for PCR are shown. The longer white and black arrows indicate the position of the initiation ATG codon and termination codon, respectively. A and B are expected DNA fragment sizes after two rounds of PCR. The oligonucleotides used for fragment A are 1+2 (first round of PCR), and 1+3 (second round of PCR) and for fragment B are 4+5 (first round of PCR) and 4+6 (second round of PCR), respectively. Primer 7 is the antisense primer used for first strand cDNA in some studies.

fication from all four individuals tested, whereas the antisense primer resulted in successful amplification of target cDNA from three of five individuals (figure 3). Also, the specific oligonucleotide-primed cDNA yielded less amplification product than random hexamer-primed cDNA.

Fragments A and B from the same individual were cloned. Three clones were obtained, one of which was a short form that requires further study. The sequence analysis of the full-length clones showed that the correct identification of nucleotides 1389 and 1390 of the originally published B_c cDNA sequence is TC (data not shown) instead of the CT (4), resulting in a single amino acid change from threonine (ACT).

In our preliminary studies, several thermostable DNA polymerases, as well as several techniques, including "RNA-PCR" and "Hot Start" kits, were tested for amplification of the β chain gene. None were able to support consistent amplification of full-length (3 kb) β_c cDNA. The success of the B_c cloning reported here is probably the result of a combination of alterations including using the high-efficiency SUPERSCRIPT RT, random hexamers, an aliquot of the RT reaction, and 2 rounds of PCR.

In our previous experiments of cDNA clones for the α chain of the GM-CSF receptor, the use of random primers in RT increased the yield of RT-PCR products although both oligo(dT) and antisense primers also resulted in successful amplification of the target cDNA (data not shown). These data led us to select random hexamers as primers for reverse transcription for the B_c chain instead of oligo(dT) or specific antisense oligonucleotides. Also, the use of oligo(dT)-primed cDNA has the disadvantage that the reverse transcription may not reach the 5'-end of the mRNA for long mRNAs. Specific primers may be useful for obtaining a specific transcript, but may suffer from inefficient annealing and may not reach the 5'-end due to mRNA length or secondary structure. Random primers should prime most species of mRNA present and allow the amplification of any desired target sequence from target RNA.

In our experiments, no β_c cDNA was identified from first-round PCR, and no amplification of β_c cDNA occurred with oligo(dT)

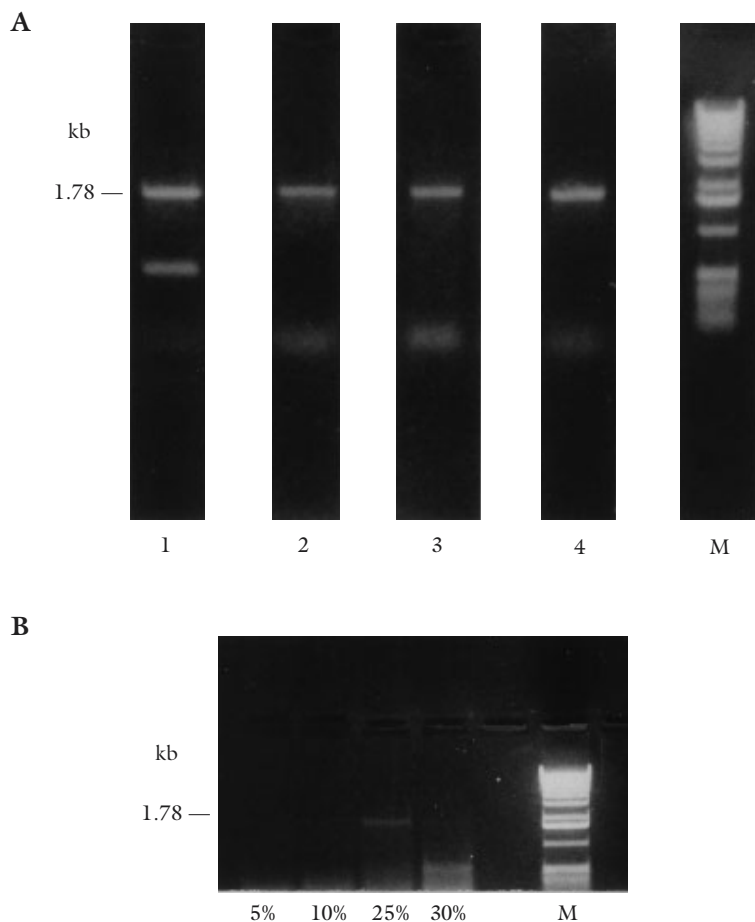


FIGURE 2. Agarose gel analysis of RT-PCR products. Panel A. Reverse transcription was performed with random hexamers. 25% (5 μ l) of the RT product was used for PCR with primers 1 and 2. About 3% (3 μ l) of the first-round PCR products were used as templates for second-round PCR with primers 1 and 3. The samples in lanes 1–4 are from 4 individuals. Panel B. 5–30% (1–6 μ l) of RT products were used for the amplification of fragment A with two rounds of PCR.

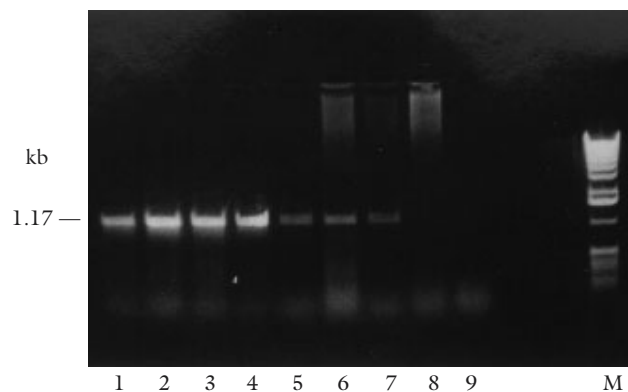


FIGURE 3. The effect of using a sequence-specific primer and random primers in RT on the amplification of fragment B. The cDNAs from random hexamer primed (lanes 1–4) and specific antisense (lanes 5–9) oligonucleotide-primed RNA were amplified by two rounds of PCR for the 1.17-kb fragment B.

priming of reverse transcription. Nested PCR was necessary for amplification of cDNA to useful levels. The amount of RT-derived template used for PCR also was important for isolation of β_c cDNA, with either too little or excessive amounts of template yielding less than optimal results. In our experience, the use of "one-tube" methods limited the choice of template amount and did not improve the results.

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EXON TRAPPING FROM A YEAST ARTIFICIAL CHROMOSOME (YAC) THAT CONTAINS THE *C-FOS* GENE

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The identification of expressed sequences in a defined chromosomal location is a crucial step in the positional cloning of genes associated with human disease. Several strategies have been employed to address this problem (1-5), one of which, exon trapping, has previously been shown to be an efficient means of isolating coding sequences from genomic DNA of relatively high complexity (6) and has been successfully employed to isolate genes associated with Menkes disease and with Huntington's disease (7,8).

Exon trapping is based on the direct isolation of transcribed sequences from cloned genomic DNA through the selection of 5' and 3' splice sites that are functional in COS-7 cells. Spliced sequences are then amplified by RT-PCR from the COS-7 cell RNA. Exons have been identified from large regions of defined

genomic DNA in single cosmids (5,6) or pools of cosmids (6), but there has been little information on the efficiency of exon trapping from YACs. Using a YAC contig for the Alzheimer's disease candidate region (C. Van Broeckhoven and A.M. Goate, unpublished), we analyzed the feasibility of exon trapping from YACs as a means of identifying transcribed sequences in this region of chromosome 14. To quantitate the efficiency of the method, we initially performed exon trapping on a nonchimeric chromosome 14 YAC (460 kb) that contains the *c-fos* gene and detected amplified internal *c-fos* exons in the total splice products by hybridization.

METHODS

Shotgun subcloning of the c-fos YAC into pSPL3. The chromosome 14 *c-fos* YAC (460 kb) was separated from host yeast chro-

mosomes by pulsed-field gel electrophoresis. The YAC DNA was excised in an agarose gel slice and treated with β -agarase. The partially purified YAC DNA was digested with *Pst* I (60 units) in ~5 ml total volume at 37°C for 6 h and then concentrated using a column with silica-based resin followed by ethanol precipitation. The YAC DNA (~100 ng) was ligated to 100 ng of the exon trapping vector pSPL3 that had been digested with *Pst* I and dephosphorylated. To examine the effects of input DNA complexity on exon trapping efficiency, the ligation was transformed into two 100- μ l aliquots of GIBCO BRL DH10B™ competent cells. One transformation (B) was diluted in 10 ml SOB+ampicillin media and grown overnight at 37°C. The other transformation (A) was subdivided into 5 aliquots (A1–A5), which were each grown overnight in 2 ml SOB+ampicillin media. The YAC shotgun libraries used in this study had an average insert size of ~1.0 kb and contained sufficient primary recombinants to give greater than 10-fold coverage of the YAC.

Exon trapping. Miniprep plasmid DNA (1 μ g) from both the total YAC/pSPL3 shotgun library (B) and the 5 subdivided library aliquots (A1–A5) was transfected into COS-7 cells using the LIPOFECTACE™ Reagent as described in the GIBCO BRL Exon Trapping System. Total RNA was prepared 24 h post-transfection using the GIBCO BRL TRIzol™ Reagent. RT-PCR of ~3 μ g RNA from the YAC/pSPL3 library (B), each of the 5 library aliquots (A1–A5), and a pool of the 5 subdivided library RNAs (termed Ax) was performed as recommended by the manufacturer.

UDG ligase-free cloning. Secondary PCR products (A1–A5, Ax, and B) were electrophoresed through a 1% agarose gel and blotted onto nylon membrane. The membrane was then probed with a 30-base oligonucleotide specific for exon 2 in the *c-fos* gene (11). The Ax secondary amplification products (derived from the pooled A1–A5 RNAs) were then cloned into the pAMP10 vector using uracil DNA glycosylase (UDG) cloning with the GIBCO BRL CLONEAMP® System. 5 μ l of the PCR product was cloned directly into pAMP10; the remainder was digested for 3 h with 20 U of *Bst*X I prior to cloning. An aliquot (25 μ l) of this second *Bst*X I reaction was also separated on a low-melting-point agarose gel,

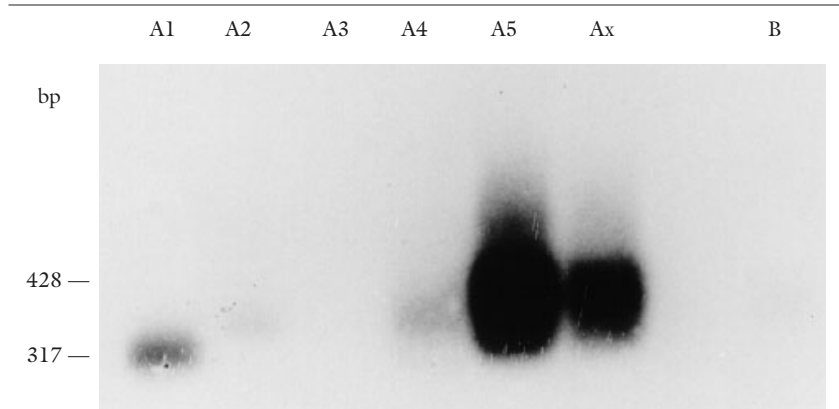


FIGURE 1. The effect of YAC/pSPL3 library complexity on the efficiency of exon trapping. Hybridization signals obtained when a blot of amplified splice products was probed with a ³²P-labeled oligonucleotide specific for exon 2 of the *c-fos* gene. DNAs from a subdivided YAC/pSPL3 library (lanes A1–A5), a total YAC library (lane B), and a pool of the A1–A5 RNAs (lane Ax). The sizes of the full-length (428 bp) and truncated (317 bp) *c-fos* exon 2 amplification products are indicated.

and DNA fragments larger than the pSPL3 vector-only-derived splice product (177 bp) were gel purified, cloned into pAMP10, and transformed into GIBCO BRL SUBCLONING EFFICIENCY DH5 α ™ cells. To assess the number of internal *c-fos* exons present in the cloned secondary PCR products, membrane replicas of the transformants (~400 colonies) were prepared and probed with the *c-fos* exon 2 oligonucleotide. The identity of positives was confirmed by DNA sequencing.

Insert size and sequence analysis. To further analyze the cloned splice products, the insert sizes of 100 recombinants were determined by colony PCR followed by agarose gel electrophoresis. Products were then sorted into size groups, and clones from each group were subjected to DNA sequencing. Each sequence was compared with the DNA databases using the BLAST network service of the National Center for Biotechnology Information (10).

RESULTS

Secondary PCR products from exon trapping experiments on the chromosome 14 *c-fos* YAC appeared as smears on a 1.5% agarose gel with the majority of the amplified DNA in the size range 250 bp to 450 bp. This corresponds to exon sizes of 73 to 273 bp, excluding the 177 bp of vector-derived sequence that is present on each splice product. Weak bands could be discerned in the smear at ~180 bp (which was presumed to be derived from vector-only splicing) and at ~280 bp. When a Southern blot

TABLE 1. Analysis of cloned splice products.

	<i>c-fos</i> YAC (chr. 14)	Cosmid Pools (chr. 9) ^a
DNA complexity	460 kb	385 kb
Sequences isolated	21	17.6
HIV/repeat artifacts	5	1.8
Yeast-derived sequences	1	—
Yield total putative exons	15	15.8
Yield kb input DNA/exon	30.7	24.4

Results of sequence analysis of cloned trapped exons from the *c-fos* YAC. Inserts from 100 recombinant clones were determined by colony PCR followed by electrophoresis through a high-resolution 2% METAPHOR™ gel. Products were then sorted into size groups and a number of clones from each group were subjected to DNA sequencing. Different sequences were compared with DNA databases using the BLAST computer programs.

^aData from chromosome 9 cosmid pools from Church *et al.* (6); figures were averaged from 8 pools containing a total of 88 cosmids.

of the agarose gel was probed for exon 2 of the *c-fos* gene (figure 1), strong hybridization signals were obtained from splice products derived from A1 and A5 of the subdivided YAC shotgun library aliquots and from products derived from the pooled A1–A5 RNAs. In contrast, hybridization signals obtained from products derived from the entire YAC library were very weak (lane B). Interestingly, the *c-fos* probe detected at least two splice products—one corresponding to the expected size of the exon (251 bp) and a second of shorter length. Subsequent sequence analysis demonstrated that the shorter product diverged from the *c-fos* exon at an internal *Pst* I site in exon 2 (data not shown). Presumably this shorter fragment was generated from clones containing only an intact 5′ splice acceptor site that was combined with a cryptic splice donor site in the pSPL3 vector.

Hybridization of the *c-fos* probe to membrane replicas of DH5α colonies containing cloned putative exons (derived from the Ax splice products) gave 5 positives when the secondary amplification reaction was redigested with *Bst*X I and then size-selected on an agarose gel. One positive was obtained when the products were simply redigested with *Bst*X I; however, no positives were obtained when an aliquot of the secondary reaction was cloned directly into pAMP10 without any pretreatment.

Analysis of the insert sizes of 100 cloned putative exons revealed at least 21 different splice products (table 1). Sequence analysis indicated that ~25% of the 100 clones (3 different sequences) were derived solely from HIV-*tat* sequences—an artifact that has been

previously observed with this technique and vector (6). Two other sequences contained only repetitive elements, while another was derived from a yeast gene. The remaining 15 sequences were considered to be putative chromosome 14-derived exons. One of these sequences was identical to the entire length of exon 2 in the *c-fos* gene (the truncated product was not present), while two other sequences displayed weak similarity to known sequences using the BLASTN and BLASTX programs (p value <0.01).

DISCUSSION

This report describes the use of exon trapping to isolate expressed sequences from a chromosome 14 YAC containing the *c-fos* gene. Detection of the internal *c-fos* exon 2 was employed as a means to determine the efficiency of the protocol.

The complexity of the target DNA has previously been suggested to significantly affect the yield of trapped exons (6), and our initial experiments therefore focused on the nature of this observation and its significance for exon trapping from the *c-fos* YAC (460 kb). Transfection of shotgun-cloned DNA from the entire YAC resulted in much lower levels of *c-fos* exon trapping than obtained by transfecting the YAC in five pools of shotgun clones and analyzing each pool individually. A more surprising observation was that combining the RNAs isolated from COS-7 cells that had been transfected separately with one of the five pools of YAC clones also resulted in a large increase in *c-fos* exon isolation when compared with RNA isolated from cells transfected with the entire YAC. This suggests that increasing target DNA complexity not only “dilutes” a particular exon but also reduces the efficiency of splicing in the transfected COS-7 cells. The mechanism of such a reduction in splicing efficiency is unclear, but we have observed the effect consistently with this YAC and with a second overlapping YAC of similar size (data not shown).

The exon trapping system includes a *Bst*X I digestion of primary PCR products (6) to reduce pSPL3 vector-vector splice products. Despite this step, a significant amount of vector-only-derived artifacts was seen in our splice products. By digesting secondary PCR products with *Bst*X I and size-selecting on agarose gels,

the proportion of *c-fos* exons in the cloned splice products increased from less than 1 in 400 to ~1 in 100. Another problem with exon trapping from this YAC was products derived from cryptic splicing of the HIV *tat* sequences in the pSPL3 vector. These represented ~25% of the cloned sequences. It is highly likely that both these vector-derived artifacts were due to the presence of a high proportion of small (<1.0 kb) inserts in the YAC/pSPL3 shotgun library, the majority of which will not contain exons. We are currently evaluating the use of size-selected YAC DNA as a means to improve the efficiency of exon isolation.

Sequence analysis of the cloned splice products revealed a total of 15 different putative chromosome 14 exons that did not contain either HIV or repetitive sequence. These data suggest that an exon was recovered for every 30.7 kb of input YAC DNA. This figure for exon yield is similar to that obtained from pools of chromosome 9 cosmids (6, table 1), whose total complexity was similar to the *c-fos* YAC; however, it is significantly lower than for single cosmids (6). It is likely, however, that the number of exons trapped from the YAC is considerably higher than this initial study would suggest, since a significant number may have been missed because they are of similar size to other exons. To resolve this question, we are currently sequencing a large number of cloned splice products at random.

In summary, this report demonstrates that it is possible to use YACs as the input DNA for exon trapping. If a contig of nonchimeric YACs is available, it is likely that direct exon trapping coupled to large-scale sequencing of the products may prove the fastest means of generating

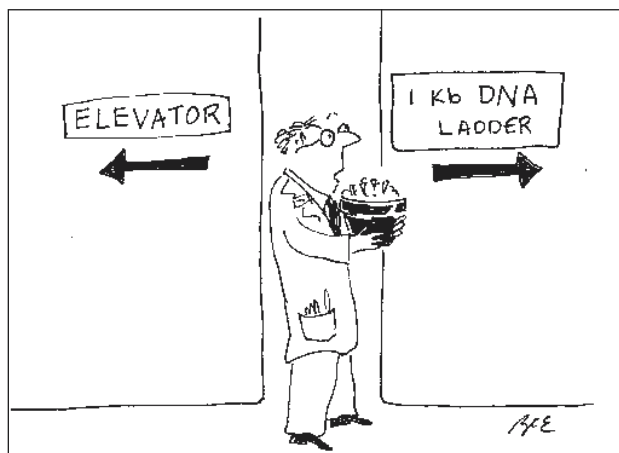
a first-pass transcription map of a particular chromosomal location.

ACKNOWLEDGEMENTS

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GEL ELECTROPHORESIS WITH SEPARIDE™ GEL MATRIX

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SEPARIDE Gel Matrix is a blend of polysaccharides used to prepare gels for the electrophoretic separation of low-molecular-weight DNAs. The gels are made by dissolving SEPARIDE Gel Matrix in TAE buffer with heating and then pouring the hot solution into a horizontal mold for gel formation and electrophoresis. The gels can be electrophoresed with ethidium bromide present, or the DNA can be visualized by staining after electrophoresis. The gels are mechanically strong, which facilitates handling.

Figure 1 shows the electrophoretic resolution of DNA in SEPARIDE gels of different concentration. As expected, with increasing gel concentration the mobilities decreased overall, and mobility ratios between successive bands increased. In each case, the DNA signal was sharp with low background. The 2% and 3% SEPARIDE gels effectively resolved the closely

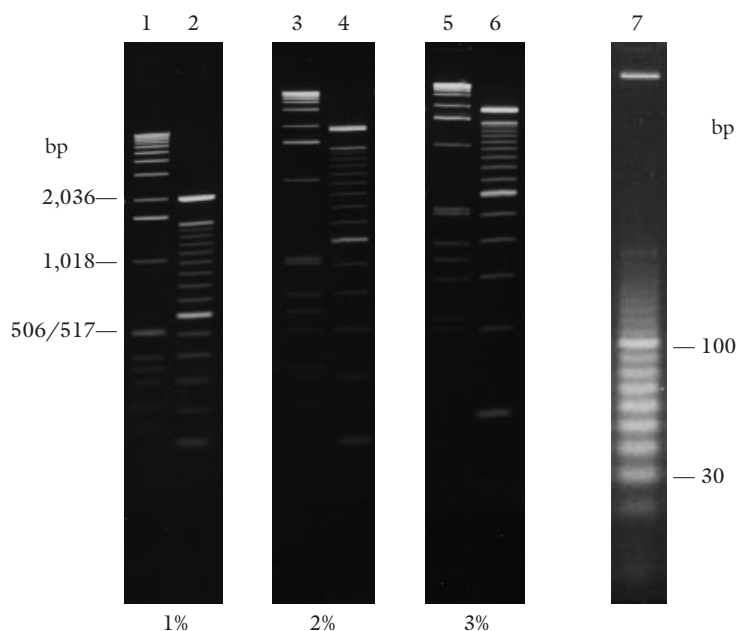


FIGURE 1. Electrophoretic resolution of DNA in SEPARIDE gels. The GIBCO BRL 1 Kb DNA Ladder (200 ng) and 100 bp DNA Ladder (200 ng) were electrophoresed in 1% (lanes 1 and 2), 2% (lanes 3 and 4), or 3% (lanes 5 and 6) SEPARIDE gels. The GIBCO BRL 10 bp DNA Ladder (750 ng) was electrophoresed on a 4% SEPARIDE gel (lane 7). The gels were electrophoresed in a GIBCO BRL HORIZON® 58 Apparatus at 130 V (lanes 1–4) or at 110 V (lanes 5–7), using TAE buffer. The DNA was stained with ethidium bromide after electrophoresis.

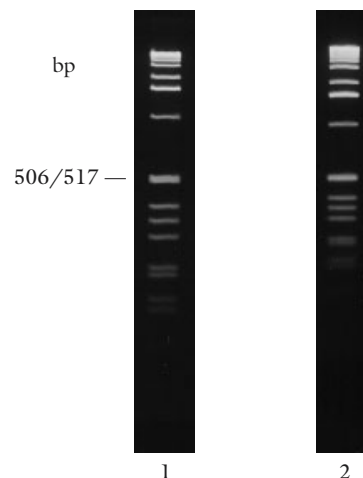


FIGURE 2. Comparison of gels made from SEPARIDE matrix and from NUSIEVE-agarose. 300 ng of the 1 Kb DNA Ladder was electrophoresed on a 2% SEPARIDE gel (lane 1) or a 2% NUSIEVE-agarose (3:1) gel (lane 2). The gels were electrophoresed in a HORIZON 58 Apparatus at 135 V using TAE buffer. The DNA was stained with ethidium bromide after electrophoresis.

comigrating 506-bp and 517-bp fragments, even on a small gel (5 cm × 8 cm). Good resolution of the 10 bp DNA Ladder was achieved in a 4% SEPARIDE gel.

Figure 2 shows a comparison of a 2% SEPARIDE gel and a 2% NUSIEVE®-agarose gel. Overall, the shorter fragments were resolved better in the SEPARIDE gel, and, in particular, the 506-bp and 517-bp fragments were resolved in the SEPARIDE gel, but not in the NUSIEVE-agarose gel.

SEPARIDE gels should not be prepared with TBE buffer. Care should be taken to ensure that the SEPARIDE matrix is completely dissolved in the TAE before pouring the gel and that no bubbles are introduced during the pouring process.

SEPARIDE gels can be used simply and effectively to give sharp, rapid DNA resolution. They give very good electrophoretic separation over the size range of interest for most PCR applications (generally 100 bp to 1,000 bp). The SEPARIDE gel concentration can be adjusted to optimize resolution in the desired size range.

PHAGEMID NESTED DELETIONS FROM DELETION FACTORY® SYSTEM VERSION 2.0

The process of sequencing large pieces of DNA can be significantly streamlined by using the DELETION FACTORY System (1). This is a transposon-based system for generating nested deletion subclones *in vivo* from cloned inserts as large as 40 kb. Selectable and contra-selectable (conditionally lethal) markers are used together to select and to sort deletions progressing from either end of the insert. Through this technology, separate nested deletion sets traveling in each direction across the insert are retrieved for use in sequencing or mapping.

The amount and quality of data obtained from a given sequencing reaction are highly dependent on the template. Single-stranded DNA generally gives longer and more accurate sequence readings (2), while double-stranded DNA is simpler to prepare. Phagemids incorporate an M13-like f1 origin of replication into an ordinary plasmid vector to provide the flexibility to prepare either double-stranded or single-stranded DNA templates without recloning (3). From a phagemid, double-stranded DNA is isolated by standard plasmid miniprep procedures, or single-stranded DNA is produced with the aid of an M13 helper phage and an *E. coli* carrying the F' episome. Inserts were shown to be more stable in phagemids than in M13 (4).

Recently, we modified the DELETION FACTORY System, creating a new version that takes advantage of phagemids. A new cloning vector, pDELTA 2, was constructed to improve the selection conditions and to produce deletion end products that are phagemids. As such, these deletion subclones can be sequenced as either single-stranded or double-stranded templates. For a set of deletion subclones from a given selection condition, the order of data assembly is derived simply from the deletion subclone size. We demonstrate the use of this new system to generate several single-stranded deletion subclones of a 20-kb genomic DNA insert for sequencing.

METHODS

Generating nested deletions. A 20-kb *Not* I fragment of human genomic DNA was cloned into the *Not* I site of pDELTA 2 to create pYA100. pYA100 was subjected to the GIBCO BRL DELETION FACTORY System Version 2.0 (Cat. No. 10224) to generate bidirectional sets of nested deletions. The system was used as previously described (5) except for necessary changes in the selection conditions as required for pDELTA2. The selections were made by

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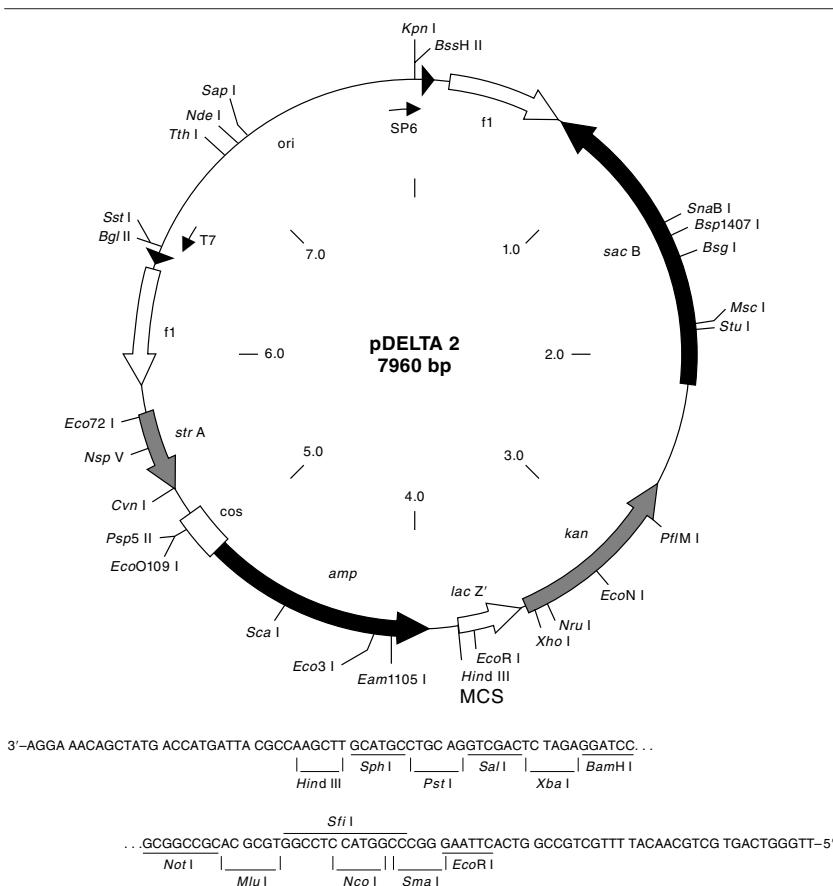


FIGURE 1. Map of plasmid pDELTA 2 and sequence of the MCS. The map is based on assembly of known DNA sequences used in the construction of the vector. Solid arrowhead, $\gamma\delta$ transposon end; small arrow inside circle, SP6 or T7 promoter sequence; solid arrows, marker pair used in isolating clockwise deletions; and shaded arrows, marker pair used in isolating counterclockwise deletions. Restriction endonucleases with single cleavage sites in the vector are indicated. The sequence of the MCS shown spans the *Eco* R I and *Hind* III sites of the pDELTA 2 map.

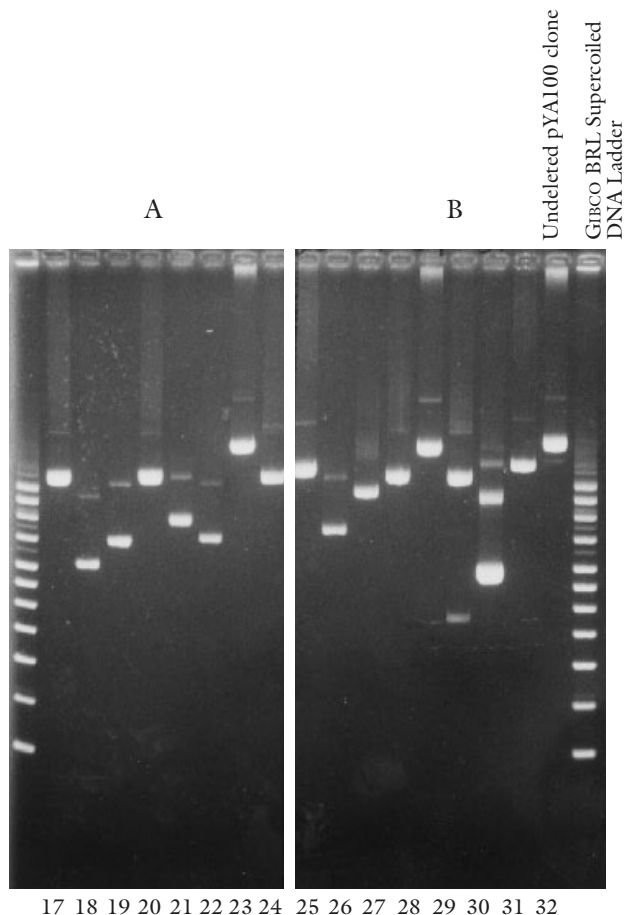


FIGURE 2. Agarose gel electrophoretogram of pYA100 deletion clone DNAs. Supercoiled DNAs were obtained from deletion subclones of pYA100 by miniprep. Panel A. DNAs from eight individual clones recovered from sucrose/ampicillin selection. Panel B. Eight DNAs recovered from streptomycin/kanamycin selection. Samples were electrophoresed through a 0.8% (w/v) agarose gel in TAE buffer. Numbers below each lane indicate individual clones. The lower band in lane 30 is the transposase-containing plasmid from the DF1 cells sometimes recovered, but which does not interfere in the analysis.

plating transformed cells on LB agar plates containing 5% (w/v) sucrose plus 100 µg/ml ampicillin, and on LB agar plates containing 100 µg/ml streptomycin plus 50 µg/ml kanamycin.

Preparation of single-stranded DNA. To obtain single-stranded DNA for sequencing, GIBCO BRL LIBRARY EFFICIENCY DH5αF™ Competent Cells were transformed with selected deletion phagemids. Two milliliters of autoclaved 2X YT broth [1% (w/v) yeast extract, 1.6% (w/v) tryptone, and 0.5% (w/v) sodium chloride (pH 7.5)] containing the appropriate selective antibiotic were inoculated with a single colony and incubated with shaking overnight at 37°C. The next day, 20 µl of the culture was diluted to 2 ml with fresh 2X YT broth containing the appropriate selective

antibiotic and incubated with shaking at 37°C for 1 to 2 h. M13K07 Helper Phage was added to a multiplicity of infection of about 10. The sample was returned to the 37°C shaking incubator for 1 h. Kanamycin was then added to the culture to a final concentration of 25 µg/ml to eliminate any uninfected cells. The sample was returned to the 37°C shaking incubator for an additional 5 to 6 h. The isolation of phage and purification of single-stranded DNA were as described (6). The final pellet was dissolved in 10 µl of 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.4). Three to seven microliters were used in the sequencing reaction.

DNA sequencing. Sequencing was performed using the GIBCO BRL dsDNA Cycle Sequencing System with either the T7 Promoter Primer or the SP6 Promoter Primer. [γ -³²P]ATP (5,000 Ci/mmol) from Amersham was used within two weeks of receipt for end labeling of primers. GEL-MIX® 6 was used to prepare sequencing gels that were electrophoresed on a Model S2 Sequencing Gel Electrophoresis System. The dried gel was exposed to x-ray film.

RESULTS AND DISCUSSION

The DELETION FACTORY System Version 2.0 employs a unique cloning vector, pDELTA 2 (figure 1), from which deletions are generated. pDELTA 2 is a cosmid containing the recognition sequences required for $\gamma\delta$ transposition. These recognition ends flank the origin of replication from pBR322. The transposase enzyme to form deletions is provided in *trans* by *E. coli* DF1 competent cells. A unique multiple cloning site (MCS) is embedded in the *lacZ* gene to allow for blue/white screening of colonies during the cloning process. Pairs of selectable and contra-selectable markers straddle the MCS and are used to select for deletions and to sort them according to the direction of the deletion. *SacB*, conveying sensitivity to sucrose, and *Amp* are used to select for clockwise deletions, while *StrA*, conveying sensitivity to streptomycin, and *Kan* are used to select for counterclockwise deletions.

DELETION FACTORY System Version 2.0 is similar to the original version except in the cloning vector. pDELTA 1 was modified to create pDELTA 2, which has the following new characteristics:

1. Two copies of the fl origin of replication have been inserted in opposing directions. As deletions occur, either clockwise or counterclockwise from the $\gamma\delta$ transposon ends, only one of the two fl origins remains, creating a phagemid from the resulting deletion subclones. pDELTA 2 itself is not a phagemid.
2. The origin of replication derived from pBR322 replaces that from pUC to lower the copy number. This increases the recovery and improves the stability of larger clones (7) and improves the size distribution of deletion subclones (8).
3. The *Amp* gene has been substituted for the *Tet* gene. Since membrane permeability is impeded by the *Tet* gene product and tetracycline is light sensitive, *Tet* was not ideal as a selection marker (9).
4. The *Xba* I site in the MCS is now unique to the vector.

The new DELETION FACTORY System was tested with pYA100. Double-stranded miniprep DNAs from 24 sucrose/ampicillin-selected deletion phagemids and 24 streptomycin/kanamycin-selected deletion phagemids derived from pYA100 were examined by agarose gel electrophoresis. These deletion subclones ranged in size from 4 to 26 kb. The supercoiled DNAs from eight clones recovered from each selection condition are shown in figure 2. From the 48 examined, six clones, covering a range of sizes, were selected to be sequenced in single-stranded form (table 1). Sequence was obtained in all cases. Figure 3 shows the sequence from the two largest clones of 14 and 18 kb.

In addition to sequencing, nested deletions are also valuable for mapping special regions within a stretch of DNA. Exons or factor binding sites could be easily localized using nested deletion sets. Once identified, single-stranded DNA from the phagemids could be used to

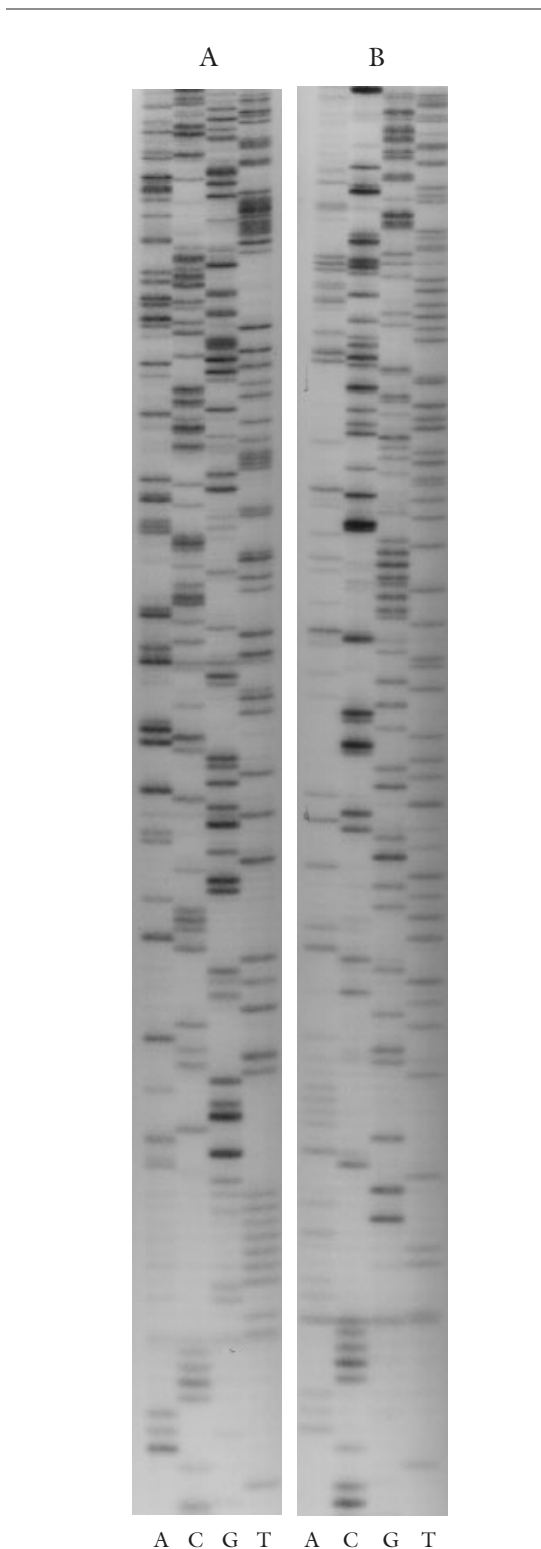


FIGURE 3. Sequence autoradiogram of 14-kb and 18-kb deletion phagemids. Deletion phagemids derived from pYA100 were sequenced as single-stranded DNA isolated as described in *Methods*. Panel A. Sequence data from clone 27 (14 kb). Panel B. Sequence data from clone 38 (18 kb). Both were sequenced with ^{32}P end-labeled T7 Promoter Primer.

TABLE 1. pYA100 deletion phagemids.

Selection Condition	Clone	Size (kb)
sucrose/ampicillin	11	8.0
sucrose/ampicillin	10	9.5
sucrose/ampicillin	9	10.5
sucrose/ampicillin	7	12.0
streptomycin/kanamycin	27	14.0
streptomycin/kanamycin	38	18.0

readily create point mutations at key sites for further study.

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COLONIES CONTAINING RECOMBINANT PLASMIDS CAN BE USED AS STARTING MATERIAL FOR PCR SITE-DIRECTED MUTAGENESIS

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The PCR Site-Directed Mutagenesis System combines PCR and UDG cloning to efficiently generate DNA containing a desired mutation (1). Mutagenesis is performed using four oligonucleotide primers. The gene is amplified in two segments and the PCR products are then cloned into an appropriate vector. Two of the primers overlap and one or both of these gene-specific primers contain the desired mutation. Two primers defined by the vector are used in combination with the gene-specific primers. The amplified fragments can be combined by overlap extension (2,3) or inclusion of deoxyuracil (dU) residues in place of deoxythymidine (dT) in the overlapping region of the mutagenesis primers (1,4,5). The vector primers are designed for UDG cloning of the combined PCR fragments (6,7). Due to the efficiency of UDG cloning, products can be generated with few cycles of amplification, using conditions designed for high fidelity of polymerization by *Taq* DNA polymerase.

This method permits the use of a double-stranded recombinant plasmid as the template DNA in PCR. Ideally, this plasmid is purified DNA, although DNA prepared by miniprep also works well. Using the *lacZ* α gene, we demonstrate that colonies containing recombinant plasmids can be used effectively as starting material for mutagenesis.

METHODS

Mutagenesis. The mutagenesis assay, including PCR, *Dpn* I treatment, UDG cloning, and transformation, was performed as described in the GIBCO BRL PCR Site-Directed Mutagenesis System (1). For site-directed mutagenesis using bacterial colonies as the source of starting DNA template, individual colonies of DH5 α cells containing pLAC-201 were picked, placed immediately into 10 μ l of 1X PCR buffer, incubated at 95°C for 5 min, and placed on ice. Five microliters of the sample were placed in each of two tubes in which fragments 1 and 2 of the *lacZ* α gene were amplified separately. To each tube, appropriate ingredients for PCR in a final volume of 50 μ l were added. Amplifications were performed for 12 cycles. In addition, 25- μ l aliquots were removed from the reactions containing colonies 1 and 2 after 8 cycles and placed on ice.

PCR was also performed on plasmid pLAC-201 DNA prepared by a miniprep procedure (8), and with DNA purified on CsCl gradients. Both of these plasmid preparations were linearized with *Sca* I. Five nanograms of the linear DNA were amplified for 8 cycles in two separate reactions. The two amplification products were combined, and the mutagenesis assay and cloning were completed. After transformation, colonies were scored on the basis of phenotype.

Primers. Oligonucleotide primers 1242, 1319, 1979, and 1801 were derived from the *lacZ* gene of pUC19 (referred to, respectively, as CP1b, CP2a, CP2b, and CP1a in ref. 1). Primers 1801 and 1242 generated fragment 1 (277 bp), upon amplification using pLAC-201 DNA. Primers 1979 and 1319 produced fragment 2 (236 bp). Primers 1242 and 1319 overlapped by 12 bp, and primer 1242 contained the single base substitution within its sequence (figure 1).

RESULTS AND DISCUSSION

The *lacZα* gene, residing in plasmid pLAC-201, served as a template for a single base substitution (1). This plasmid was derived from pUC19 by site-specific, PCR-based mutagenesis at base 89 of the *lacZα* structural gene sequences (4,9). The G→A substitution resulted in the creation of a termination codon (opal) that prevents proper production of the *lacZα* complementation peptide, resulting in a white colony phenotype in *E. coli*.

In this report, base 89 was reverted to wild-type by site-specific mutagenesis. The reversion of base 89 from A→G was accomplished by PCR site-directed mutagenesis and cloned. In the presence of the *lacZ* inducer IPTG, and X-gal, the phenotype of colonies that contained the properly mutagenized plasmid was blue due to the restoration of the wild-type (pUC19) sequence, whereas the plasmid template pLAC-201 resulted in a white colony phenotype.

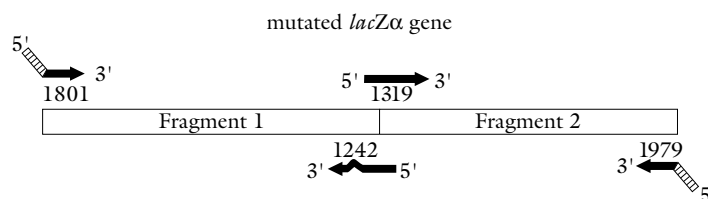
Table 1 summarizes the results of site-directed mutagenesis using a number of templates. When purified plasmid pLAC-201 was used in the mutagenesis assay, 90% of the colonies recovered harbored recombinant plasmids containing the mutation. The use of linearized DNA prepared by a miniprep procedure reduced the efficiency only slightly, to 84%. The efficiency of mutagenesis is dependent on the number of amplification cycles (data not shown). When colonies were used directly as a source of DNA, and amplification was again performed for 8 cycles, the efficiency was drastically reduced, to only 43% or 18%. This can be attributed to two factors. First, the amount of plasmid DNA present in the colony that is transferred to the reaction mix is highly variable. Second, the plasmid DNA present in these

colonies is intact, rather than linear. In general, amplification from intact plasmid templates is less efficient than from linearized DNA templates. By increasing the number of cycles of amplification from 8 to 12, these factors can be overcome. The efficiencies of mutagenesis ranged from 83% to 93%.

A low number of cycles (*e.g.*, 8), combined with the use of reaction components that are recommended for high-fidelity activity by *Taq* DNA polymerase (10), minimizes the possibility of random misincorporation of bases by the enzyme. A larger number of recombinants may then need to be screened in order to identify the desired plasmid.

The total number of transformants obtained when colonies were used as starting material for mutagenesis was variable (table 1). The difference between 8 and 12 cycles is reflected not only in the percentage of mutants, but also in the total number of recombinant-containing colonies obtained. The number of colonies shown in the table represented the recovery from one transformation. If all the available amplified material was used for transformation, 10-fold more colonies would be expected. For most purposes, sufficient colonies were obtained to locate the desired mutant.

In summary, plasmid-containing colonies can be used as the source of DNA for PCR site-directed mutagenesis. Due to the variability possible in the amount of DNA recovered from any one colony, it is advisable to select several colonies. The efficiency of mutagenesis is more variable with colonies than when purified plas-



Primer 1242 AAC GUC GUG ACU GGG AAA ACC CTG G
Primer 1319 AGU CAC GAC GUU GUA AAA CGA CGG C
Primer 1979 CAU CAU CAU CAU GCG CAA CGC AAT TAA TGT GAG
Primer 1801 CUA CUA CUA CUA AGC GGG TGT TGG CGG GTG

FIGURE 1. Placement of primers for PCR site-directed mutagenesis. The *lacZα* gene was amplified in two fragments. Hatched lines denote dU-containing regions of the primers, “^” represents the location of the mutation. The nucleotide representing the desired mutation is underlined in the sequence of primer 1242.

mid DNA is used as the template for PCR, but this can be largely overcome by increasing the number of cycles of amplification.

TABLE 1. Use of plasmid-containing colonies and purified plasmid DNA in PCR site-directed mutagenesis. DNA for amplification was derived from either colonies, minipreps, or CsCl-purified preparations. The efficiency of mutagenesis indicated is the percentage of colonies exhibiting a blue phenotype.

Starting Template	Cycles	Efficiency of Mutagenesis	Colonies Recovered from 1 ml
Purified plasmid DNA	8	90%	2,283
Miniprep plasmid DNA	8	84%	1,000
Colony No. 1	8	43%	1,435
Colony No. 2	8	18%	315
Colony No. 1	12	93%	12,020
Colony No. 2	12	83%	4,027
Colony No. 3	12	87%	3,487
Colony No. 4	12	91%	2,987
Colony No. 5	12	87%	2,733
Colony No. 6	12	89%	6,013
pAMP2 Vector control	—	—	14

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LIPOFECTAMINE™ REAGENT EFFICIENTLY TRANSFERS GENES INTO MAMMALIAN CELL LINES

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The transfer of recombinant genes into mammalian cells or tissue where the gene is expressed is a powerful tool in studying the function and control of these genes. However, many researchers find transfection of cell lines to be the most troublesome facet of their studies. Each cell line has unique properties able to affect gene transfer making certain cell lines intrinsically easier to transfect while others are more difficult. This is true for cell lines of the same tissue or even different clones of the same cell line. Therefore, selection of a transfection technique with a high probability of effecting successful gene transfer into the target cell line is important. We have found LIPOFECTAMINE Reagent to be able to transfect even the most impervious cell lines. We report here successful gene transfer into 17 cell lines of various species and tissues using LIPOFECTAMINE Reagent with a standard protocol.

METHODS

Luciferase reporter gene vectors driven by no promoter (negative control), the SV40 viral promoter/enhancer, or the bovine MHC Class I promoter/enhancer BL3-6prmt (GenBank L19193) were transfected into 17 different cell lines. Cell lines were obtained from ATCC except BL3.1 (1). Cells were transfected using GIBCO BRL LIPOFECTAMINE Reagent following the manufacturer's recommended protocol. Adherent cells were plated onto 6-well plates and grown until 70% to 80% confluent. Cells were then washed with 2 ml/well OPTI-MEM® I Reduced Serum Medium. After discarding the wash, 0.8 ml/well OPTI-MEM I was added. Non-adherent cells in exponential growth phase were pelleted and washed with OPTI-MEM I, then pelleted and resuspended in OPTI-MEM I at a concentration of 3×10^6 cells/ml. Cells were plated at 0.8 ml/well. For each transfection (well), 1 µg of plasmid DNA in 100 µl

OPTI-MEM I was mixed with 6 μ l LIPOFECTAMINE Reagent in 100 μ l OPTI-MEM I. The DNA/lipid mix was left to incubate at room temperature for at least 15 min before being added (0.2 ml/well) to the cells. After 3 h incubation at 37°C in a humidified 5% CO₂ incubator, 1 ml/well of media + 20% FBS was added. Sixteen hours later, cell extracts were prepared (200 μ l/well) and 20 μ l of the extract were analyzed via a luciferase assay system for 30 s on a Monolight 2010 luminometer. Gene transfer of each plasmid was performed in triplicate for each cell line. A standard curve of relative light units (RLU) vs. luciferase was made (figure 1) using purified firefly luciferase.

RESULTS AND DISCUSSION

Although there was enormous variability in transfection efficiencies between the different cell lines, the results indicate that by using LIPOFECTAMINE Reagent, we could transfect all the cell lines tested (table 1). However, we recommend that transfections be optimized for individual cell lines with transgene expression <5 times background. We also used the same transfection protocol for stable gene transfer integrating various recombinant gene constructs into the genome of A20.J, P815, RAW 264.7, COLO 320HSR, SW 837, BL3.1, and FLK cells (data not shown).

To study transgene function and control, the proper choice of transfection method is necessary for efficient gene delivery into the cell. We report here the ability of LIPOFECTAMINE Reagent to transfect all 17 cell lines tested. These cell lines are in addition to 7 other cell types transfected using LIPOFECTAMINE Reagent (2). High efficiency of transient or stable gene delivery to a wide variety of cell types with a minimum of labor makes LIPOFECTAMINE Reagent an excellent choice for a transfection technique.

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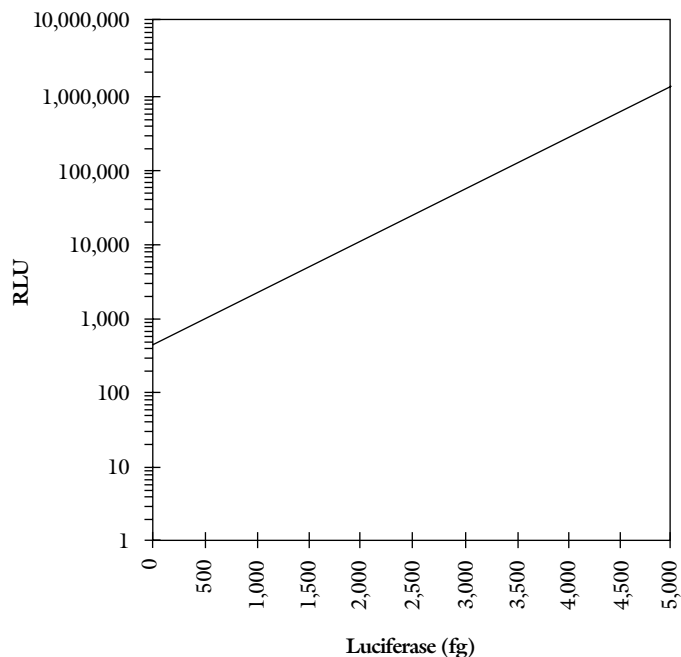


FIGURE 1. Luciferase standard curve. Known amounts of luciferase were assayed in triplicate with less than 5% coefficient of variation. Femtogram amounts of luciferase can be calculated based on the number of Relative Light Units (RLU) presented in table 1.

TABLE 1. Luciferase production in cell lines transfected by LIPOFECTAMINE reagent.

Species	Cell Line	Tissue	Mean RLU/20 ml sample ^a	
			SV40	BL3-6prmtr
Mouse	A20.J	B-Lymphoma	46,350 ^b	52,339
	C ₂ C ₁₂	Myoblast	168,745	7,954
	G8	Myoblast	977,224	35,826
	J774A.1	Monocyte	938	765
	P815	Mastocytoma	77,239	37,257
	RAW 264.7	Macrophage	6,767	9,809
Human	COLO 320HSR	Colon carcinoma	2,090,087	3,640,021
	JAR	Choriocarcinoma	145,248	4,678
	JEG-3	Choriocarcinoma	2,009,976	50,973
	LCL721.221	B-Lymphoblast	8,826	2,351
	SW 837	Rectal carcinoma	1,889	1,584
Cow	BL3.1	B-Sarcoma	48,298	239,506
	MDBK	Kidney	1,501	1,542
Dog	D17	Osteosarcoma	397,883	177,653
Monkey	Vero	Kidney	432,226	77,423
Rat	NMU	Mammary	389,923	11,298
Sheep	FLK	Kidney	2,365,004	144,557

^aLuciferase expression was driven by either the viral SV40 or mammalian BL3-6prmtr promoter/enhancer.

^bMean RLU of three transfections with measurements on 20 μ l/200 μ l total lysate. Mean RLU of negative control transfected cell lysate was ~460 for all cell lines. Signal saturation level of the luminometer was $\sim 2 \times 10^6$ RLU. For cell lines expressing $> 2 \times 10^6$ RLU, dilutions of lysate were used to measure within the linear range, and results were multiplied by the dilution factor so cell lines could be directly compared. Coefficient of variation was less than 10%.