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Development of episomal expression systems for genetically engineering human hematopoietic cells: Model analyses of the M-CSF:M-CSF receptor pair

Groger, Richard Kevin, Ph.D.
Case Western Reserve University (Health Sciences), 1990

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DEVELOPMENT OF EPISOMAL EXPRESSION SYSTEMS
FOR GENETICALLY ENGINEERING HUMAN HEMATOPOIETIC CELLS:
MODEL ANALYSES OF THE M-CSF:M-CSF RECEPTOR PAIR

by
RICHARD KEVIN GROGER

Submitted in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

Thesis Advisor: Mark Louis Tykocinski, M.D.

Department of Pathology
CASE WESTERN RESERVE UNIVERSITY
August 13, 1990
CASE WESTERN RESERVE UNIVERSITY

GRADUATE STUDIES

We hereby approve the thesis of

Richard Kevin Groger

candidate for the Ph.D.
degree.*

Signed:  

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DEVELOPMENT OF EPISOMAL EXPRESSION SYSTEMS
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MODEL ANALYSES OF THE M-CSF:M-CSF RECEPTOR PAIR

Abstract
by
RICHARD KEVIN GROGER

This thesis encompasses two linked sets of studies. One set focuses on novel developments in the field of stable gene transfer technology, particularly applicable to human cells, and the other employs some of these technological developments for model analyses of selected hematopoietic molecules with regulatory functions.

The gene transfection studies have culminated in both the introduction of a series of highly utilitarian episomal expression vectors and in an expansion of the repertoire of gene transfer strategies available for genetically engineering eukaryotic cells. Specific accomplishments include: (i) the assembly of a versatile array of episomal vectors, based upon Epstein-Barr virus (EBV) and BK virus replicative motifs and incorporating alternative promoters and selectable markers; (ii) the demonstration of the effectiveness of such vectors for
stable sense and antisense RNA expression, as well as for sequential or simultaneous gene cotransfection; (iii) the inception of an episome-based directional antisense cDNA cloning and expression technology; and (iv) the elucidation of structural rules governing episomal replicative capacity, providing general principles that can guide artificial episome assembly.

In parallel studies, episomal expression vectors were employed to study one specific hematopoietic ligand:receptor pair, namely monocyte/macrophage colony stimulating factor (M-CSF) and the M-CSF receptor (M-CSFR), together implicated in the regulation of monocytopenesis. Two specific unresolved issues pertaining to the functional repertoire of this molecular pair were addressed, exploiting the technical capacity to coordinately engineer, and manipulate the expression of, both molecules. Sense transfection analyses led to two principal findings: (i) the function of the M-CSF:M-CSFR pair is, at least in part, cell type- and lineage-restricted; and (ii) M-CSF, when artificially tethered to cell surfaces by a glycoinositolphospholipid membrane anchor, can function as an adhesin, extending the scope of possible functions associated with molecules of this class.
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CHAPTER 1: EPISOMAL VECTORS.

1.A. INTRODUCTION.

Several classes of vectors have been introduced for gene expression purposes in eukaryotic cells. These include retroviral integrating, non-retroviral integrating, and episomal non-integrating vectors. The latter, episomal vectors, are defined as expression vectors that function as episomal replicons, circular DNA elements that replicate extrachromosomally in eukaryotic cell nuclei. Most episomal vectors that have been developed can be considered shuttle vectors in that they incorporate replicative signals and selectable markers for their maintenance not only in eukaryotic (e.g. human), but also in prokaryotic (e.g. E. coli), cells. Episomal replicons tend to have limited host ranges, and specific episomal expression vectors have been developed for cells of species ranging from single cell eukaryotes, such as yeast and slime mold, to higher eukaryotes, such as rodents and primates.

In principle, episomal (extrachromosomally-replicating) expression vectors offer significant advantages over integrating ones for achieving high level sense and antisense RNA transcription in cells: (i)
Episomal vectors, by virtue of their self-replicating property, afford an expeditious means for amplifying exogenously introduced transcriptional cassettes; (ii) Episome-based expression of transfected genes is not subject to positional effects at chromosomal integration sites, and hence, episomal vectors are expected to permit more reproducible levels of gene expression than do integrating ones; (iii) Episomal vectors avoid complications of host sequence mutagenesis at chromosomal integration sites; (iv) Higher gene transfection efficiencies can generally be achieved with episomal, as opposed to integrating, vectors (Belt et al., 1989), presumably as a consequence of obviating the chromosomal integration step; and (v) Episomal vectors can be conveniently recovered from cells by the method of Hirt (1967), a feature of such vectors that is of considerable value for both confirming the integrity of transfected genes and recovering individual cDNAs from cells transfected with sense or antisense cDNA clonal pools (see Chapter 2). The major disadvantage of episomal, as opposed to integrating, vectors is that the former segregate randomly during cell division; hence, selective pressure must be continued on an ongoing basis in order
to ensure maintenance of the episome in the cell population.

The replicative signals of episomal vectors are largely derived from DNA viruses that themselves normally replicate extrachromosomally in the cell nucleus. These replicative signals encompass both cis-acting sequences, e.g., a eukaryotic origin of replication (ori), as well as sequences encoding trans-acting nuclear factors required for ori's replicative function. Such trans-acting factors can either be encoded by sequences on the episome itself, or alternatively, may be provided in trans by the host cell. The host range specificity seen for viral replicative signal functioning is probably a consequence of host cell-derived factors, and in turn, the host range of episomal vectors likely reflects the host range specificity of the viral replicative signals from which they are derived.

The growing list of viruses used in generating episomal vectors includes simian virus 40 (SV40) (Mellon et al., 1981), bovine papillomavirus (BPV) (Law et al., 1983), BK virus (BKV) (Milanesi et al., 1984), and Epstein-Barr virus (EBV) (Yates et al., 1985). Both EBV- and BKV-based episomes have been used for stable gene expression in human cells (vide infra), and BPV has been
used to stably express genes in rodent cells. SV40, which replicates to levels that rapidly lead to host cell death, has been widely used for transient gene expression purposes in primate cells. Specific episomal vectors have also been developed for use in lower eukaryotic cells, such as those of yeast and slime mold.

1.B. EBSTEIN-BARR VIRUS-BASED EPISOMAL VECTORS.

The EBV genome replicates extrachromosomally in human B lymphocytes as a 172 kb, double-stranded, closed DNA circle. A 1.8 kb segment from this viral genome, designated oriP (for origin of plasmid replication), can confer to any circular DNA element the ability to replicate extrachromosomally in eukaryotic cells, as long as they simultaneously expressing the virally-encoded EBV nuclear antigen-1 (EBNA-1) (Yates et al., 1984). The EBV oriP consists of a set of twenty 30 bp tandem repeats, separated by 1 kb from a symmetrically arranged pair of homologous repeats, referred to as the region of dyad symmetry (Reisman et al., 1985). Both sequence elements (the twenty member repeat family and the region of dyad symmetry) bind to the EBNA-1 protein (Rawlins et al., 1985), and furthermore, both are required for episomal replication. Varying the spacing between, and the
relative orientations of, these regions affects episome copy numbers in cells. Mecsas and Sugden (1987) have suggested that the EBNA-1 protein is analogous to the SV40 T antigen, in that the latter also binds to a region of dyad symmetry in its corresponding ori. The SV40 T antigen is an ATP-dependent helicase which facilitates bidirectional DNA replication (Stahl et al., 1986).

An episome constructed by Yates et al. (1985), p201, contains both the oriP region and sequences encoding the EBNA-1 protein, together sufficient for directing episomal replication in a wide variety of human cells which are not latently infected by EBV, and hence, do not have endogenous EBNA-1 protein. A variant of p201, p205, encodes a variant of EBNA-1 with a 700 bp in-frame deletion, removing a 230 amino acid long glycine/alanine-rich region; this p205 variant has been shown to replicate to twice the copy number of p201. Both p201 and p205 contain the bacterial hygromycin phosphotransferase (hph) gene (Gritz and Davies, 1983), allowing for the selection of stably transfected cells in the presence of the eukaryocidal antibiotic hygromycin B.

In order to create first generation EBV episomal expression vectors that would be generally useful for high level sense and antisense RNA expression purposes, I
inserted transcriptional cassettes, consisting of the Rous sarcoma virus (RSV) 3' long terminal repeat (LTR) (Gorman, et al., 1982), a multiple cloning site (MCS), and an SV40 late polyadenylation/termination signal (Okayama and Berg, 1983) linearly arrayed, into p220.2 (a derivative of p201 provided by B. Sugden, U. of Wisconsin). One of the resultant vectors, designated pREP3, is schematically depicted in Figure 1.1 (see Section 2.C.i and Figure 2.1 for details of the multistep assembly scheme for this episome). The RSV 3' LTR was chosen since previous studies in our laboratory (Hambor et al., 1988; Hauer et al., 1989) established the capacity of this promoter/enhancer element, when episome-based, to direct high level, constitutive expression of the prokaryotic chloramphenicol acetyl transferase (CAT) gene in transformed (U937 and K562) and nontransformed (T lymphocyte) human hematopoietic cells. pREP3, and related vectors, have been shown in our laboratory to be useful for driving high level antisense RNA transcription in human cells, thereby being effective in mediating specific inhibition of gene expression. For instance, Figure 1.2 demonstrates the successful use of pREP1 (differing from pREP3 only in the MCS), to achieve specific antisense RNA-mediated inhibition of CD8
Fig. 1.1. pREP3. The hph gene is flanked by the herpes simplex virus type 1 thymidine kinase promoter and termination signals (stippled). The 20 member family of 30 base pair repeats and the region of dyad symmetry within the Epstein-Barr virus origin of replication (EBV oriP) are indicated. B, BamHI; Bs, BstEII; EBNA-1, Epstein-Barr virus nuclear antigen-1; hph, hygromycin phosphotransferase; K, KpnI; Nh, NheI; No, NotI; P, PvuII; PA, SV40 late polyadenylation/termination sequences; pBR, pBR322 origin of replication and ampicillin-resistance gene; RSV, Rous sarcoma virus 5' long terminal repeat; S, SalI; Sf, SfiI; Ss, SstI; Xh, XhoI.
Fig. 1.2. Selective antisense RNA-mediated inhibition of surface CD8 expression on 8L2 cells. pα-CD8/REP1 and pRSVαCATα/220.2 8L2 transfecants and parental 8L2 cells were stained on day 46 with OKT8 (anti-CD8), OKT3 (anti-CD3), and OKT11 (anti-CD2) (stippled areas) or normal mouse IgG (Miles; open areas) as primary antibodies, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Miles) was used as the secondary antibody (Hambor et al., 1988).
expression on a CD8⁺ cloned T cell line, 8L2. In this case, a 459 bp segment of the CD8 coding sequence was inserted in an antisense orientation into pREP1, generating pα-CD8/REP1. pα-CD8/REP1 8L2 transfectants demonstrated greater than 95% inhibition of CD8 expression, without affecting CD2 or CD3 expression (Hambor et al., 1988a). In a similar fashion, greater than 97% inhibition of CD8 was achieved in a second CD8⁺ T cell clone, JH.ARL.1 (Hambor et al., 1988b), establishing the generality of the earlier finding with 8L2. This work represented the first application of episomal vectors to antisense RNA expression work, highlighting the effectiveness of inhibition that can be achieved with such vectors and contrasting with the variable antisense RNA-mediated inhibition achieved by others with integrating vectors. Additionally, this work provided the first reported stable gene transfer into nontransformed, cloned human T lymphocytes, perhaps reflecting a special advantage of EBV episomal vectors in this cellular context. Subsequent studies in the laboratory, with both T and non-T (e.g., K562 erythroleukemia and KM-102 bone marrow stromal) cells, have employed pREP3 for equally effective inhibition of other genes, such as cell surface-associated CD2 and soluble
GM-CSF. Moreover, pREP vectors have been shown to be effective for stable sense RNA expression. For instance, Figure 1.3 demonstrates the use of pREP2 for the sense expression of a CD8/DAF chimeric polypeptide in K562 and U937 cells (Tykocinski et al., 1988).

1.C. POLYOMA VIRUS-BASED EPISOMAL VECTORS

Milanesi et al. (1984) showed that a DNA fragment from the BK papovavirus, containing the BKV origin of replication (BKV ori) and BK large T antigen, can confer to any circular DNA element the ability to replicate extrachromosomally in human cells. Grossi et al. (1988) have recently reported the construction of a G418-selectable episomal cDNA expression vector, termed pRP-c, incorporating the minimal BK virus sequences required for extrachromosomal maintenance. Similar work in our laboratory (R. Groger, C. Hauer, and M. Tykocinski, unpublished observations), performed contemporaneously with the work of Grossi et al., has shown that BKV-based episomal replicons can be used to mediate efficient transfection of, and high level reporter gene expression in, the human leukemic cell line K562. Our first BKV-based episomal expression vector, designated pRBK, is shown in Figure 1.4. Unlike vectors based upon the
Fig. 1.3. Sense expression pCD8·DF2/REP2 transfected K562 and U937 cells. pCD8·DF2/REP2-transfected U937 cells (Left) and pCD8·DF2/REP2- and pCD8/REP2-transfected K562 cells (Right) were treated with phosphatidylinositol-specific phospholipase C (b and d) or buffer alone (a and c) and analyzed by flow cytometry (as in Fig. 2) using the anti-CD8 monoclonal antibody OKT8; mean fluorescence intensities (X) (channel numbers) are given. The dotted lines correspond to controls consisting of the various combinations of transfectants and monoclonal antibodies (mAB) indicated in the figure. Approximately $10^6$ cells were used for each analysis (Tykocinski et al., 1988).
Fig. 1.4. pRBK. The hph gene is flanked by the herpes simplex virus type 1 thymidine kinase promoter and termination signals (stippled). The darkly shaded region within the BK viral sequences is the 68 bp minimal origin of replication (BK ori). hph, hygromycin phospho-transferase; Nh, NheI; No, NotI, PA, SV40 late polyadenylation/termination sequences; pBR, pBR322 origin of replication and ampicillin-resistance gene; RSV, Rous sarcoma virus 5' long terminal repeat; S, SalI; Sf, SfiI; Xh, XhoI.
highly homologous SV40 virus, which replicate to copy numbers that kill their host cell, BKV-based episomes replicate to a lower and self-limited copy number (1-400 copies per cell); hence, the latter, like EBV-based episomes, can be used to derive stable transfectants. The BKV ori consists of a minimal region of 76 bp, containing an inverted repeat and a 20 bp AT block (Deyerle, et al., 1989). The BK large T antigen is a helicase homologous to the SV40 large T antigen, and may function in a manner analogous to the latter and the EBNA-1 gene product.

The following chapters describe further enhancements of the EBV and BKV episomal vector systems, with adaptations for specialized gene transfer applications, analyses of primary sequence determinants of episome function, and model applications of such vectors in molecular studies in human hematopoietic cells. More specifically, the next chapter details a novel strategy for isolating unknown genes mediating defined cellular functions, through gene inhibition with the randomly generated clones of an episome-based antisense cDNA expression library. Chapter 3 examines how altering the configuration of DNA sequence elements in EBV- and BKV-based episomes affects episome stability and reporter
gene expression. Chapter 4 discusses the development and pairwise use of independently-selectable episomal vectors, providing the first efficient stable cotransfection system for eukaryotic cells. The second part of this thesis, (Chapters 6-9) deals with the specific application of our episomal expression system to study the actual and potential functional repertoire of M-CSF and its receptor in human hematopoietic cells.
CHAPTER 2: DIRECTIONAL ANTISENSE AND SENSE cDNA CLONING USING EPSTEIN-BARR VIRUS EPISOMAL EXPRESSION VECTORS.

2.A. ABSTRACT.

A set of Epstein-Barr virus (EBV) episomal expression vectors, incorporating either the Rous sarcoma virus 3' long terminal repeat or the human metallothionein IIa gene promoter, were constructed. The transcriptional cassettes encompassed by these vectors were designed to permit both antisense and sense RNA transcription. A novel methodology was developed for directional cDNA cloning using an oligodeoxynucleotide adapter, and the EBV episomal vectors alternatively enabled the insertion of cDNA segments in antisense or sense orientations. We propose a strategy for random antisense RNA mutagenesis exploiting this vector system and a method for episome-based directional antisense cDNA cloning and expression, permitting the rapid identification of genes mediating selectable cellular functions.
2.B. INTRODUCTION.

Cellular mutagenesis strategies offer important experimental means for genetically dissecting cellular physiological processes. Mutagenesis techniques resulting in gross chromosomal alterations, such as those employing ionizing radiation (Chu et al., 1961) and insertional mutagenesis (King et al., 1985), permit, in principle, the identification of the affected genes responsible for the phenotypic alterations in mutant cells. In the case of ionizing radiation-induced DNA damage, translocations or deletions may point to the chromosomal location of the gene of interest, and thereby provide an important clue for its identification. For insertional mutagenesis, the mutagenizing DNA segment tags the affected gene, facilitating its recovery from genomic DNA libraries produced from the mutant cell. These DNA-based mutagenesis strategies have limitations for somatic cell genetics, including the multiplicity of mutagenic events per cell, the relatively cumbersome experimental stratagems required for retrieval and identification of affected genes, and the presumed requirement, in many cases, for inactivation of both alleles of codominantly expressed diploid genes and all loci of duplicated genes.
The development of antisense RNA technology in recent years has introduced an additional experimental approach for cellular mutagenesis (Inouye, 1988). Cellular phenocopies of null mutations can be generated through the introduction, into target cells, of promoter-DNA cassettes which direct specific antisense RNA transcription (Izant and Weintraub, 1984; Kim and Wold, 1985). To date, this and other (Toulmé and Hélène, 1988) antisense RNA techniques have been used to inhibit the expression of specific, preselected genes. We have been intrigued by the alternative method of performing antisense RNA mutagenesis in a random context to generate arrays of antisense cellular mutants. Such an RNA-based strategy would avoid the potential limitations, of DNA-based approaches resulting from genetic diploidy and duplication.

The utilization of episomal replicons, as vectors for antisense RNA transfection work, has added a new dimension to this technology. Episomal replicons are circular DNA elements containing viral or other nucleotide sequences that permit them to replicate extrachromosomally to high copy numbers in eukaryotic nuclei (Milanesi et al., 1984; Yates et al., 1985). Hence, expression vectors fashioned from episomal repli-
cons permit the amplification of transfected genes, and at the same time bypass cis effects upon promoter function at chromosomal integration sites. In a recent study, we (Hambor et al., 1988) and have demonstrated the utility of episomal vectors based upon Epstein-Barr virus (EBV) for attaining effective antisense RNA-mediated inhibition of a preselected gene in human cells. Episomal vectors would confer a special advantage to an antisense RNA-based random mutagenesis strategy, since the episome-based gene segments corresponding to affected genes can be readily retrieved from mutant cells by the Hirt (1967) procedure.

In this study, we report the assembly of a set of EBV episomal vectors designed for high-level antisense or sense RNA transcription in human cells. Three of these EBV episomal vectors have been specifically engineered to alternatively permit directional antisense and sense cDNA cloning. We further introduce the use of oligodeoxyribonucleotide (oligo) adapter addition for directional cDNA cloning and employ this method to generating episome-based, directional cDNA libraries.
2.C. MATERIALS AND METHODS.

2.C.i. Plasmid constructions.

The construction schemes for pREP2 and pREP3 is outlined in Figure 2.1 and the corresponding legend. For the assembly of pMEP4, pREP4, and pREP5 vectors, p220.2/B⁻ and pRSVPA2 (see Fig. 2.1) served as the starting points. p220.2/B⁻ was converted into p220.2/XS in two steps. p220.2/B⁻ was first linearized with HindIII, and the cleaved site was filled-in using PolIk, generating the intermediate plasmid p220.2/B'H⁻. The NheI site created by fill-in and recircularization was then eliminated by SalI+NheI digestion, PolIk fill-in, and recircularization, resulting in p220.2/XS. pRSVPA2 was converted into pHS1PA4 by an indirect approach in the following five steps: (1) pRSVPA2 to pMMTVPA2, replacing the RSV 3' LTR (HindIII-HindIII) in pRSVPA2 with the MMTV 5' LTR, using a MMTV 5' LTR (HindIII-HindIII) obtained from our derivative of pMSG (Pharmacia, Inc., Piscataway, NJ), in which the SmaI site upstream of the MMTV 5' LTR has been changed to a HindIII site with an oligo linker (New England Biolabs, Inc.); (2) pMMTVPA2 to pHSV1PA2, replacing the MMTV 5' LTR (HindIII-KpnI) in pMMTVPA2 with the human metallothionein II₄ (hMTII₄) promoter (HindIII-
Fig. 2.1. Assembly scheme for pREP2 and pREP3 vectors. The RSV 3' LTR and SV40 late polyadenylation/termination sequences were derived from the plasmids RSVCAT (Gorman et al., 1982a) and pCDVI (Okayama and Berg, 1983), respectively. Plasmid p220.2 was provided by Dr. B. Sugden and is a derivative of p201 (Yates et al., 1985), in which a multiple cloning site (MCS; BamHI, XbaI, SalI, PstI, HindIII), derived from pUC12, has been inserted into the NarI site of the herpes simplex virus 1 (HSV1) thymidine kinase-encoding gene termination sequence (B. Sugden, personal communication). The transcriptional cassette in the plasmid intermediate pRSVPA2 was transferred into p220.2/B', a modified version of p220.2. The sequences of the two strands of the oligo adapter (KpnI and BamHI cohesive ends) used to convert pREP2 to pREP3 are: 5'-CAGCTGCTAGGCGCCTCGAGGCAGGCAAGGCCG-3' and 5'-GGATCCGCCCTTCGAGGCGCCGCTAGCAGCTGTTAC-3'. B, BamHI, pBT, pBluescript; C, ClaI; H, HindIII; K, KpnI; Klenow, PolIk; N, NdeI; Na, NaeI; Nh, NheI; No, NotI; Pv, PvuII; RV, EcoRV; S, SalI; Sf, SfiI; T, TthIII-1; Xh, XhoI.
KpnI) obtained from our derivative of pHS1CAT (Scholer et al., 1986), in which the BamHI site downstream of the hMTII A promoter has been changed to a KpnI site with an oligo linker; (3) pHS1PA2 to pHS1PA3, generating a MCS downstream from the hMTII A promoter by inserting the pREP3 MCS (KpnI-BamHI; Fig. 2.1) between the corresponding sites in pHS1PA2; (4) HS1PA3 to HS1BglII, eliminating the ClaI and HindIII sites immediately upstream of the hMTII A promoter by digesting pHS1PA3 with ClaI+HindIII, blunting by PolIk fill-in, and religating with a BglII oligo linker; and (5) pHS1BglII to pHSVPA4, adding a HindIII site to the MCS by digesting with NheI, filling-in with PolIk and religating with a HindIII linker (two NheI sites flank the new HindIII site). All other construction steps are as depicted in Figure 2.2. The sequences of the two strands of the oligo adapter (KpnI and BamHI cohesive ends) used to convert pREP4 to pREP5 are: 5'-GATCGGTACCAGCTGAAGCTTGGCTAGGCGCGCCTCGAGGCACGCGATCCGATAC-3' and 5'-GGATCCGGCTTGC-CGGCGCTGAGGCGCGCTAGGACAGCTTGCAGCCGTTAC-3'. Oligos were synthesized by Dr. P. deHaseth (Case Western Reserve University) using the phosphoramidite method (Beaucage and Caruthers, 1981).
Fig. 2.2. Assembly scheme for pMEP4, pREP4, and pREP5 vectors. The assembly of the intermediate vectors p220.2/XS and pHSIPA4 is described in section 2.C.i. All other construction steps are depicted. The Nael site in the MCS is not unique. RSV 3' LTR (hatched); SV40 late polyadenylation/termination sequences (stippled); hMTII_A promoter (alternate hatched); Bg, BglII; Xb, XbaI. For other symbols, see Fig. 2.1 legend.
The plasmid pβJW102/X2 was derived by inserting an XmnI linker (5'-GAAAATTTTC-3') into the PvuII site of our plasmid pβJW102/GEM. This plasmid was derived previously from pβJW102 (Wilson et al., 1978) by transferring the HindIII-BamHI segment, encompassing part of the human β-globin-encoding gene, into pGEM-1 (Promega Biotec Inc., Madison, WI).

2.c.ii. cDNA cloning.

Total cellular RNA from human myeloid leukemia (HL-60) cells induced with 10⁻⁷M vitamin D for 2 h was extracted by the guanidinium thiocyanate/CsCl method (Chirgwin et al., 1979); poly(A)⁺RNA was purified (x2) by oligo(dT)-cellulose chromatography. Poly(A)⁺RNA (15 μg) was heated to 65°C for 15 min, and first strand synthesis was then primed with a 1:1 (w/w) ratio of oligo(dT)₁₂₋₁₈ to poly(A)⁺RNA, using 150 units of avian myeloblastosis virus (AMV) reverse transcriptase Biorad, Inc., Richmond, CA; 42°C, 45 min, 100 μl reaction volume, in a buffer consisting of 50 mM Tris·HCl, pH 8.3 at 42°C/10 mM MgCl₂/10 mM dithiothreitol/140 mM KCl/0.5 mM of each deoxynucleotide triphosphate (Pharmacia, Inc.)/150 μCi [α-³²P]dCTP). The reaction was stopped with 100 mM EDTA, pH 8.0/0.2% sodium dodecyl sulfate, and the mixture was
passed over a 1-ml G-50 Sephadex (Pharmacia, Inc.) column in a syringe to eliminate free nucleotides. Alkaline hydrolysis (70 mM NaOH, 65°C, 1 h, 175 µl reaction volume) was followed by neutralization with 75 mM glacial acetic acid and precipitation with 2 vols. ethanol. Second strand synthesis was accomplished by a two step procedure to maximize first-strand utilization. This entailed a primary synthetic reaction using 200 units AMV reverse transcriptase (42°C, 1 h, 150 µl reaction volume, in the same buffer used for first strand synthesis), followed by a secondary synthetic reaction which was initiated by adding 25 units of PolIk (NEB, Inc.) to the reaction mixture (37°C, 1 h). After passage over a 1-ml G-50 Sephadex column, the cDNA was phenol-chloroform (1:1)-extracted, chloroform-extracted, and precipitated with 0.1 vol. NaOAc, pH 5.0/2 vols. ethanol. The hairpin loop was cleaved and the cDNA ends were blunted with 20 units Mung bean nuclease (Stratagene, Inc., San Diego, CA; 30°C, 30 min, 200 µl reaction volume, in a buffer consisting of 30 mM NaOAc, pH 5.0/50 mM NaCl/1 mM ZnCl₂/5% glycerol). Following extraction and precipitation, HindIII sites within the double-stranded cDNA inserts were protected using 7.5 units of M·AluI methylase (NEB, Inc.; 37°C, 30 min, 30 µl reaction
volume, in a buffer consisting of 50 mM Tris·HCl, pH 7.5/10 mM EDTA/5 mM 2-mercaptoethanol/80 μM 5-adenosylmethionine). Following extraction and precipitation, a PolIk reaction was repeated to maximize blunting of cDNA insert ends, using 2.5 units of PolIk (25°C, 15 min, 30 μl reaction volume, in a buffer consisting of 7 mM Tris·HCl, pH 7.5/7 mM MgCl₂/1 mM DTT/20 μM of each dNTP). PolIk was heat-inactivated (65°C, 10 min), and the reaction mixture was then extracted and precipitated. The oligo adapter depicted in Fig. 2.3, consisting of a phosphorylated 10-mer annealed to a nonphosphorylated 14-mer, was ligated to both ends of cDNA inserts using 400 units of T4 DNA ligase (NEB, Inc.; 12°C, 16 h, 20 μl reaction volume). Following heat inactivation (65°C, 10 min), HindIII digestion (200 units, 37°C, 2 h, 150 μl reaction volume) was performed. CL6B-Sepharose chromatography (2-ml column, 200-μl fractions) was used to remove free oligos. cDNA inserts were then phosphorylated with T4 polynucleotide kinase (NEB, Inc.) and inserted between the phosphatased BamHI and HindIII sites of either pBluescript (Stratagene, Inc.) or our EBV episomal cloning vectors pREP4, pMEP4, and pREP5.
2.C.iii. Bacterial transformation.

The bacterial strains ER1451 (JM107 background; EcoK R'R', McrA') and ER1562 (MM294 background; EcoK R'M', McrA'B') were obtained from NEB, Inc. and bacteria were made competent for transformation by the RbCl₂ procedure distributed by the company.

2.D. RESULTS AND DISCUSSION.

2.D.i. Assembly of EBV episomal expression vectors for directional cDNA cloning.

In previous studies we have shown that the RSV 3' LTR, when based in an EBV episome, directs high level expression of transfected genes in human T-cell clones (Hambor et al., 1988) and human myeloid leukemia cells (Hauer et al., 1989). Consequently, in designing a set of EBV episomal expression vectors for human cells, we chose the RSV 3' LTR as promoter. The construction of our RSV 3' LTR-based EBV episomal expression vectors pREP2 and pREP3 is depicted in Figure 2.1. To assemble pREP2, a transcriptional cassette, consisting of the RSV 3' LTR, unique KpnI and BamHI subcloning sites, and the simian virus 40 (SV40) late polyadenylation/termination signals arranged in a linear array (represented in the
plasmid intermediate pRSVPA2), was inserted into the EBV episomal replicon p220.2, a derivative of p201 (Yates et al., 1985). Plasmid p220.2 carries the (oriP), the EBNA-1-encoding gene, whose product transactivates oriP's replicative function in human cells (Reisman and Sugden, 1986), the *Escherichia coli* hph gene which confers resistance to the eukaryocidal antibiotic hygromycin B (Gritz and Davies, 1983), and prokaryotic gene sequences, which confer to it a eukaryotic-prokaryotic shuttling capacity. The effectiveness of the transcriptional cassette configured into the pREP2 vector was established in our recent human CD8 expression study (Tykocinski et al., 1988). To facilitate subcloning of DNA segments into the transcriptional cassette, pREP3 was derived by introducing an oligo adapter between the KpnI and BamHI sites of pREP2, thereby providing additional unique subcloning sites.

Our oligo adapter-addition directional cDNA cloning methodology (see section 2.D.ii, below) requires the presence of unique BamHI and HindIII subcloning sites in the MCS of the cloning vector. While pREP2 and pREP3 possess unique BamHI sites in their MCSs, two HindIII sites are present outside of, in addition to the one inside of, their respective MCSs. The construction of
EBV episomal vectors (pREP4, pMEP4, pREP5) that contain unique BamHI and HindIII sites in their MCSs is depicted in Figure 2.2. The starting points for this assembly scheme were the plasmid intermediates pRSVPA2 and p220.2/B' depicted in Figure 2.1. The unique HindIII and BamHI sites in the pREP4 (pMEP4, the inducible hMTII_A promoter) and pREP5 vectors are arrayed in alternative orientations to permit antisense and sense directional cDNA cloning, respectively.

2.D.ii. Directional cDNA cloning into prokaryotic and pREP vectors.

We developed a procedure for directional cDNA cloning into plasmid vectors (Fig. 2.3) in which we modified the directional cDNA cloning procedure reported by Dorssers and Postmes (1987) and Meissner et al. (1987) by substituting adapters for linkers. According to this general method, directionality is achieved by creating a HindIII site at the 3'-end of each cDNA insert. This is accomplished by linking an oligo starting with GCTT to both ends of cDNA inserts, generating HindIII sites (AAGCTT) at the 3' ends of all cDNAs derived from poly(A)'mRNAs, but at the 5'-ends of only those one in 16 cDNAs starting with the TT dinucleotide. HindIII
Fig. 2.3. Procedure for directional cDNA cloning into EBV episomal vectors using an oligo adapter. Poly(A)"RNA (blackened bars) is converted into double-stranded cDNA (stippled bars), and the oligo adapter (designated in upper-case letters) is ligated to both ends of the cDNA. Following HindIII digestion and kinasing, the cDNA is directionally inserted into either pREP4 or pREP5. See section 2.C.ii, for details of the experimental protocol.

The hph gene is flanked by the HSV-1 thymidine kinase promoter and termination signals (stippled). R.T., AMV reverse transcriptase; pBR ori, pBR322 origin of replication; amp, ampicillin-resistance gene; P, phosphate moiety. For other symbols, see Figure 2.1 legend.
digestion generates a HindIII cohesive end at the cDNA 3' end::oligo-adapter junction, but leaves the BamHI cohesive end of the adapter in place at the 5' terminus of the cDNA. Alternative ordering of the HindIII and BamHI sites in the MCSs of pREP4 and pREP5, enables antisense and sense cDNA cloning, respectively. Use of phosphorylated 10-mer and nonphosphorylated 14-mer strands in the adapter prevents oligo concatenation.

We incorporated some redundant steps into our methodology (described in detail in section 2.C.ii) to maximize cloning efficiencies (Fig. 2.3). Second-strand synthesis was accomplished with two enzymatic reactions performed in tandem, using AMV reverse transcriptase and PolIk. These two enzymes complement each other in overcoming DNA secondary-structural constraints. Blunting of cDNA insert ends was attained through the use of both mung-bean nuclease and PolIk fill-in reactions to maximize the amount of blunt-ended double-stranded cDNA substrate available for adapter ligation.

We first used a test double-stranded DNA insert to determine whether directionality could in fact be achieved by our oligo adapter-addition methodology and to assess the efficiency of the M·AluI (HindIII) methyla-
tion, adapter ligation, restriction-site cleavage, and
vector ligation steps of this procedure. An 1813-bp *XmnI* DNA fragment, which encompassed an asymmetric internal *HindIII* site and a stretch of four A's at one end to mimic the poly(A) stretch at the end of cDNA inserts, was excised from our plasmid βJW102/X2 and used as the test insert (Fig. 2.4a). This test insert was processed as indicated in Figure 2.3, starting with the methylation step, and ligated into either pBluescript or pREP4 vectors. To avoid restriction due to *AluI* methylation, *McrB* bacterial strains (ER1451 and ER1562) were used. All pBluescript-based clones examined (twelve of twelve in ER1451, and 17 of 17 in ER1562; data not shown) and all pREP4-based clones (26 of 26 in ER1562; Fig. 2.4b) contained the insert in the correct orientation with the internal *HindIII* site intact, establishing the efficiency of this directional cloning methodology.

We next used poly(A)+RNA from the HL-60 line to generate a cDNA library in the pBluescript vector, by the method outlined in Figure 2.3. To definitively establish the directionality of cDNA clones in this library, eight were sequenced from the flanking *SalI* site in the pBluescript MCS, just beyond the predicted A·T stretches. As expected, a poly(A) stretch was visualized within the cDNA contributing to a newly constituted *HindIII* site in
Fig. 2.4. Use of a test DNA insert to establish that
directionality can be achieved by the adapter-addition
methodology. (a) The schematic illustrates the array of
restriction endonuclease sites that are expected
following addition of the oligo adapter (see Fig. 3) to
the ends of the 1.8 kb XmnI test insert derived from
pβJW102/X2 (stippled) and subsequent directional
subcloning of this adapter-modified insert into the
vector pREP4 (10.1-kb) (hatched). HindIII and BamHI
digestions are expected to yield DNA segments of 706-bp
and 1763-bp in length, respectively, when this test
insert is correctly oriented in the vector. The test
insert was mobilized from pβJW102/X2 by XmnI digestion,
and this 1813-bp fragment contained a HindIII site 707 bp
from the new XmnI site, ending in the dinucleotide AA.
(b) Oligo adapter-modified test insert was subcloned into
pREP4, and the orientation of the test insert in
independent subclones (subclones Nos. 1–9 shown) was
determined by restriction endonuclease digestion with the
enzymes indicated and analysis on 0.7% agarose gels. B,
BamHI; H, HindIII; U, uncut; X, XhoI; Xm, XmnI. DNA
sizes (in kb) are shown on the right margin.
A

B
six of eight clones (Fig. 2.5). The remaining two cDNAs also displayed the expected HindIII site and the oligo adapter sequence, but instead of a poly(A) stretch, they exhibited A + T-richness in this vicinity, raising the possibilities of internal priming or A-tail truncation, but not ruling out incomplete methylation of an internal HindIII site. These findings, along with XhoI and HindIII+BamHI analytic digests (data not shown), established that all eight clones were appropriately oriented. Furthermore, parallel experiments indicated the feasibility of directionally cloning cDNA inserts into pREP4 with cloning efficiencies comparable to those seen for pBluescript (data not shown).

2.D.iii. Conclusions

(i) Circular DNA elements incorporating the oriP and EBNA-1-encoding genes can replicate extrachromosomally to high copy numbers in a wide array of human cell types (Yates et al., 1985). We (Hambor et al., 1988; Tykocinski et al., 1988), and others (Reisman and Sugden, 1986; Drinkwater and Klinedinst, 1986; Shimizu et al., 1986; Margolskee et al., 1988; Young et al., 1988; Lotteau et al., 1989) have recently demonstrated that such EBV episomal replications can serve as vectors for
Fig. 2.5. Demonstration of the directional orientation of cDNA inserts within clones. Randomly selected pBluescript-based cDNA clones were linearized at their SalI sites, end-labeled by a PolIk fill-in reaction using \([\alpha-^{32}\text{P}]\text{dTPP}\) (New England Nuclear, Inc.) and sequenced by the Maxam-Gilbert chemical cleavage method (Maxam and Gilbert, 1980), including a fifth confirmatory reaction as previously described (Tykocinski et al., 1984). The poly(A) stretch of a representative cDNA clone is shown to join with the oligo adapter to create a \textbf{HindIII} site (AAGCTT).
efficient sense and antisense RNA expression. In this report, we describe the assembly of a series of highly utilitarian EBV episomal vectors, termed pREP and pMEP, which incorporate the constitutive RSV 3' LTR and inducible (heavy metals or glucocorticoids) hMTII₄-encoding gene promoters, respectively. The MCSs in the transcriptional cassettes of these various pREP and pMEP vectors confer subcloning flexibility, and, by virtue of their compatibility with the restriction endonuclease sites in commonly used prokaryotic vectors, permit the assembly of episomes for specific gene expression in simplified two-step constructions.

A significant feature of the pREP and pMEP vectors is their modularity. The entire transcriptional cassette can be mobilized from each vector with SalI and transferred into other plasmids to create new vectors. For instance, these transcriptional units can be readily introduced into a BPV episomal replicon (Law et al., 1983; Elbrecht et al., 1987) to create vectors suited for antisense or sense expression in murine cells. Similarly, the pREP and pMEP transcriptional cassettes can be transferred to a BK episomal replicon (Milanesi et al., 1984) to establish an alternative human episomal expression vector. Other sequence elements in these vectors
can also be replaced in a modular fashion, such as the EBNA-1/orip unit (ClaI, XbaI), the promoter (XbaI, MCS), and the Hyg\textsuperscript{R} gene (one of two NruI sites, MCS).

(ii) In this study, we describe a novel directional cDNA cloning methodology. Directionality in directional cDNA cloning procedures has previously been achieved through the use of vector-primers (Okayama and Berg, 1983; Alexander et al., 1984; Kowalski et al., 1985; Lang and Spritz, 1985), adapter-primers (Coleclough and Erlitz, 1985; Krawinkel and Zoebelein, 1986; Palazzolo and Meyerowitz, 1987), or linker-addition (Helfman et al., 1983; Dorssers and Postmes, 1987; Meissner et al., 1987). We now introduce the use of adapter-addition as a highly efficient alternative for directional cDNA cloning. Our method is a variation of a previously reported linker-addition cDNA cloning strategy (Dorssers and Postmes, 1987; Meissner et al., 1987), which, through the use of an oligo adapter, simplifies the cloning protocol of the latter by bypassing the requirements for BamHI methylation and digestion reactions.

A heterogenous array of vectors have been employed in directional cloning studies. Since we had previously demonstrated the utility of EBV episomal replicons for effective antisense RNA-mediated gene inhibition and for
high level sense RNA expression, we chose to exploit EBV episomes as cDNA cloning vectors. In a recent study, Margolskee et al. (1988) described a methodology for transferring directionally cloned cDNA inserts (oriented for sense expression) from a primary cDNA cloning vector into an EBV episomal replicon to create episome-based cDNA libraries. In contrast to this approach, our methodology entails the cloning of cDNA inserts directly into episomal vectors, simplifying constructions and minimizing potential library-skewing artifacts.

Three episomal vectors (pREP4, pMEP4, pREP5) were specially engineered to contain unique BamHI and HindIII sites within their MCSs, which permits directional cloning of cDNA inserts by our adapter-addition method. Alternative ordering of the unique BamHI and HindIII restriction sites within the MCSs of the pREP4 (pMEP4) and pREP5 vectors permits both antisense and sense cDNA cloning, respectively, using the same preparation of cDNA inserts. Furthermore, the incorporation of KpnI (3' protruding) and NheI (3' recessive) restriction enzyme sites upstream of the HindIII cloning site in pREP4 offers the option of eliminating A·T stretches upstream from cDNA inserts in pREP4 (pMEP4) through a unidirectional exonuclease cleavage and religation
strategy (Henikoff, 1984), a step which may be required for optimal antisense RNA activity if the presence of U stretches on antisense transcripts proves problematic.

An McrB\(^+\) phenotype, which characterizes many commonly used bacterial strains (Raleigh and Wilson, 1986; Meissner et al., 1987), leads to restriction at AluI methylated sites. Consequently, we used McrB\(^-\) strains for our cloning procedure, since it includes an AluI methylation step. The modest transformation efficiencies obtained with these strains (approximately \(10^7\) colonies per \(\mu\)g) limited our cDNA cloning efficiencies (\(6 \times 10^4\) clones per \(\mu\)g cDNA insert), and work is currently directed at maximizing these efficiencies for McrB\(^-\) strains.

(iii) Directional cDNA cloning methodologies for eukaryotic expression studies have, in all instances to date, focused upon sense expression. In this report, we have introduced the notion of directional cDNA cloning for antisense RNA expression, and we have described vectors and methods suited for this purpose. Our demonstration of the feasibility of antisense cDNA library assembly lays the groundwork for random mutagenesis strategies founded upon an antisense RNA concept. Specifically, pools of random antisense cDNA
clones can be stably transfected into target cells, and arrays of cellular phenocopies of null mutations can thereby be generated. Transfectant cells displaying a specific mutated phenotype can be selected from amidst the transfectant pools, and in turn, the antisense expression construct responsible for the mutated phenotype in these cells can be retrieved. An episome-based expression system offers particular advantages in this context, since it facilitates the retrieval process. Hence, the application of an episome-based random antisense RNA mutagenesis strategy to the identification of unknown genes mediating defined cellular functions now seems feasible.
CHAPTER 3: FACTORS AFFECTING TRANSFECTION EFFICIENCY AND PLASMID STABILITY FOR EPSTEIN-BARR VIRUS-BASED EPISOMAL REPPLICONS.

3.A. ABSTRACT.

Our previous investigations have established the utility of a set of Epstein-Barr virus (EBV) episomal vectors for stable sense and antisense RNA expression in human cells (Groger et al., 1989). In this report, we demonstrate that the relative orientation of specific sequence components within such episomes dramatically affects their utility for stable gene transfer and expression. Specifically, an episomal configuration which situates a strong eukaryotic enhancer/promoter, the Rous sarcoma virus 3' long terminal repeat (RSV 3' LTR), adjacent to the EBV origin of replication (oriP), significantly decreases the stability of the episome, as compared to an episome in which the RSV 3' LTR is more distant from the oriP. A compilation of our data with other analogous data in the literature suggests that interference between exogenously introduced enhancer/promoters with the replicative function of neighboring ori's may represent a more general phenomenon. Our findings indicate that a study of the
factors which govern the stability of EBV-based episomes, and of the levels of gene expression by the transcriptional cassettes they contain, may lead to the elucidation of rules dictating episomal replicative capacity and provide guidelines for the design of other classes of episomal replicons with optimal stability and function.

3.B. INTRODUCTION.

The tight clustering of cis- and trans-acting sequences in episomal vectors raises the possibility of interference between the disparate DNA sequence elements present on such episomes. In the case of our work with EBV-based episomes, striking differences in apparent transfection efficiencies (based on the number of hygromycin B-resistant cells proliferating after transfection and selection in hygromycin B-containing medium) were observed for alternative p220.2-based EBV episomal vectors, incorporating distinct promoter and reporter gene sequences. An initial correlative analysis of episomal configurations and relative transfection efficiencies, along with previous data published for other episomal systems, pointed to one sequence alignment feature that might be of particular importance for EBV
episomes in this regard, namely, the distance between the promoter/enhancer element of the transcriptional cassette and the Epstein-Barr virus origin of replication (oriP). In the present study, we provide data supporting this hypothesis, specifically demonstrating that when a transcriptional cassette is oriented in a way such that its promoter is proximal to, and the transcriptional direction is away from, oriP (α-orientation), the apparent efficiency of stable transfer is significantly lower than it is in the case of the corresponding vector in which the promoter is distal to, and the transcriptional direction is towards, oriP (β-orientation). We further probe EBNA-1 sequence determinants of EBV episome replicative efficiency. These studies provide important insights into critical primary sequence determinants of episomal replicative capacity of relevance to understanding the biology of both natural (e.g., viral) and artificial episomes.
3.C. MATERIALS AND METHODS.

3.C.i. Plasmid Construction.

The plasmid pREP7α was constructed as follows: The BstEII to SstI fragment of p205 was ligated into the corresponding sites of pREP4α (Groger et al., 1989). To generate pREP7β, the transcriptional cassette of pREP7α was mobilized by SalI digestion and religated into pREP7α linearized with SalI, subsequently isolating the β-oriented construct. Plasmids pCD8α/REP4α, pCD8α/REP4β, pCD8α/REP7α, and pCD8α/REP7β (Fig. 3.1) were created by transferring an RSV.CD8.pA transcriptional unit, obtained from a partial SalI digest of pCD8α/REP7α, into SalI-digested pREP4α and pREP7α.


K562 cells (Lozzio and Lozzio, 1975) obtained from the American Type Culture Collection, were grown in complete medium consisting of RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine (2 mM; M.A. Bioproducts, Walkersville, MD), and gentamicin sulfate (40 μg/ml) in a 5% CO₂ environment. Culture medium was refreshed every 3-4 days, and cells were maintained in a logarithmic phase of growth at all times.
Fig. 3.1. pCD8α/REP4α, pCD8α/REP4β, pCD8α/REP7α, and pCD8α/REP7β. The hph gene is flanked by the herpes simplex virus type 1 thymidine kinase promoter and termination signals (stippled). EBNA-1 dl 7 (in pCD8α/REP7α and pCD8α/REP7β) contains a 700 base pair in-frame deletion of the IR3 region of EBNA-1 (see section 1.B). B, BamHI; EBNA-1, Epstein-Barr virus nuclear antigen-1; hph, hygromycin phosphotransferase; K, KpnI; oriP, Epstein-Barr virus origin of replication; PA, SV40 late polyadenylation/termination sequences; pBR, pBR322 origin of replication and ampicillin-resistance gene; RSV, Rous sarcoma virus 5' long terminal repeat.
3.C.iii. Lipofection.

30 μl lipofectin (BRL) was diluted in 1.5 ml Opti-MEM (Gibco) and mixed with 10 μg plasmid DNA diluted in 1.5 ml Opti-MEM. The lipofection/DNA mixture was allowed to stand for 10' at room temperature, and it was then combined with 10^6 cells that had been previously washed three times and resuspended in 0.5 ml Opti-MEM. In all cases, plasmid DNAs used in a given experiment were prepared in parallel by the miniprep method and carefully semiquantitated to ensure that cells were transfected with the same quality and amount of plasmid. Cells were incubated for 3h at 37°C in 5% CO₂, and then 3 ml of complete medium was added. Following an overnight incubation, cells were centrifuged and resuspended in complete medium. Three days after lipofection, cells were transferred to medium containing hygromycin B as indicated (5 x 10⁶ cells/ml). Cells were resuspended in fresh complete medium, supplemented with the hygromycin B every four days.
3.D. RESULTS.


With the goal of defining primary sequence features of EBV episomes that influence their functional capabilities as vectors, we first focused on the orientation of the transcriptional cassette within EBV vectors as a potentially critical parameter. As a prelude to this analysis, two paired sets of EBV episomal expression constructs, each comprised of episomes with alternative transcriptional cassette orientations, were assembled. For all of these episomal constructs, the lymphoid cell surface-associated glycoprotein CD8α, whose expression levels can be readily semiquantitated by indirect immunofluorescence and flow cytometric analysis, served as a reporter gene. One paired set consisted of the episomes pCD8α/REP4α and pCD8α/REP4β. In pCD8α/REP4α, the CD8α coding sequence has been subcloned into our vector pREP4α (Groger et al., 1989), characterized by an α-oriented transcriptional cassette, i.e., the RSV 3' LTR promoter is proximal to the EBV oriP. pCD8α/REP4β was generated by inverting pCD8α/REP4α's transcriptional cassette, placing the RSV
3' LTR in a position distal to the EBV oriP. The second α/β paired episomal set consisted of pCD8α/REP7α and pCD8α/REP7β. The pREP7 series of vectors differ from pREP4 vectors in their EBNA-1 sequences; pREP7 vectors have the truncated EBNA-1 variant that is present in p205 and has been reported to attain twice the episome copy number seen for p220.2 (Yates et al., 1985). Otherwise, the pREP7 pair parallels the REP4 pair, with the transcriptional cassettes pCD8α/ REP7α and pCD8α/REP7β inverted relative to each other.

K562 myeloid leukemia cells were lipofected with DNA from the 2 α-oriented (pCD8α/REP4α and pCD8α/REP7α) and 2 β-oriented (pCD8α/REP4β and pCD8α/REP7β) EBV episomal expression constructs. Stable K562 transfecants (5x10⁶/ml) were selected in 200 μg/ml hygromycin B, starting at 72 hours post-transfection (with medium changes every 4 days), and viable cell numbers were determined 22 days post-transfection. For each of the α-oriented episomes (pCD8α/REP4α and pCD8α/REP7α), fewer than 5x10⁶ hyg² transfecants/10 ml were present. In contrast, with the β-oriented episomes pCD8α/REP4β and pCD8α/REP7β, there were 4.7x10⁶ and 4.6x10⁶ hyg² transfecants, respectively. A similar finding of an apparently higher transfection efficiency with EBV
episomes containing β-oriented, as opposed to α-oriented, transcriptional cassettes was observed when pHEBo-based plasmids (Fig. 3.2), which are entirely missing the EBNA-1 coding sequence, were transfected into EBV-transformed (EBNA-1') B cells (J. Yates, Roswell Park, unpublished observations).

To assess the functionality of the α- and β-oriented transcriptional cassettes, expression of the CD8α reporter was analyzed by indirect immunofluorescence and flow cytometry. As seen in Fig. 3.3, high CD8α epitope densities are apparent for both pCD8α/REP4α and pCD8α/REP4β K562 transfectants, with no significant differences between the two. Hence, the pREP4β vector not only yields substantially higher transfection efficiencies than its β-oriented counterpart, but also maintains its utility as a gene expresser.


An early study by Sugden and coworkers (Yates et al., 1985) demonstrated that higher episome copy numbers can be achieved with a truncated EBNA-1 variant with a 700 bp in-frame deletion, eliminating a 230 amino acid long glycine alanine-rich region. pREP7 episomes
Fig. 3.2. α- and β-orientations in pHEBo based plasmids.
The hph gene is flanked by the herpes simplex virus type 1 thymidine kinase promoter and termination signals (stippled). hph, hygromycin phosphotransferase; oriP, Epstein-Barr virus origin of replication; pBR, pBR322 origin of replication and ampicillin-resistance gene (John Yates, personal communication).
pHEBo-based α-oriented construct

pHEBo-based β-oriented construct
Fig. 3.3. Comparison of CD8 expression in pCD8α/REP4α and pCD8α/REP4β transfectants. Three weeks after transfection, approximately $5 \times 10^5$ cells were stained with OKT8 (anti-CD8), or normal mouse IgG (Miles; open areas) as primary antibodies, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Miles) was used as the secondary antibody. For each figure, cell number (vertical axis) is plotted against log fluorescence (horizontal axis).
K562 Transfectants

CD8α/REP4α

CD8α/REP4β
incorporate this truncated EBNA-1 variant and thereby permitted us to specifically address the possibility that vectors so modified would permit enhanced gene expression. A comparison of the stable transfection efficiencies obtained with pCD8α/REP7β versus pCD8α/REP4β (data cited above) indicated that, at least in the case of K562 cells, the EBNA-1 truncation does not make a significant difference. To ascertain whether there is an effect upon gene expression, immunostaining was performed following 25 days of hygromycin B selection, comparing the level of surface CD8α epitope expression by cells stably transfected with pCD8α/REP4β and pCD8α/REP7β (Fig. 3.4). An approximately two-fold excess in CD8α surface expression was noted for the pCD8α/REP7β (EBNA-1-truncated) variant. The explanation for the advantages associated with the EBNA-1 truncation remain to be determined, but may stem from non-specific cellular toxicities associated with the intact EBNA-1 DNA-binding protein.
Fig. 3.4. Effect of deletion of the IR3 region of EBNA-1. Three weeks after transfection, approximately $5 \times 10^5$ cells were stained with OKT8 (anti-CD8), or normal mouse IgG (Miles; not shown) as primary antibodies, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Miles) was used as the secondary antibody. Cell number (vertical axis) is plotted against log fluorescence (horizontal axis).
CD8 Staining
1) CD8α /REP4β
2) CD8α /REP7β

We derived a new set of stable K562 transfectants with pCD8α.REP4α and pCD8α.REP4β. To permit the efficient generation of α-oriented (as well as β-oriented) transfectants, we used a less rigorous selection system than the one described above. Namely, cells were grown in six well plates in the presence of 200 μg/ml hygromycin B, and half the medium was withdrawn and replaced with fresh medium every four days. Immunostaining performed after three weeks of this selection scheme revealed comparable levels of CD8α epitope expression for pCD8α/REP4α and pCD8α/REP4β transfectants. Cells were then grown in the presence (Fig. 3.5) or absence (Fig 3.6) of hygromycin B and immunostained for CD8α expression after 1, 2, 3, 4, 5, and 14 weeks. While there was a dramatic loss of CD8α expression (by week 6) in the population of pCD8α/REP4α K562 transfectants, even in the continuous presence of hygromycin B, pCD8α/REP4β K562 transfectants maintained strong CD8α expression. Moreover, in the absence of hygromycin B selection, the rate of loss of CD8α expression for α-oriented episomal transfectants was
Fig. 3.5. CD8 expression in pCD8α/REP4α and pCD8α/REP4β transfectants maintained in 200 μg/ml hygromycin B. At the times indicated after the beginning of the experiment, approximately $5 \times 10^5$ cells were stained with OKT8 (anti-CD8), or normal mouse IgG (Miles; open areas) as primary antibodies, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Miles) was used as the secondary antibody. For each figure, cell number (vertical axis) is plotted against log fluorescence (horizontal axis).
Fig. 3.6. CD8 expression in pCD8α/REP4α and pCD8α/REP4β transfectants maintained in the absence of hygromycin B. At the times indicated after the beginning of the experiment, approximately $5 \times 10^5$ cells were stained with OKT8 (anti-CD8), or normal mouse IgG (Miles; open areas) as primary antibodies, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Miles) was used as the secondary antibody. For each figure, cell number (vertical axis) is plotted against log fluorescence (horizontal axis).
significantly greater than it was for the β-oriented ones. These findings point to a striking degree of instability for α-oriented episomes.

3.2. DISCUSSION.

3.2.1. In this study, we have systematically tested, and substantiated, our hypothesis that transcriptional cassette orientation within EBV episomal vectors markedly influences their overall effectiveness. Specifically, our data, employing paired sets of EBV episomal expression constructs, have demonstrated that episomal vectors with α-oriented transcriptional cassettes are associated with dramatically lower stable transfection efficiencies, as compared to vectors with β-oriented transcriptional cassettes. Moreover, populations of transfectants bearing α-oriented episomes develop phenotypic loss (but still hyg^R) variants at a substantially higher frequency in the presence of hygromycin B selection, as well as exhibit a more rapid phenotype loss in the absence of hygromycin B selection. Taken together, the data point to greater episome instability, and an enhanced propensity for episome loss, for α-oriented episomes. Nonetheless, at early stages
post-transfection, α-oriented episomes provide for levels of gene expression that are equivalent to those seen for their β-oriented counterparts.

3.E.ii. Several hypotheses can be considered in explaining the α/β orientation effects. One set of hypotheses are predicated upon the notion that α-oriented episomes are less effective in their replicative capacity. Possibilities in this category center around effects upon the episome's replicative signals and include: (i) interference with oriP's replicative function, perhaps mediated by the RSV 3' LTR promoter/enhancer element; and (ii) interference with EBNA-1 gene expression. In either case, decreased episomal replicative capacity would necessarily result in decreased production of the hph-encoded phosphotransferase, thereby providing a reasonable explanation for lower stable transfection efficiencies and greater propensities for episome loss. Another set of hypotheses are predicated upon the alternative notion that the disadvantages noted for α-oriented episomes do not in fact stem from deficits in their replicative potential per se. Possibilities in this category include: (i) direct interference with hph gene
expression, blocking phosphotransferase production. This could be a consequence of the opposing directions of transcription for the reporter and \textit{hph} genes; and (ii) \textit{\alpha}-oriented episomes might confer some proliferative advantage to transfected cells that is independent of both \textit{hph} gene expression and of the episomes' inherent replicative capacity.

While providing a definitive explanation for the \(\alpha/\beta\) difference is beyond the scope of the present study, we currently favor the first hypothesis cited, relating to \textit{oriP} interference. The unpublished observation (J. Yates, personal communication) of similar \(\alpha/\beta\) differences with pHEBo-based plasmids, which both lack an EBNA-1 gene and in which the transcriptional directions for reporter \textit{hph} genes are reversed (as compared to our vectors), argue against the hypotheses centered around a role for EBNA-1 or \textit{hph} deficits in the observed \(\alpha/\beta\) differences. Moreover, our present finding of rapid \(\alpha\)-oriented episome loss following hygromycin B withdrawal argues against a pivotal role for \textit{hph} gene expression itself in this setting. Lastly, while we cannot formally exclude the last cited hypothesis, namely that cells bearing \(\beta\)-oriented episomes are endowed with proliferative advantages that do not stem from differential episomal
replicative capacity, we deem this possibility unlikely for the following reason. One would predict that in the absence of selective pressure, any such proliferative advantage should result in a shorter doubling time for cells transfected with β-oriented episomes, as compared to α-oriented ones. Such a difference in proliferative rates was not observed in the hygromycin B withdrawal experiments; however, small differences in cell doubling times could have escaped our detection. Any proliferative advantage would have to be relative, rather than absolute in nature, since even the β-oriented episomes were overtaken by their non-transfected counterparts when selective pressure was withdrawn.

If a common mechanism explains the differences in transfection efficiency between α- and β-oriented reporter gene-containing p220.2- and pHEBo-based plasmids, the most likely possibility is that the strong promoter/enhancer element driving transcription of the reporter gene somehow interferes with the function of oriP. This notion is substantiated by the fact that analogous results have now been obtained with two different promoter/enhancer elements driving reporter gene transcription (RSV 3' LTR and CMV IE promoter [unpublished results from both our lab and J. Yates]),
with two different reporter genes (CD8 and CAT [data not shown]), as well as with three different selectable markers (hygromycin phospho-transferase, neomycin phosphotransferase [data not shown] and histidinol dehydrogenase [discussed in Chapter 4]). Hence, the $\alpha/\beta$ difference for EBV vectors applies to a broader set of promoters and selectable markers, and is not simply a peculiarity of one particular set of episomal constructs. The relevance of these observations to other eukaryotic ori's (such as BK virus or bovine papillomavirus ori's) remains to be demonstrated; ongoing work to study this phenomenon in BK virus-based plasmids is discussed in Chapter 5.

We and a few of the over 100 investigators to whom we have now provided our EBV vectors have noted the occasional emergence of hyg$^R$ phenotype loss variants among transfected cells maintained in long term culture. The oriP interference cannot in and of itself explain such phenotypic changes noted. It is possible that propensities for chromosomal integration of episomes and intra-episomal rearrangements, may account for this sort of episomal instability; both would be selected for during long term culture. Transfectants, even if arising rarely, bearing integrated episomes (with either an
intact or disrupted reporter gene) or episomes with internal rearrangements that give them a replicative advantage, will have a selective advantage over cells bearing an unperturbed episomal element. Such cells are likely to have a greater probability of passing their selectable marker gene on to their progeny, and hence are expected to eventually overtake the population. Consistent with the occurrence of episomal integration is our finding (unpublished observations) that early-passage transfectants lose their episome-directed phenotype when grown for several weeks in the absence of selection, while late-passage transfectants often fail to revert to their non-transfected phenotype following the withdrawal of selective pressure. Consistent with the occurrence of intra-episomal rearrangements is our present finding that even in the presence of ongoing selective pressure, cells sometimes lose their episome-mediated phenotype. We have yet to obtain direct evidence (genomic vs. low molecular weight DNA blotting experiments) which would support or discount our hypothesis.

In addition to the obvious desirability of a more stable phenotype, additional advantages would result from the greater number of independent transfectants obtained following transfection.
3.E.iii. As discussed in section 3.E.ii, the most attractive hypothesis accounting for the α/β difference is that the promoter/enhancer element of the transcriptional cassette interferes with oriP's replicative function. This conception is consistent with a broader set of phenomenological observations in the literature, coming from other groups investigating both EBV-based and polyoma virus-based episomes and these will be discussed in this section.

Lupton and Levine (1985) and Chittenden et al. (1989) have demonstrated that an SV40 promoter/enhancer element adjacent to, but directed away from, the region of dyad symmetry in the EBV oriP could interfere with its function. This inhibitory effect was diminished when the distance between the region of dyad symmetry and the SV40 promoter/enhancer was increased. In the case of the mouse polyoma virus, a similar interference between an SV40 promoter/enhancer and the polyoma ori was reported, although in this case, the interference could be overcome by removing the α region of the polyoma ori (Campbell and Villarreal, 1986). This latter finding is notable since it suggests that interference between certain viral promoter/enhancer elements and viral ori's requires the presence of particular subsequences within the ori. From
a model proposed below, we postulate that the α region of the polyoma ori might represent an enhancer which is necessary for the normal function of the ori, but whose loss can be compensated for by an adjacent SV40 enhancer.

Another pertinent finding was related to the SV40 ori. Binninger et al. (1989) have reported that an RSV LTR enhancer inhibited the replication of plasmids containing the SV40 ori. Furthermore, Kumar et al. (1986) reported that multiple copies (4 or more) of the SV40 72 bp enhancer greatly reduced DNA replication, while increasing the levels of transcription of adjacent genes. Since multiple copies of the 72 bp repeats result in a nucleosome gap (Jongstra et al. 1984), Kumar et al. suggested that this inhibition of plasmid replication may be due to an alteration of chromatin configuration at the ori. Multiple copies of a 21 bp repeat from the early promoter region did not affect DNA replication. The authors further proposed that the low number of enhancer repeat elements found in animal viruses (two 72 bp repeats in SV40; three 68 bp repeats in BK virus) reflects a need to balance the requirements of RNA transcription and DNA replication.

In the case of the pREP system, we speculate that placing a heterologous enhancer near the oriP, which
itself has EBNA-1 dependent enhancer activity (Reisman and Sugden, 1986), may parallel the effect seen when multiple copies of the SV40 72 bp repeat are present at the SV40 ori. Since the effect of an enhancer on a promoter element tends to decrease as the distance between these elements increases (Jongstra et al. 1984), it is possible that the greater plasmid stability seen with β-oriented EBV-based constructs, as compared with the α-oriented constructs, is the result of increased distance between the RSV 3' LTR and oriP in the former. Moreover, perhaps it is the presence of extra enhancer element sequences in the pREP episomes which explains why the favored orientation in pREP is one in which the directions of DNA and RNA synthesis are opposed; by contrast, in naturally-occurring episomes, the direction of leading strand DNA synthesis and RNA transcription tend to coincide (Brewer, 1988). In E. coli, transcription of the most actively transcribed genes is directed away from the ori. In the polyoma viruses, which have a bidirectional ori, transcription is directed away from the ori, terminating a point opposite the ori, coincident with the termination of DNA replication. EBV replication is predominantly unidirectional, originating within the region of dyad symmetry, but blocked in one
direction at the region of direct repeats, and proceeding around the plasmid, terminating at the region of direct repeats (Gahn and Scildkraut, 1989). In the EBV genome, transcription of the TR gene, the first gene encountered during leading strand synthesis, is in the same direction as plasmid replication.

3.E.iv. Based on the present study, we propose a set of design principles (vide infra) for the production of efficient episomal expression vectors. High transfection efficiencies are of particular importance in several experimental contexts. For instance, in many situations in which individual gene expression is modified through sense or antisense RNA expression, a significant advantage of a highly efficient transfection system is that one can study a large mixture of independent transfectants rather than one or a few clones, thereby reducing the possibilities of artifactual results stemming from the clonal variation of cells. Furthermore, high transfection efficiencies are a prerequisite for transfection work (Chapter 2). The design principles that we offer are as follows: (i) if the enhancer element driving the promoter for the transcriptional cassette is distinct from the enhancer
activity present in the ori, an optimal configuration of the episome would place that enhancer at as great a
distance as possible from the ori; (ii) if the enhancer activity within the ori is used to drive an enhancerless
promoter for the transcriptional cassette, the promoter should be situated as close as possible to the ori; and
(iii) only if the episome is configured in a way so that any heterologous enhancer element is situated at a
sufficient distance from the ori to prevent interference with its function, would a configuration allowing DNA
synthesis and RNA transcription to coincide be favored. These hypothetical principles are experimentally
testable. The next chapter outlines those experiments which are currently in progress to test and extend this
model.
CHAPTER 4: EXPERIMENTS IN PROGRESS: 1) DETERMINING WHETHER AN RSV 3' LTR PROMOTER/ENHANCER ELEMENT INTERFERES WITH THE FUNCTION OF A BK (POLYOMA) VIRUS ORIGIN OF REPLICATION IN BK VIRUS-BASED VECTORS; AND 2) TESTING FURTHER REFINEMENTS OF OUR EXISTING EPSTEIN-BARR VIRUS-BASED VECTORS.

The experiments described in Chapter 3 suggested that an RSV 3' LTR can interfere with the function of an EBV oriP. In order to determine whether this type of interference is specific for EBV-based episomes, or instead, whether this type of interference represents a more general phenomenon, we have constructed a set of vectors based on BK polyoma virus replicative motifs. Reports in the literature (Campbell and Villarreal, 1986; Kumar et al., 1986) have in fact suggested that a strong viral enhancer element can interfere with the ori of a polyoma virus and our goal is to specifically test this for BKV.

BKV-based expression constructs, as schematically depicted in Figure 4.1, incorporating CD8α as a reporter gene, were assembled. Two of these episomes, pCD8α/RBK.1α and pCD8α/RBK.1β, have the BK ori situated adjacent to the transcriptional cassette, and hence are
close analogues of pCD8α/REP4α and pCD8α/REP4β (Chapter 3), respectively. Experiments are currently underway to compare both the transfection efficiencies and episomal stabilities of these two episomes, testing for inhibitory effects of proximal enhancer elements on episomal replication. These experiments will parallel those described in Chapter 3 which examined the functionality of EBV-based episomal vectors. The other two BKV episomes depicted in Figure 4.1, pCD8α/RBK.2α and pCD8α/RBK.2β, have been assembled in order to test our prediction (Chapter 3) that it will be more advantageous to have the direction of transcription from a distal promoter/ enhancer coincide with the direction of DNA synthesis (as is the case with pCD8α/RBK.2α), as opposed to creating a maximal distance between the RSV promoter/enhancer and the BK ori (as is the case with pCD8α/RBK.2β), at the expense of having the direction of transcription from the RSV 3' LTR promoter/enhancer oppose the direction of DNA synthesis.

The model proposed in Chapter 3 and being tested, in part, by the preceding BKV-based experiment, predicts that for optimal utility, an episome should have its ori shielded by as great a distance as possible from any strong promoter/enhancer element, and that if possible,
the direction of RNA transcription should coincide with the direction of DNA transcription. According to this model, and with respect to EBV-based vectors, if the increased stability of pCD8α/REP4β, as compared to pCD8α/REP4α, is a direct consequence of the greater distance in the former plasmid between the RSV 3' LTR and oriP, one would predict that the advantages of pREP4β over pREP4α would diminish as the size of the insert in the multiple cloning site decreases. This would be particularly relevant to those antisense RNA-mediated gene inhibition applications in which only a short antisense oligonucleotide is being expressed. To test this prediction, and to prepare a potentially improved alternative to pREP7β, pREP11β (Fig. 4.2) was constructed. In pREP11β, the orientation of the EBNA-1/oriP-containing fragment has been reversed relative to its orientation in pREP7β. This EBV episomal vector: (i) possesses an in-frame deletion of the IR3 region of the EBNA-1 gene (expected to increase episome copy number); (ii) maximizes of the distance between the RSV 3' LTR and oriP; and (iii) provides an episomal configuration in which the directions of transcription for the reporter, EBNA-1, and hph genes, all coincide.
with the direction of leading strand DNA synthesis (unidirectional in the case of EBV).

The initial test of pREP11β will be by lipofecting identical amounts of pREP11β and pREP7β (no reporter genes in either case) into K562 cells and subsequently selecting transfectants in the presence of hygromycin B. The number of viable hygromycin B-resistant cells at a specified time following transfection will be counted to assess: (i) the capacity of pREP11β to function in human cells; and (ii) any difference in the apparent transfection efficiencies of the two episomes. If pREP11β is functional, and if it indeed has a greater apparent transfection efficiency than does pREP7β, then the relative utility of pREP7β and pREP11β will be assessed in a model antisense experiment using a short antisense oligonucleotide. Our a priori prediction is that pREP11β will turn out to be the optimal EBV episomal expression vector.
Fig. 4.1. pCD8α/RBK.1α, pCD8α/RBK.1β, pCD8α/RBK.2α, and pCD8α/RBK.2β. The hph gene is flanked by the herpes simplex virus type 1 promoter and termination signals (stippled). B, BamHI; BK ori, BK virus 68 bp minimal origin of replication; hph, hygromycin phosphotransferase; K, KpnI; PA, SV40 late termination/polyadenylation signals; pBR, pBR322 ampicillin resistance gene and origin of replication; RSV, Rous sarcoma virus 5' long terminal repeat; X, XbaI.
Fig. 4.2. pREP7β and pREP11β. The 30 bp repeats and region of dyad symmetry within the Epstein-Barr virus origin of replication (EBV oriP) are indicated. EBNA-1 dl 7 contains a 700 bp in-frame deletion of the IR3 region of Epstein-Barr virus nuclear antigen-1 (see section 1.B). The hph gene is flanked by the herpes simplex virus type 1 promoter and termination signals (stippled). B, BamHI; Bs, BstEII; H, HindIII; hph, hygromycin phosphotransferase; K, KpnI; Mh, NheI; No, NotI; P, PvuII; PA, SV40 late termination/polyadenylation signals; pBR, pBR322 ampicillin resistance gene and origin of replication; RSV, Rous sarcoma virus 5' long terminal repeat; Sf, SfiI; Ss, SstI; Xb, XbaI; Xh, XhoI.
CHAPTER 5: STABLE GENE COEXPRESSION USING EPSTEIN-BARR
VIRUS-BASED AND BK VIRUS-BASED VECTORS.

5.A. ABSTRACT.

In our previous investigations, we have established the utility of a set of Epstein-Barr virus (EBV) episomal vectors for stable sense and antisense RNA expression in human cells (Groger et al., 1989). To enable stable coexpression of transfected genes in human cells, we have now developed a series of other episomal expression vectors, incorporating alternative viral replicative signals (EBV; BK virus [BKV]), selectable markers (hygromycin phosphotransferase [hph]; neomycin phosphotransferase [neo]; or histidinol dehydrogenase [hisD]), and promoter/enhancer elements (Rous sarcoma virus 3' long terminal repeat [RSV 3' LTR]; cytomegalovirus [CMV]). We have achieved stable cotransfection through the sequential or simultaneous use of pairs of EBV-based episomes, combining hph and hisD, or alternatively hph and neo selectable markers. Unexpectedly, we noted that cotransfection using two independently selectable EBV episomes is significantly more efficient than cotransfection using a pair of episomes with different (EBV and BKV) replicative
signals. This first efficient technology for stable gene cotransfection should facilitate the study of multisubunit molecules, as well as enable the coordinate expression of sense and antisense RNA in individual transfected cells.

5.B. INTRODUCTION.

Stable gene transfer technology has been largely confined, to date, to the manipulation of single genes through sense or antisense production. Only limited attention has been directed towards developing stable gene cotransfer capabilities which would allow the manipulation of more than one gene simultaneously. Egelhoff et al. (1989) reported the development of an episomal vector for use in Dictyostelium, with the hph gene as the selectable marker, and these investigators suggested that this vector could be used to coexpress genes in cells already transfected with a neo-containing plasmid. By contrast, transient gene coexpression has been extensively applied to the study of a broad set of genes, serving to highlight the importance of cotransfection strategies. Extending the cotransfection approach to stable contexts would open up applications,
such as antisense RNA mutagenesis, that are relatively inaccessible through transient gene cotransfer.

In previous work, we and others have demonstrated the utility of EBV episomal expression vectors for high level sense and antisense RNA expression in human cells (Tykocinski et al., 1988; Hambor et al., 1988; Hauer et al., 1989). The first set of vectors we produced and evaluated all shared the EBV origin of replication (oriP) and EBV nuclear antigen-1 (EBNA-1) gene as replicative signals, the hph gene as a selectable marker, and the RSV 3' LTR as a constitutive promoter. The vectors of this early series were designated pREP1-pREP5 (Groger et al., 1989). The high transfection efficiencies and gene expression levels that have been achieved with these episomal vectors, along with their substantial advantages in situations where extrachromosomal replication is preferable, make them an attractive vector system to build upon in the development of a utilitarian stable gene cotransfection technology.

In this report, we describe the assembly of a series of episomal vectors incorporating alternative replicative signals, selectable markers, and promoter/enhancer elements. We further report their utility, in conjunction with our previous vectors, for sequentially
or simultaneously cotransflecting two independently-selectable episomes into a single cell. In the first set of studies described with *hph*- and *hisD*-selectable episomes, a heterogeneous set of independently-expressed reporter genes are employed (CD8 α-chain, M-CSFR, and M-CSF:DAF (see Chapter 9). In the later experiments, the α- and β-chains of CD8 are used as reporter genes; since the surface expression of CD8β on the cell surface is dependent upon the expression of the CD8α (DiSanto et al., 1988; Norment and Littman, 1988), the observation of CD8β on the cell surface provides strong experimental evidence that a given cell has been stably transfected by both episomes. Lastly in these studies, we also compare the utility of episomal pairs incorporating distinct replicative signals for coexpression work.

5.C. MATERIALS AND METHODS.

5.C.i. Plasmid Construction.

The assembly of pCD8α/REP7β (Fig. 5.2) and pM-CSF:DAF/REP4α are detailed in sections 3.C.i and 8.C.i, respectively.

pM-CSFR was assembled by transferring the M-CSFR cDNA coding sequence from BamHI-digested pM-CSFR/BT (see
section 8.C.1) into the BamHI subcloning site of pREP3 (Groger et al., 1989).

pCD8α/RBK was constructed as follows: pBK-1 (Milanesi et al. 1984) was linearized with EcoRI, blunted by polIk, and religated with a BamHI oligo linker. A BamHI fragment of this plasmid, containing the BKV replicative sequences, was mobilized and religated into pREP4BglIII digested with BglII. The latter plasmid, pREP4BglIII, had been constructed by digesting pREP4α (Groger et al., 1989) with ClaI and XbaI, filling-in with PolIk, and religating with an oligo BglII linker). The resultant plasmid, pRBK, is depicted in Figure 1.4. To generate pCD8α/RBK (Fig. 5.2), the CD8α cDNA coding sequence was mobilized from pCD8/BT (Tykocinski et al., 1988), digested with XhoI and BamHI, and ligated into the corresponding sites in the multiple cloning site of pRBK.

pCD8α/REP8.2α and pCD8α/REP8.2β were generated as follows: pSV2his (provided by Richard Mulligan [Hartman and Mulligan, 1988]) was linearized with BamHI, filled-in with polIk, and religated, to generate pSV2hisB−. p220.2 was digested with HindIII and NruI, filled-in with polIk, and religated with BglII linkers to generate p220BglIII. The HisD containing EcoRI-AccI fragment of pSV2hisB− was filled-in with polIk, religated with BglII linkers,
digested with BglII, and then religated into the BglII-digested of p220BglII, to generate p220his.2. pCD8α/REP8.2α and pCD8α/REP8.2β (Fig. 5.1) were created by transferring an RSV·CD8·pA transcriptional unit, obtained from a partial SalI digest of pCD8α/REP7α (section 3.C.i), into SalI-digested p220his.2.

pCD8B/CEP9B (Fig. 5.2.) was constructed as follows: p462.8 (provided by John Yates) was linearized with HindIII, filled-in with polIk and religated with a KpnI oligo linker. The CMV IE regulatory region was mobilized from this construct by digestion with BamHI and KpnI and ligated into the BglII and KpnI sites of pMEP4 (described in Fig. 2.2), generating pCEP4α. The plasmid pCEP4β was created by transferring the CMV·MCS·pA transcriptional unit from pCEP4α, mobilized with SalI, into SalI-digested pREP9, and subsequently isolating the β-oriented construct. pREP9 was constructed by ligating a ClaI to BglII fragment of pREP4BglII, containing the EBV sequences and the RSV·MCS·pA transcriptional unit, to the ClaI to BamHI fragment of p293, (provided by John Yates) which contains the neo gene and pBR322 sequences. To generate pREP4αBglII, the RSV·MCS·pA transcriptional unit from SalI-digested pREP7α (see section 3.C.i.) was transferred into SalI-digested p220BglII. The EcoRI
Fig. 5.1. pCDφ/REP8.2α and pCDφ/REP8.2β. The histidinol dehydrogenase gene (*hisD*) is flanked by SV40 early promoter and termination signals (stippled). B, *BamHI*; EBNA-1, Epstein-Barr virus nuclear antigen-1; His D, histidinol dehydrogenase; K, *KpnI*; *oriP*, Epstein-Barr virus origin of replication; PA, SV40 late polyadenylation/termination sequences; pBR, pBR322 origin of replication and ampicillin-resistance gene; RSV, Rous sarcoma virus 5' long terminal repeat.
pCD8α/REP8.2α

pCD8α/REP8.2β
Fig. 5.2. Plasmids used in cotransfection experiments. The plasmid pairs (A) pCD8α/REP78 and pCD8β/CEP98 or (B) pCD8α/RBK and pCD8β/CEP98 were used for cotransfection experiments. Both the hph and neo genes are flanked by the herpes simplex virus type 1 thymidine kinase promoter and termination signals (stippled). EBNA-1 dl 7 contains a 700 base pair in-frame deletion of the IR3 region of EBNA-1 (see section 1.B). B, BamHI; BK ori, BK virus 68 base pair minimal origin of replication; CMV, cytomegalovirus immediate early enhancer/promoter; EBNA-1, Epstein-Barr virus nuclear antigen-1; H, HindIII; K, KpnI; neo, neomycin phosphotransferase; oriP, Epstein-Barr virus origin of replication; PA, SV40 late polyadenylation/termination sequences; pBR, pBR322 origin of replication and ampicillin-resistance gene; Xh, XhoI.
fragment of a human CD8 β-chain cDNA in pUC19 (provided by Jane Parnes) (Shiue et al., 1988) was first transferred into the EcoRI site of pBluescript, generating pCD88/BT, recovered, by HindIII and BamHI digestion, and then ligated into the corresponding sites of pCEP9B, generating pCD88/REP9, or pREP9, generating pCD88/REP9.

5.C.ii. Lipofection.

Lipofection and cell culture were performed as described in sections 3.C.ii. and 3.C.iii. Cells used were either K562 cells obtained from the American Type Culture Collection, or a K562 subline, K.48, provided by T. Rosenberry (CWRU). Hygromycin B was obtained from Calbiochem, G418 (Geneticin) from Gibco, and L-histidinol from Sigma.

5.C.iii. Antibodies used in flow cytometric analysis.

Rat monoclonal anti-M-CSF and anti-M-CSFR antibodies were obtained from Oncogene Sciences. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat IgG and goat anti-mouse IgG antibodies were obtained from Miles ICN. OKT8 was obtained from Ortho. An anti-CD8 β-chain-specific monoclonal antibody 2ST8-5H7 was a gift of Dr.
E. Reinherz (Dana-Farber Cancer Institute) (DiSanto et al., 1987).

5.D. RESULTS.

5.D.i. K562 double transfectants using hph and hisD.

In order to establish the feasibility of coselecting two episomes bearing independent selectable markers, we secondarily transfected stable transfectants bearing an episome carrying an hph selectable marker with an episome carrying a hisD selectable marker. Specifically, pM-CSF-DAF/REP4α or pM-CSFR/REP3 hygK.48 transfectants (maintained in continuous culture) were lipofected with pCD8α/REP8.2α or pCD8α/REP8.2β. The pREP8 series of vectors incorporate the hisD gene as a selectable marker. Cells were coselected in medium containing both 300 μg/ml hygromycin B and 5 mM L-histidinol. Stable cotransfectants were successfully obtained when either the pM-CSF-DAF/REP4α or the pM-CSFR/REP3 hygK transfectants were cotransfected with pCD8α/REP8.2β. This was substantiated by immunofluorescence and flow cytometry which documented surface coexpression of the reporter genes encoded by each of the two cotransfected episomes (data not shown). However, several attempts to
stably cotransfect the pM-CSF·DAF/REP4α or pM-CSFR/REP3 K.48 transfectants with α-oriented pCD8α/REP8.2α failed. This result is consistent with our findings, discussed at length in Chapter 3, that β-oriented transcriptional cassettes in EBV-based episomal vectors can be transfected into cells with a markedly higher efficiency than their α-oriented counterparts.

Once cotransfectants were derived, an hph and hisD withdrawal experiment was conducted to determine whether the cotransfected plasmids were indeed maintained episomally. For this experiment, one of the cotransfected lines, the pM-CSFR/REP3 (hygβ) + pCD8α/REP8.2β (hisβ) K.48 cotransfectant, was cultured in the absence of either hygromycin B or L-histidinol for four weeks, with the reciprocal antibiotic maintained in each case. Flow cytometric analysis (data not shown) revealed that when L-histidinol was withdrawn, CD8α expression was lost, confirming pCD8α/REP8.2β's episomal nature. However, when hygromycin B was withdrawn, M-CSFR expression was unaffected, suggesting that pM-CSFR/REP3 had integrated into the chromosomal DNA. In order to minimize the occurrence of chromosomal integration, we avoided long-term passaging of transfectants in our subsequent cotransfection work.

Our next focus was on coselection with episomal pairs bearing a different set of selectable markers, namely hph and neo. Both of these genes encode phosphotransferases which confer resistance to their respective eukaryocidal antibiotics. In this set of experiments, we used the reporter genes CD8α and CD8β to permit a more elegant assessment of cotransfection success (vide supra).

To enable the demonstration of the feasibility of using the hph and neo pair for stable cotransfection, we first established the selection conditions for cotransfecting with these selectable markers. pCD8α/REP7B K562 transfectants (in medium containing 200 μg/ml hygromycin B) were subjected to varying concentrations of G418 (a neomycin analogue). The toxicity profile generated in this way indicated that 1.0 mg/ml G418 was required to kill the cells within a week to 10 days. However, only 0.6 mg/ml G418 was required to kill nontransfected cells (in the absence of hygromycin B). Hence, there may be some cross-resistance, whereby the hph gene confers some resistance to G418.

Once we had established the appropriate selection conditions, we secondarily transfected pCD8α/REP7B K562 transfectants with pCD8β/REP9. Stable cotransfectants
were derived following selection in 200 μg/ml hygromycin B and 1.0 mg/ml G418. Flow cytometry (not shown) demonstrated that all cells coexpressed both CD8α and CD8β.

5.D.iii. Comparison of simultaneous versus sequential cotransfection efficiencies with EBV- and BK-virus episomal vectors.

In the experiments described thus far, the feasibility of sequentially transfecting individual members of an episomal pair into cells was established. We next determined: (i) the feasibility of simultaneously cotransfecting two episomes with distinct selectable markers in a single transfection procedure; and (ii) whether it is advantageous to use episomal pairs for such cotransfections in which each episome incorporates distinct viral replicative signals. To address this first issue, K562 cells were simultaneously lipofected with the episomal combination pCD8α/REP7β and pCD8β/CEP9β (see Fig. 5.2). Unexpectedly, large numbers of viable cells grew out after selection in 200 μg/ml hygromycin B and 1.0 mg/ml G418 when this episomal pair was used; in this case, both of the episomes incorporate EBV replicative signals. Immunostaining and flow
cytometry (Fig. 5.3) confirmed that these transfectants coexpressed the two encoded genes, CD8α and CD8β. The efficiencies of transfection observed for this simultaneous lipofection approach were consistently within an order of magnitude of that obtained using the same two episomes with a sequential lipofection approach. This striking finding establishes that multiple episomes can consistently establish themselves in a stable mode when simultaneously lipofected.

To compare EBV/BK versus EBV/EBV episomal pairs in a simultaneous cotransfection context, K562 cells were lipofected with the pCD8α/RBK and pCD8/CEP98 episomes, bearing BKV and EBV replicative signals, respectively. The number of doubly resistant cells obtained with this BKV/EBV pair was at least 250-fold lower than the number obtained with the pCD8α/REF78 + pCD8β/CEP98 pair (EBV/EBV). This surprising result contrasted with our consistent ability to use pRBK-based episomes, including pCD8α/RBK, for transfecting K562 cells with a high efficiency. Eventually, hphe-neo resistant cells grew out of from the pCD8α/RBK + pCD8β/CEP98 cotransfection.
Fig. 5.3. K562 cells stably cotransfected with pCD8α/REP7β and pCD8β/CEP9β. Two weeks after lipofection of K562 cells with pCD8α/REP7β and pCD8β/CEP9β, and selection in 200 μg/ml hygromycin B and 1.0 mg/ml G418, cells were stained with for CD8 α-chain expression, using OKT8 (top panel) or CD8 β-chain, using 2ST8-5H7, as primary antibodies. A Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was used as the secondary antibody.
Stained for CD8 $\alpha$-chain

Stained for CD8 $\beta$-chain
5.E. DISCUSSION.

In this report, we describe an episome-based technology for efficient stable gene cotransfer in human cells. Specific episomal vectors that can be employed in pairwise combinations for cotransfection purposes were assembled and their utility confirmed. Selectable marker pairs shown to be efficiently coselectable with these episomes were hph/hisD and hph/neo. Furthermore these episomes could be efficiently coselected when introduced into cells either sequentially or simultaneously.

A hygromycin B and L-histidinol withdrawal experiment with one of our cotransfectant lines revealed that while the L-histidinol-selectable episome was indeed episomal, the hygromycin B-selectable episome had integrated into the chromosome. Clearly, there is nothing in EBV episomes blocking integration, and probably, the integration complication can best be avoided by working with early passage cells.

Using a paired combination of EBV-based episomal vectors containing hygromycin B- and G418-selectable markers, we have shown that K562 cells can be cotransfected not only by sequential transfection, but even when both episomes are simultaneously transfected into nontransfected cells. The high stable
cotransfection efficiency observed in this experiment (within a log order of sequential cotransfection efficiencies) was unexpected. This finding likely reflects the efficiency with which episomes assume a stable form when introduced into eukaryotic cell nuclei. Whether this high efficiency is unique to the lipofection modality remains to be determined.

We predicted that cotransfection efficiencies could be increased by using episomes whose respective replicative machinery employ distinct and independent viral replicative signals, since this would obviate competition for common cell-derived trans-activating factors. Surprisingly, the data supported the opposite conclusion, with a BKV/EBV mixed episomal pair demonstrating at least 250-fold lower efficiency as compared to an EBV/EBV pair. Recent experiments (R. Groger et al., unpublished observations) suggest that this lower transfection efficiency with the BKV/EBV pair applies to sequential cotransfections as well.

While we had originally postulated that a pair of episomes based on different viral replicative signals might be less likely to compete for limiting cellular factors, or might be less likely to be adversely affected by a double dose of some episome-encoded factor, such as
EBNA-1 or BK T antigens, we must now generate new hypotheses. Our data suggest that either a combination of EBV and BK replicative signals somehow inhibits stable cotransfection or that two EBV-based episomes may cooperate to cotransfect cells with a higher frequency than would be expected based upon the efficiencies obtained when either is used individually. One possible explanation for this latter hypothesis is that EBNA-1 produced from one episome may facilitate the replication of the second episome. Further experiments are required to examine the reasons for the low cotransfection efficiency when the BKV/EBV combination is used. It will be of particular interest to determine whether similar results are obtained when optimal BKV- and EBV-based episomes (as generated by the experiments proposed in Chapter 4) are used in cotransfection experiments.

The major advantages for using episomal, as opposed to integrating, vectors for stable gene transfection and cotransfection derive from the increased transfection efficiencies that such vectors provide. This increased efficiency, particularly as documented here for simultaneous cotransfections, may be especially valuable for studies with primary cell cultures, in which the low transfection efficiencies associated with integrating
vectors, combined with limited proliferative capacity of such cells, together make sequential cotransfections with integrating vectors impractical. The introduction of vectorology and methodology for efficient stable gene cotransfer will enable numerous previously inaccessible cellular engineering applications. Particularly intriguing is the possibility of engineering a cell by inhibiting the endogenous gene product, through antisense RNA expression (directed at a non-coding region), and replacing it with an altered form, through sense RNA expression. Through such new strategies, the potential of gene transfer to provide clearer insights into molecular function in endogenous cellular settings will be appreciably enhanced.
CHAPTER 6: BACKGROUND ON M-CSF AND M-CSF RECEPTOR.

6.1. INTRODUCTION.

The processes of proliferation and differentiation are normally coupled in hematopoietic progenitor cells. In leukemic cells, however, these processes are uncoupled, with cells proliferating but not differentiating. An array of human leukemic cell lines can be induced to resume their differentiation program in response to specific chemical stimuli. The chemically induced differentiation program of such leukemic cells may be driven by both intracellular and extracellular molecular signals some of which may mimic those operative in normal differentiating progenitor cells.

Episomal expression systems developed in our laboratory (Chapters 1-5) now permit us to molecularly dissect the differentiation program of human leukemic cells in two alternative ways. On the one hand, specific gene products can be introduced by stably expressing a sense counterpart of the corresponding gene in the cell. On the other hand, the expression of specific genes can be blocked through antisense RNA-mediated gene inhibition. These stable gene transfer capabilities can be exploited to investigate the roles of specific growth
factors in the chemically-induced differentiation programs of human leukemic cell lines. A model system, in which these powerful transfection tools can be applied, is offered by monocytic differentiation programs of human myeloid leukemic cell lines. Based upon the hypothesis that molecules involved in normal hematopoiesis may play significant roles in leukemic hematopoiesis, our initial focus has been on a ligand:receptor pair previously implicated in normal monocytopenesis, namely, monocyte/macrophage colony stimulating factor (M-CSF) and its receptor (M-CSFR).

6.B. M-CSF.

M-CSF or (CSF-1) was independently identified based on its effects on mature macrophages and its effects upon colony formation in bone marrow culture. Virolainen and Defendi (1967) identified a factor (macrophage growth factor or MGF) released by fibroblasts, which stimulated DNA synthesis and cell division in mouse peritoneal macrophages in vitro. Other groups, using an assay for substances which can stimulate colony formation by mouse bone marrow cells in culture (Bradley and Metcalf, 1966; Ichikawa et al., 1966), identified a colony stimulating factor (CSF) present in human urine (Stanley and Metcalf,
1969), mouse embryonic cell conditioned medium (Bradley and Sumner, 1968), and mouse L cell conditioned medium (Austin et al., 1971). Stanley and co-workers (1970) showed that rabbit antisera against human urinary CSF inhibited the colony stimulating activity present in medium conditioned by mouse embryonic cell conditioned medium. Stanley and co-workers (1976) later demonstrated the identity of MGF and CSF by showing that antibodies against purified human urinary CSF (now known to be M-CSF) also neutralized MGF. M-CSF has been isolated from a wide variety of mesenchymal cells, including peripheral blood monocytes (low level expression is dramatically increased following TPA-treatment) (Horiguchi et al., 1986) and bone marrow stromal cells (Quessenberry et al., 1985). The M-CSF receptor, however, is found only in cells of the monocyte/macrophage lineage and placental cells (Woolford et al., 1985; Rettenmier et al., 1986).

The recent isolation of cDNAs corresponding to both the human and mouse forms of M-CSF have permitted primary structural determinations. Secondary structure has been studied for M-CSF purified from mouse L cell conditioned media and from human urine. M-CSF is a glycoprotein of apparent molecular weight 44-70 kDa, containing two identical 14.5 kDa polypeptide chains. The dimeric
structure is apparently required for biological activity, as reduction and alkylation, even under mild conditions, results in the both the dissociation of the dimer and the loss of biological activity (Das and Stanley, 1982). Contrary to the generally accepted model in which the two polypeptide chains are held together by disulfide bonds, Burgess and co-workers (1985) have provided evidence that the chains of the dimer are held together by non-covalent forces, since they can be dissociated under non-reducing conditions in the presence of isopropanol or acetonitrile at pH 2.1. The dissociation of the polypeptide chains which occurs following reduction and alkylation probably results from the breaking of intramolecular, rather than intermolecular, disulfide bonds.

Two lines of evidence have established that mature M-CSF contains asparagine (N)-linked oligosaccharides. First, neuraminidase (which specifically releases sialic acid residues) plus endo-β-N-acetylgalcosaminidase-D (which specifically releases N-linked oligosaccharides of the acidic complex type) decreased the molecular weight of M-CSF. Neuraminidase treatment alone produces only a small decrease in molecular weight. The decrease in molecular weight is greater if M-CSF is reduced and alkylated prior to endoglycosidase treatment, suggesting
that the native structure of the M-CSF molecule shields
some of the N-linked oligosaccharides from
endoglycosidase action. Second, M-CSF isolated from
mouse L cells grown in the presence of tunicamycin (which
inhibits the formation of N-glycosidically-linked
oligosaccharides) has a reduced molecular weight. There
is no evidence for O-linked glycosylation since treatment
of reduced and alkylated M-CSF with endo-α-N-β-
acetylgalactosaminidase does not affect its apparent
molecular weight (Das and Stanley, 1982).

The glycosylation of the mature M-CSF molecule may
be important for its stability rather than for its
biological activity. M-CSF isolated from human urine is
more susceptible to pronase digestion than the more
heavily glycosylated M-CSF found in mouse L cell
conditioned medium (Austin et al., 1971). Additionally,
M-CSF produced by mouse L cells grown in the presence of
tunicamycin is significantly less heat stable than is M-
CSF produced in the absence of tunicamycin (Tsuneoka et
al., 1981). However, neither the removal of
oligosaccharides by endoglycosidase treatment nor
prevention of oligosaccharide addition by tunicamycin
affect the biological activity of M-CSF (Das and Stanley,
1982).
The purification of M-CSF and subsequent N-terminal amino acid sequencing has allowed the isolation of M-CSF cDNAs (Ladner et al., 1987; DeLamarter et al., 1987; Rajavashisth et al. 1987; Wong et al., 1987) enabling the study of the molecular biology of M-CSF. M-CSF is transcribed from a single copy gene located on chromosome 5, band q33.1, in humans (Le Beau et al., 1986). Analyses of RNA from human MIAPaCa-2 pancreatic carcinoma cells and mouse L cells, both of which secrete biologically active M-CSF, have shown several discrete bands ranging from 1.4 to 4.5 kb in size. These different sized RNA species presumably result from differential splicing of a single mRNA transcript. More than one of these transcripts may give rise to biologically active M-CSF, as two distinct clones isolated from a MIAPaCa-2 cDNA library encode 256 and 554 amino acid long proteins, respectively (Ladner et al., 1987). Both clones yield biologically active M-CSF when expressed in COS cells. However, not all of these mRNA species give rise to biologically active M-CSF. Liver, for example, produces large amounts of a 1.4 kb M-CSF transcript, but no M-CSF activity can be measured in liver conditioned medium (Rajavashisti et al., 1987).
The cloning of an M-CSF cDNA facilitated the study of the synthesis and intracellular processing of M-CSF by allowing high level expression of that protein. Sherr and co-workers (Rettenmeir et al., 1987a) studied the synthesis and processing of M-CSF in NIH 3T3 cells cotransfected with retroviral vectors containing the human c-fms (M-CSFR) and 1.6 kb M-CSF cDNA (which encodes the 256 amino acid form of M-CSF). By pulse labelling the cotransfected NIH 3T3 cells with $^{35}$S-methionine and immunoprecipitating M-CSF at various time points, they were able to follow the maturation and secretion of newly synthesized M-CSF. The newly synthesized peptide is rapidly glycosylated and assembled into a dimer. Initial glycosylation adds 8 kDa to the apparent molecular weight of the reduced monomer (31 kDa in the absence of tunicamycin, 23 kDa in its presence), suggesting that both canonical N-linked oligosaccharide acceptor sites (Asn-X-Thr/Ser) are used. The 31 kDa protein is endoglycosidase H-sensitive and neuraminidase-resistant, consistent with the occurrence of N-linked oligosaccharide addition in the endoplasmic reticulum. Within 1 hour, the apparent molecular weight increases to 34 kDa and the glycoprotein becomes endoglycosidase H-resistant, neuraminidase-sensitive, consistent with
sialic acid addition in the golgi. Immunoprecipitable M-CSF can be sedimented with membranous organelles, but is not found in the cytosol.

Proteolytic cleavage of a cell surface form of M-CSF releases a 44 kDa homodimer into the medium starting 4h after a radiolabel pulse. A biologically active form of soluble M-CSF can also be recovered following trypsinization of these cells. Cifone and Defendi (1974) had observed that trypsinization of mouse L cells results in the release of a biologically active form of M-CSF. Sonicated S phase or mitotic L cells have a macrophage stimulatory activity which can be completely eliminated by prior trypsinization. While these findings suggest that the membrane-bound form of M-CSF is biologically active, it is possible that a protease present in the macrophage culture cleaves an inactive membrane-bound form, releasing an active, soluble growth factor. Hence, the actual functional significance of the membrane-bound form of M-CSF is unknown. More recently, Sherr and co-workers have found that the 554 amino acid long M-CSF precursor, encoded by the 4 kb cDNA, is efficiently cleaved within the cell, yielding a larger form of secreted M-CSF than is produced from the 256 amino acid precursor (Rettenmier et al., 1987b). In Chapter 9, we
describe our protein engineering strategy for creating, and expressing high levels, of a membrane-associated form of M-CSF. Our findings establish an additional, novel function associated with membrane-associated M-CSF.

6.C. M-CSF RECEPTOR.

The M-CSF receptor is a glycoprotein of apparent molecular weight 150 kDa (in TPA-induced HL-60 cells), with tyrosine kinase activity (Rettenmier et al., 1986). The M-CSF receptor is identical to the cellular homologue (c-fms) of the transforming gene (v-fms) of the McDonough strain of feline sarcoma virus (Sherr et al., 1985). Evidence for identity between M-CSF receptor and the c-fms protein comes from Sherr and co-workers (1985) who demonstrated that a rabbit antiserum against a recombinant v-fms protein precipitates a 165 kDa polypeptide from lysates of murine macrophage cell lines; immunoprecipitates with this anti-serum contained tyrosine kinase activity, and the 165 kDa protein was phosphorylated when M-CSF-dependent cells were incubated with M-CSF in the presence of gamma $^{32}$P ATP. Additionally, $^{125}$I-labeled M-CSF protein co-precipitated with the 165 kDa protein following incubation with rabbit anti-fms antisera.
The c-fms cDNA and genomic sequences have been cloned. c-fms is transcribed from a single gene located in the vicinity of the M-CSF gene on chromosome 5, band q33.2-33.3 (Le Beau et al., 1986). A 4 kb cDNA isolated from a human term placental cDNA library, by virtue of homology to a v-fms probe, encodes a 972 amino acid long protein characteristic of a membrane spanning glycoprotein (Coussens et al., 1986). Following cleavage of a putative 19-23 amino acid signal sequence, the predicted relative molecular mass (Mr) of the (non-glycosylated) protein is about 105 kDa. Eleven N-linked glycosylation sites are present in the 512 residue amino terminal half of the molecule. Two clones isolated from the placental library differ in their 3' untranslated regions. The transforming v-fms differs from the c-fms homologue at several sites, and unlike its cellular counterpart, protein kinase activity occurs in the absence of bound M-CSF ligand.

6.D. M-CSF/M-CSFR INTERACTION.

Studies with bone marrow-derived macrophages have shown that ¹²⁵I-labelled M-CSF bound to the cell surface is rapidly internalized (t 1/2 = 90 seconds) and is degraded in the lysosomes (t 1/2 = 3.5 hours) (Guilbert
and Stanley, 1986). The internalization and degradation of M-CSF by mature macrophages may provide a mechanism for feedback regulation of less mature, more proliferative cells of the monocyte lineage (Chen et al., 1987). Sherr and co-workers (1985) showed that binding of M-CSF to its receptor in the presence of gamma $^{32}$P ATP stimulates autophosphorylation of tyrosine. Since M-CSF can stimulate autophosphorylation in isolated macrophage membranes, it is unlikely that internalization of the M-CSF/M-CSF receptor complex is necessary for kinase activity. Orloffsky and Stanley (1987) examined the effect of M-CSF on specific mRNA transcription in an M-CSF-dependent murine macrophage line previously arrested by M-CSF starvation. Transcription increased for three genes, c-fos, c-myc, and JE, all of which are transcribed when GM-CSF is substituted for M-CSF. Transcription of a fourth gene, KC, was found to be increased in M-CSF-treated, but not GM-CSF-treated, cells, suggesting that M-CSF can also induce the transcription of some genes not associated with cellular proliferation.
6.2. M-CSF STIMULATION OF MACROPHAGE END-CELL FUNCTIONS.

M-CSF has been shown to stimulate a number of end-cell functions in mature macrophages, including mannose-inhibitable binding and killing of *Candida albicans* (Karbassi, et al., 1987), enhanced release of oxygen reduction products (Wing et al., 1985), and secretion of β-interferon (Fleit and Rabinovitch, 1981; Warren and Ralph, 1986), interleukin 1 (Moore et al., 1980), tumor necrosis factor (Warren and Ralph, 1986; Ralph and Nakoinz, 1987), and prostaglandin E (Moore et al., 1984). Macrophages derived from mouse bone marrow cultured with M-CSF secrete β-interferon when stimulated with LPS, poly I:C, or Newcastle virus (Fleit and Rabinovitch, 1981). Warren and Ralph (1986) showed that interferon secretion by human peripheral blood monocytes pre-incubated with M-CSF increases 22 fold following poly I:C treatment. Additionally, M-CSF preincubation increases the LPS- and TPA-stimulated secretion of tumor necrosis factor by a factor of twelve. Ralph and Nakoinz (1987) reported that murine peritoneal exudate macrophages incubated with M-CSF alone develop moderate tumoricidal activity against TU5 sarcoma cells, and that this tumoricidal activity is greatly enhanced if the cells are then incubated with lymphokine isolated from concanavalin A treated C3H/HeN
cells. In light of such effects of M-CSF on macrophage and cell functions, one can postulate two roles for M-CSF and its receptor in the chemically-induced differentiation of certain leukemic cell lines, whereby this molecular pair is not only driving differentiation but is also functionally modifying the differentiated leukemic cells.

6.7. M-CSF AND M-CSF RECEPTOR EXPRESSION DURING MONOCYTOIC DIFFERENTIATION.

The leukemic cellular expression of M-CSF and its receptor has been most intensively studied in HL-60 cells, a human promyelocytic leukemia cell line which can be induced to differentiate into cells resembling monocytes (1,25 dihydroxyvitamin D₃), macrophages (TPA), or granulocytes (retinoic acid or DMSO) (Collins, 1987). Neither M-CSF nor its receptor are detected in uninduced HL-60 cells. Twenty four hours following TPA treatment of HL-60 cells, a 4.6 kb M-CSF transcript is seen and the level of this transcript continues to increase at 48 and 65 hours. An M-CSF-specific radioimmunoassay shows that production of M-CSF protein parallels, but lags behind, the induction-specific expression of M-CSF mRNA. By comparison, the level of M-CSF receptor transcription
peaks at 24 hours, but declines by 48 and 65 hours. Neither CSF-1 nor c-fms transcription are detected when HL-60 cells are induced along the granulocytic lineage with DMSO (Horiguchi et al., 1986). The induction of c-fms is also seen during vitamin D₃-induced HL-60 monocytic differentiation. The induction of c-fms during monocyte/macrophage differentiation requires prior protein synthesis, as the inhibition of protein synthesis by cycloheximide prevents the TPA-induced expression of c-fms. In an HL-60 subline resistant to inducers of monocytic or granulocytic differentiation, c-fms transcription does not increase, suggesting that increased c-fms expression is linked to the differentiation process (Sariban et al., 1985).

Induction of c-fms transcription is also seen following TPA-induced differentiation of ML-1 cells along the monocyte lineage, but not when these cells are induced to differentiate along the granulocyte lineage by DMSO (Woolford et al., 1985), and during TPA-induced monocytic differentiation of U937 cells (Sariban et al., 1985).

M-CSF and M-CSF receptor inducibility in HL-60 cells make these cells interesting candidates for transfection studies. However, initial attempts in our laboratory to derive stable hygromycin B-resistant transfectants of HL-
60 cells using our EBV episomal replicons have been unsuccessful; other investigators have also found these cells difficult to transfect. Hence, our experimental strategy has centered around the study of the M-CSF:M-CSFR pair in U937 cells (Chapter 7).

6.C. THE U937 CELL LINE.

The U937 cell line was isolated from the pleural fluid of a patient with diffuse histiocytic lymphoma (Sundstrom and Nilsson, 1976). Phenotypically, these cells resemble blast cells of the monocyte lineage. The phenotypic changes that occur following treatment of these cells with phorbol ester were described in detail by Minta and Pambrun (1985). Uninduced U937 cells grow in suspension as a homogeneous population of single, round cells, with a doubling time of approximately 24 hours. Electron microscopic analyses reveal round cells with ruffled membranes, containing a large round immature nucleus and basophilic cytoplasm with small vacuoles and prominent granules. Histochemical staining shows that the cells lack transglutaminase and myeloperoxidase and that 60–70% of the cells stain weakly for non-specific esterase. They do not stain with monoclonal antibodies to the human peripheral blood monocyte/macrophage-
specific antigens Leu-M2 and Leu-M3, while they do stain weakly with monoclonal antibodies to HLA-DR antigens.

Within 48 hours after TPA treatment (162 nM), nearly all of the cells stop dividing and become adherent. Electron microscopy shows that these TPA-treated cells become highly vacuolated, extend numerous filamentous filapodia, and become either rounded, spindle-shaped, or stellate in appearance. The cytoplasmic to nuclear ratio is increased. TPA-treated cells stain strongly for transglutaminase and non-specific esterase, but not myeloperoxidase, and they also stain with antibodies for the Leu M-2, Leu M-3, and HLA-DR antigens. Functional assays show that these cells develop chemotactic responsiveness to C5a, the ability to mediate antibody-dependent cell-mediated cytotoxicity (ADCC), increased phagocytic activity, and superoxide anion production following TPA induction. Interestingly, the rate of appearance of the differentiated phenotype was accelerated when higher concentrations of cells were exposed to TPA. This is consistent with a model in which the cells stimulate the differentiation of neighboring cells through the release of paracrine factors.

β-interferon, whose production by macrophages is stimulated by M-CSF, may play a role in the TPA-induced
differentiation of U937 cells. Interferons are a class of proteins originally discovered on the basis of their antiviral activity, but which have later been found to mediate a wider range of functions. Treatment of U937 cells with exogenous α- or β-interferon induces some of the same phenotypic changes as does treatment with TPA, including enhanced ADCC against chicken erythrocytes, clumping, increased cytoplasmic to nuclear ratio, enhanced prominence of cytoplasmic granules, and membrane ruffling. β-interferon-treatment also induces NBT reduction and 2'-5'-A synthetase activity, two characteristics seen following TPA induction. Other changes which mark TPA-induced differentiation were not observed following interferon treatment, including the weak cytotoxic activity against K562 cells (Hattori, et al. 1983), non-specific esterase staining, superoxide generation, and OKM1 surface antigen expression (Testa et al., 1988).

The role of endogenous interferon production in the TPA-induced differentiation of U937 cells was examined by adding antibodies to purified human β-interferon to the culture medium during TPA induction (Yarden et al., 1984). The presence of these antibodies during TPA induction prevents expression of class I MHC
genes and increase in 2'-5' A synthetase activity. The effect of β-interferon antibodies on the development of ADCC was not examined.

Further evidence suggesting a role for interferons in the differentiation of U937 cells comes from the observation that U937 cells cultured in the presence of 2'-5' ApApA show increased ADCC against chicken erythrocytes, and have increased cytoplasmic to nuclear ratio, ruffling of the plasma membrane, and increased prominence of eosinophilic granules, all markers of differentiation (Schmidt et al., 1984). As stated earlier, 2'-5' A synthetase activity is induced following treatment of U937 cells with exogenous β-interferon. The relationship between β-interferon and M-CSF expression in differentiating U937 cells has not been examined.

Northern blotting analysis has shown that interferon produced by U937 cells in response to TPA induction is related to, but not the same as, β1-interferon. A 1 kb mRNA transcript from TPA-induced U937 cells hybridizes to a β1-interferon genomic probe under low stringency conditions, but fails to hybridize under stringent conditions. No hybridization is seen using a β2-interferon probe, even under non-stringent conditions (Resnitzky et al., 1986).
Since some of the end-cell functions stimulated by M-CSF treatment of monocytes are also observed in TPA-treated U937 cells, one can postulate a role for endogenous M-CSF production in the monocytic differentiation of U937 cells. We have tested this hypothesis by gene transfection analysis (Chapter 8). If an M-CSF/M-CSF receptor interaction were sufficient to induce some part of the differentiated phenotype, these changes would be observed if a transfected U937 cell line constitutively expressing the M-CSF receptor were treated with exogenous M-CSF. If an M-CSF/M-CSF receptor interaction were necessary for any aspects of U937 differentiation, antisense RNA-mediated inhibition of M-CSF or M-CSF receptor expression should interfere with the TPA induced differentiation program. While the M-CSF/M-CSF interaction might be blocked using antibodies against either the ligand or the receptor, anti-sense RNA-mediated gene inhibition has three potential advantages over antibody-mediated neutralization of the ligand or blocking of the receptor. Antibodies against a ligand may not always block its binding to the receptor, and antibodies to a receptor may actually simulate ligand binding. Also, antibodies can not inhibit any potential interaction between receptor and ligand occurring
intracellularly and would be unable to reach sites of ligand receptor interaction between closely associated cells.
CHAPTER 7: OPTIMIZATION OF METHODOLOGIES FOR STABLE GENE TRANSFER USING EBV-BASED EPISOMAL VECTORS IN HUMAN MYELOID LEUKEMIC CELLS.

7.A. INTRODUCTION.

One principal goal of my original experimental agenda was to use antisense RNA analysis to define the roles of M-CSF and M-CSFR in certain leukemic cellular differentiation programs. Specifically, the focus of this line of experimentation was to be on human myeloid cells. While I have had success transfecting a U937 subline, which I have subsequently shown to be less inducible along the monocyte/macrophage lineage than more commonly used U937 sublines, I am reluctant to carry out experiments which might not be reproducible by other labs, or which may yield results which might represent artifacts of this particular subline. Instead, I have concentrated on trying several transfection techniques on three different TPA-inducible U937 sublines (two of which were provided by labs which have reported success in transfecting them) as well as the bipotential myelomonocytic cell line PLB985, provided by Dr. Thomas Rado (University of Alabama at Birmingham), which has
been reported to be transf ectable (Dr. Tim Ley, Washington University, personal communication).

Apart from one successful set of lipofections of a TPA-inducible U937 subline (obtained from the ATCC) with pCD8\alpha/RBK reporter gene and with pRSVCAT\alpha/220.2 (Fig. 7.4), (a success which could not be reproduced using subsequent lots of lipofectin), these attempts have been unsuccessful. Transfection methods (using several different EBV-or BK virus-based vectors) have included electroporation, lipofection, protoplast fusion, DEAE dextran, calcium phosphate precipitation, and polybrene transfection. In the case of PLB985 cells (as well as K51 and HL-60 cells), only electroporation and lipofection were tried. While control transfections of K562 cells have yielded stable transfectants using electroporation, lipofection, and calcium phosphate precipitation, none of these methods has resulted in the stable transfection of any of the myelomonocytic cell lines mentioned above. In some cases (high voltage, high capacitance), electroporation yielded a weak transient CAT assay, but did not result in the stable transfection of U937 cells. On a positive note, this extensive transfection experience has allowed us to dramatically increase the efficiency with which we can transfect K562
cells, a development which will be critical in future experiments requiring transfection of these cells with sense or antisense cDNA expression libraries.

7.B. MATERIALS AND METHODS.

7.B.i. Cell Culture.

U937 and K562 cells were grown in complete medium consisting of RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine (2 mM; M.A. Bioproducts, Walkersville, MD), and gentamicin sulfate (40 μg/ml), in a 5% CO₂ environment. Culture medium was refreshed every 3-4 days and cells were maintained in a logarithmic phase of growth at all times. Different U937 sublines were obtained from the American Type Culture Collection, J. Clements (Johns Hopkins, T. Ley (Washington University), and C. Hauer (CWRU). K562 cells used here are the K.48 subline, provided by T. Rosenberry (CWRU).

7.B.ii. Episomes.

The episomes used in these transfection experiments are pRSVCATα/220.2 and pRSVCATβ/220.2 (Hauer et al., 1989), along with p462.3 and p462.4 (referred to here as pCMVCATα/220.2 and pCMVCATβ/220.2); the latter provided
by J. Yates (Roswell Park). These four episomes are schematically depicted in Figure 7.1.

7.B.iii. Electroporation.

A diverse set of electroporation apparatuses and electroporation parameters were employed in these experiments; hence the particulars are detailed in the results section.

7.B.iv. DEAE dextran.

A DEAE-dextran transfection procedure, modified for use with suspension cells, was reported by Fujita et al. (1986). Briefly, $5 \times 10^6$ cells were resuspended in 1 ml PBS containing 10 µg RSVCATa/220.2 and 200 µg DEAE dextran (Stratagene); this cellular suspension was incubated at room temperature for 15'. Either or 0.1 ml or no DMSO was then added and the mixture swirled. After 2', PBS (15 ml) was added to stop the DMSO shock. Cells were washed three times in PBS before plating in growth medium.
Fig. 7.1. pRSVCATα/220.2, pRSVCATβ/220.2, pCMVCATα/220.2, and pCMVCATβ/220.2. The *hph* and *neo* genes is flanked by the herpes simplex virus type 1 thymidine kinase promoter and termination signals (stippled). B, *BamHI*; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus immediate early enhancer/promoter; EBNA-1, Epstein-Barr virus nuclear antigen-1; EBV *oriP*, Epstein-Barr virus origin of replication; pBR, pBR322 origin of replication and ampicillin-resistance gene; Xh, *XhoI*.

The basic protocol for protoplast fusion has been described (Sandri-Goldin et al., 1983), and we have introduced modifications for suspension cells (Hambor et al., 1988). Protoplasts were copelleted with $10^7$ logarithmically growing target cells (prewashed with PBS) three times) (5000:1, protoplast/target cell ratio) at 1700 x g in a DuPont-Sorvall HS-4 centrifuge for 20' at 4°C. Fifty percent polyethylene glycol 1540 in PBS (1 ml) was added dropwise over 1', and cells were then immediately diluted with PBS (1 ml) over 1' and more PBS (20 ml) over 3'. Cells were then pelleted, washed twice with PBS, and resuspended in complete medium.

7.B.vi. Lipofection.

Lipofection was performed as described in section 3.C.ii., with the following exceptions: The number of cells lipofected, the amount of DNA used, and the length of incubation in the presence of DNA and lipofectin before adding RPMI, containing 10% fetal calf serum, were varied as indicated in the results section.
7.B.vii. CaPO₄ precipitation.

Cells were grown in 100 mm tissue culture dishes (Falcon) (5 x 10⁶ cell in 10 ml medium). Calcium phosphate precipitation was performed using a kit from Stratagene. 500 µl of a solution containing CaCl₂ (250 mM) and 20 µg pRSVCATα/220.2 was mixed with 500 µl of a solution containing 50 mM BES, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 6.95. This mixture was allowed to stand at room temperature for 10', and then added to cells with swirling. This cellular mixture was then incubated 12-24 hours at 37°C, 5% CO₂. Dead cells were removed by density centrifugation and viable cells were resuspended in complete medium.


Chisholm and Symonds (1988) reported the use of polybrene to stably transfec HL-60 cells. According to this procedure, 30 µg polybrene (Aldrich) plus 10 µg DNA were added to 3 ml complete medium. 10⁶ cells were resuspended in this mixture and incubated for 6h at 37°C, 5% CO₂. Cells were subsequently harvested and resuspended for 1-3' in growth medium (RT) containing 30% DMSO. Cells were washed twice with, and resuspended in, complete medium.
7.B.ix. CAT Enzymatic Assay.

Cells (5 x 10^6) were harvested, washed three times in PBS, resuspended in 25 mM Tris (pH 7.8) (100 μl) and lysed by four cycles of freeze-thawing. Crude cellular extracts (20 μl per reaction mixture) were assayed for chloramphenicol acetylating activity by a standard 1hr assay as described (Gorman et al., 1982b). Enzymatic mixtures were extracted with ethyl acetate, and unacetylated and acetylated (1-acetate, 3-acetate, 1,3-diacetate) forms of [14C]-chloramphenicol were separated by ascending chloroform/methanol (95:5) thin-layer chromatography on silica gel plates (20 x 20 cm) (Whatman). Spots were visualized by overnight autoradiography (Kodak XAR film).


The basic procedure for stable transfectant derivation was described by Hauer et al (1989). Preliminary hygromycin B toxicity profiles established that optimal cell killing by 10-12 days, of U937 and K562 was achieved with 0.2 and 0.3 mg/ml of hygromycin B (Calbiochem, Inc., La Jolla, CA) respectively. Cells were aliquoted (5 x 10^4 cells/ml) into 6-well Falcon culture plates post-transfection, and one half of the
medium was replaced with fresh hygromycin B-containing medium every four days until hygromycin B-resistant clones emerged.

7.C. RESULTS.

7.C.1. Electroporation.

While I have reproducibly used electroporation to stably transflect K562 cells with pREP-based plasmids, multiple attempts to derive stable transfectants of various inducible U937 sublines using this gene transfer technique failed. For many cell lines, electroporation conditions which result in 30% early cell death provide for optimal entry of DNA into cells (C. Hauer, personal communication). Multiple attempts to electroporate U937 cells (summarized below) resulted in varying degrees of cell death, but in no case were stable hyg\(^{R}\) U937 transfectants obtained. In only one case was transient chloramphenicol acetyl transferase (CAT) activity detected. In retrospect, several factors may have accounted for the excess cell death that occurred at higher voltage and capacitance settings: (i) Toxic substances (possibly proteases released following degranulation) secreted into the medium by electroporated
cells (T. Ley, personal communication). This cause of cell death can be effectively reduced by washing cells immediately after electroporation; (ii) the oxidation of the stainless steel electrodes of the electroporation apparatus occurs rapidly, even after only a single electroporation. This oxidation is enhanced in the presence of phenol red (a pH indicator) in cell growth medium (T. Ley, personal communication). This can markedly affect current delivery to the cells. Meticulous cleaning of electrodes (with steel wool) prior to each electroporation was initiated only in later experiments; and (iii) contaminants may have been present in DNA prepared according to our earlier laboratory plasmid maxiprep procedure, perhaps preventing transfection of U937, but not the more transfecatable K562, cells. By eliminating these sources of cell death, it is likely that higher field strengths could have been employed for more effectively introducing DNA into monocytic cells. It is important to keep these factors in mind when reviewing the electroporation results presented below, which were generated over a long period of time, as our understanding of factors affecting electroporation efficiency advanced.
7.C.i.a. Effect of different electroporation media.

The nature of the electroporation medium can dramatically affect the efficiency of DNA transfer. J. Frazier (UCLA) has noted that electroporation in the presence of complete medium yields significantly higher transient expression of transfected DNA in T cells than does electroporation in the presence of PBS with 20 mM Hepes (personal communication). J. Clements (Johns Hopkins) has found that electroporation with a PBS/sucrose-based medium yields optimal transient gene expression in her U937 sublines (personal communication).

To start, I compared the effect of different electroporation media on the transient expression of transfected DNA in U937 and K562 cells. These experiments were, in part, predicated upon two assumptions: (i) conditions which result in high transient gene expression are more likely to yield stable transfectants; and (ii) conditions which yield a stronger transient CAT signal in K562 cells might also increase DNA entry into U937 cells.

A comparison of transient chloramphenicol acetyltransferase (CAT) activity in K562 cells electroporated with pRSVCA TA/220.2 in complete medium vs. PBS/20 mM Hepes revealed significantly higher CAT
activity when complete medium was used as the electroporation buffer (Fig. 7.2). Electroporation in the presence of complete medium also proved to be advantageous for the derivation of stably transfected K562 (data not shown). When U937 cells were electroporated in the presence of complete medium, however, neither transient CAT activity nor stable transfection were achieved (data not shown). I have not examined the effect of the serum component of complete medium on electroporation efficiency.

To examine the effect of ionic strength of the electroporation buffer on DNA transfer, I electroporated U937 cells using a low ionic strength buffer (272 mM sucrose, 7 mM phosphate, 1 mM MgCl₂, pH 7.4 with 10 μg pRSVCATα/220.2) and varying electroporation conditions (for BTX electroporator: 300 V or 350 V, 50μF, 3.5 mM gap electrode; for Promega Biotec electroporator: 4.0 mM gap electrode, 2.5 μs time constant) as recommended by J. Clements for the electroporation of U937 cells (vide supra). Using these conditions to electroporate U937 sublines obtained from the A.T.C.C. and J. Clements, I failed to detect any transient CAT activity and were also unable to derive stably transfected cells (data not shown). I did not examine the effect of low ionic
Fig. 7.2. Comparison of PBS/Hepes vs. complete medium as electroporation buffers. K562 cells were electroporated using an X-Cell 450 electroporator (Promega Biotech, Inc.) (4 mM gap electrode, 650 V/cm, 950 μF) in either complete medium (lane 1) or PBS with 20 mM Hepes (pH 7.2) (lane 2) and culture in complete medium for 24h prior to harvesting for CAT assays. CM, chloramphenicol.
strength buffer on transfection efficiency of K562 cells.

7.C.i.b. Effect of DNA concentrations on electroporation efficiency.

Most electroporation attempts with U937 were conducted with 10–20 µg episomal DNA. When U937 cells were electroporated with markedly higher DNA concentrations (500 µg RSVCATα/220.2; 950 µF, 400 V/cm, 4 mm gap electrode, in complete medium), a weak transient CAT signal was seen (Fig. 7.3), however, cell viability was poor, and no stable transfectants could be derived. It should be noted that cells were not washed immediately following electroporation, as was subsequently suggested by protocols provided to us by T. Ley (see below).

By contrast I also examined whether the use of smaller amounts of episomal DNA (100 ng or 1 µg pCMVCATα/220.2) for electroporations would allow for stable transfer into U937 cells, since J. Yates (SUNY Buffalo) informed us that the use of less DNA during electroporations can improve cell viability (personal communication). Again, this set of electroporations yielded neither transient CAT activity nor stably transfected cells.
Fig. 7.3. Use of a high concentration of plasmid DNA in electroporation of U937 cells. U937 cells were electroporated using 500 μg pRSVCATα/220.2 (complete medium, 950 μF, 400 V/cm) and cultured in complete medium for 24h prior to harvesting for a CAT assay (lane 1). The positive control (lane 2) is a K562 RSVCATα/220.2 stable transfectant. CM, chloramphenicol.
7.C.i.c. Use of different plasmids for electroporation attempts.

One possibility is that some component of the episome pRSVCATα/220.2 might be particularly inhibitory for U937 cells. To rule out this possibility, a variety of episomes (pRSVCATα/220.2; pRSVCATβ/220.2; pCMVCATα/220.2; pCMVCATβ/220.2; pCD8α/REP7α; pCD8α/REP7ß; pCD8α/RBK; and pCD8α/REP9α pCD8α/REP9ß) were employed. None of these episomes yielded stable hyg² transfectants.

7.C.i.d. Effect of washing cells following electroporation.

T. Ley has observed that significant cell death occurs following U937 or PLB985 electroporation if these cells are not washed immediately after electroporation (unpublished observation). In fact, if cells were so washed, higher voltages and capacitances could be successfully employed using integrating vectors. A possible interpretation of this phenomenon is that cells of the monocytic lineage may degranulate in response to electroporation, releasing proteases which result in cell death if not immediately removed.

To start I confirmed that washing of cells following electroporation significantly increases post-transfection
cell viability. When PLB985 cells were washed following electroporation (10^7 cells in 500 μl serumless RPMI/100 μl HEBS/5 μg RSVCATβ/220.2/100 μg salmon sperm DNA, using a BTX 300 electroporator, 3.5 mM gap electrode, 600 μF, 150 V, 10^7 viable cells were counted 48 hours after transfection (a significant number of dead cells were also present). In contrast, only 2 x 10^6 viable cells were counted after 48 hours if cells were not washed prior to culture. When cells were electroporated at a higher voltage, 250 V, the effect was even more dramatic. When cells were washed immediately after electroporation, 3 x 10^6 viable cells were present at 48 hours, while if they were cultured without washing, no cells survived. Despite these encouraging results, electroporation of U937 and PLB985 cells (washing cells immediately after electroporation) at 150, 200, or 250 V yielded neither detectable transient CAT signals nor stable hyg^r transfectants.

7.C.ii. DEAE-dextran.

While DEAE-dextran transfection has been used to transiently introduce DNA into cells, it is generally regarded as a poor method for stably transfecting cells. However, two lines of evidence suggest that DEAE-dextran
may be useful for stably introducing episomal vectors into cells. Innis and Scott (1983) reported that plasmids containing the SV40 ori could replicate when introduced into COS cells by DEAE-dextran. Reeves et al. (1985) showed that plasmids introduced into mammalian cells using DEAE-dextran rapidly assembled into minichromosomes. In order to test the possibility that DEAE-dextran could be used to stably transfect human cells with episomal vectors, I attempted to use a DEAE-dextran procedure, as modified by Fujita et al. (1986), to transfect K562 and U937 cells. Transient CAT activity was detected for the K562 transflectants, but not for the U937 transflectants (Fig 7.4). In the case of K562 cells, DMSO shock did not detectably increase the transient CAT activity. Selection in hygromycin B, however, failed to yield stable transflectants for either cell line. Of note is the fact that this was the only example, for all of the transfection techniques attempted, in which a transfection that resulting in a transient CAT signal for K562 cells failed to yield stable transflectants of that cell line. A subsequent attempt to introduce RSVCATα/220.2 into U937 cells using a higher concentration of DEAE-dextran (500 μg/ml) and longer incubation times (15, 30, and 60 minutes, no DMSO shock)
Fig. 7.4. DEAE-dextran transfection of K562 and U937 cells. K562 cells were transfected with pRSVCATα/220.2 using DEAE-dextran transfection without DMSO shock (lane 1) or with 2' DMSO shock (lane 2). U937 cells were transfected with pRSVCATα/220.2 without DMSO shock (lane 3) or with 2' DMSO shock. The positive control (lane 5) is a K562 cell line stably transfected with pRSVCATα/220.2. CM, chloramphenicol.
failed to yield transient CAT activity as well.

7.C.iii. Protoplast fusion.

All attempts at protoplast fusion resulted in 100% cell death in the case of U937 cells. Mock protoplast fusions, mimicking all aspects of the procedure, but with an absence of bacterial protoplasts, yielded viable cells. This result suggests that U937 cells are particularly sensitive to some bacterial component present during the protoplast fusion procedure.


One set of lipofections (vide supra) yielded both transient and stable transfectants for U937 cells (Fig. 7.5). Non-specific esterase staining following TPA induction confirmed that these transfectants were indeed capable of monocytic differentiation (not shown). All subsequent attempts at lipofection, using different DNA to lipofectin ratios, different lipofection times, and using other U937 sublines as well as other monocytic leukemia cell lines (HL-60, PLB985, KG1 [data not shown]) were unsuccessful. It should be noted that the aliquot of lipofectin used in the single successful lipofection experiment (provided as a gift by BRL) was not available
Fig. 7.5. Unrepeatable successful lipofection of U937 cells. Using lipofectin obtained as a gift from BRL, U937 cells were lipofected with pRSVCATα/220.2 (30 μg lipofectin, 10 μg DNA, 3h). Cells were harvested for CAT assay after 48h culture in complete medium (lane 1) or after 2 weeks selection in hygromycin B-containing medium (lane 2). The positive control (lane 3) is a K562 cell line stably transfected with pRSVCATα/220.2. CM, chloramphenicol.
for subsequent attempts. These subsequent, unsuccessful attempts were performed using a commercially available preparation of lipofectin from BRL. A representative from BRL informed us that a change in the way that the liposomes are prepared was implemented when the reagent became commercially available, but would not elaborate on this point to protect proprietary information.

X. Fan and B. Bloom (Albert Einstein College of Medicine) have also been successful in transfecting U937 cells using the original gift preparation of lipofectin but have not been able to stably transfect U937 using commercially available preparations and have found at least a 3- to 4-fold reduction in transient luciferase reporter gene expression when using the commercially available lipofectin as compared to the gift preparation (personal communication).

7.C.v. CaPO₄ precipitation. K562 cells were stably transfected with pRSVCA/220.2 using CaPO₄ precipitation. Parallel CaPO₄ precipitations using U937 cells failed to yield stable hyg⁺ transfectants.

Chisholm and Symonds (1988) reported the use of polybrene to stably transfect HL-60 cells. An attempt to introduce RSVCAT8/220.2 into K562 and U937 cells using this procedure (with one, two, or three minute DMSO shock) yielded neither transient CAT activity nor stable transfectants for either K562 or U937 cells.

7.D. DISCUSSION.

With one exception (vide supra) my attempts to stably transfect U937 cells with EBV- or BKV-based episome using electroporation, DEAE dextran, protoplast fusion, lipofection, CaPO₄ precipitation polybrene transfection have failed. Previous reports and personal communications suggest that the stable gene transfer into human monocytic leukemia cell lines should be possible. For instance, Kurata et al. (1989) reported the stable transfection of U937 cells using protoplast fusion. Reisman and Rotter (1989) reported the stable transfection of HL-60 cells with EBV-based episomal replicons using electroporation, suggesting that my inability to transfect monocytic cell lines is probably not due to a failure of these cells to support the replication of EBV-based plasmids. T. Ley and D. Link
have used integrating, G418-selectable vectors to stably transfec
t both U937 and PLB985 cells (personal
communication).

Possible explanations for my failure to transfec
t U937 cells by any of the means tested could be due to
three possible causes: (i) DNA may not have entered the
cell or may have been rapidly degraded; (ii) DNA may have
entered the cell nucleus, but may have been unable to
express the reporter gene or establish itself episomally.
(iii) U937 cells may be exquisitely sensitive to some
bacterial substance which may have co-purified with the
DNA, and which either inhibited DNA uptake by the cells
or else resulted in the death of cells which had
internalized DNA.

The finding of a weak transient CAT signal when U937
cells were electroporated in the presence of 500 μg
plasmid DNA warrants further investigation. Chen and
Okayama (1987) have reported the need for extra
purification steps for DNA to be used in CaPO₄
precipitation experiments. DNA was purified twice by
cesium chloride centrifugation and phenol extracted
multiple times, following the addition of 1% SDS to
remove E. coli proteins which are toxic to cells.
Failure to recover viable cells following protoplast
fusion attempts also suggests the possibility of a toxin present in bacteria, but not necessarily removed by our plasmid purification procedure. T. Ley has also reported that a phenol extractable substance associated with DNA following cesium chloride centrifugation significantly inhibits the stable transfection of U937 cells by electroporation (personal communication). J. Yates informed us (personal communication) that transfection efficiency is increased when less DNA was used in electroporation experiments; this could be due to a toxicity inherent in the DNA itself or to an accompanying impurity. The transfection procedures described above used K562 cells as a positive control, since K562 cells have been easily transfectable in our hands. The difference in transfectability of K562 versus U937 cells may be due to a higher transfection efficiency of K562 cells (possibly due to membrane properties or differences in the ability to support episome replication, or else due to decreased sensitivity of K562 cells to contaminating bacterial toxins.

Plasmid DNA used in the transfection procedures described above was not subject to the repeated phenol extractions recommended by Chen and Okayama (1987), and thus the failure of these experiments to stably introduce
episodes into U937 cells may be due, in part, to problems with the purity of the plasmids used.

I am continuing my attempts to use transfection analysis of the role of M-CSF and M-CSFR in U937 differentiation in collaboration with T. Ley, who has established conditions for stable gene transfer into the U937 and PLB935 cell line using electroporation. As of this writing, an integrating vector, pM-CSFR/Billneo appears to mediate a higher transfection efficiency (as measured by the number of G418^8 cells) than do either of the two episomes used, pCD8α/REP9α and pCD8α/REP9β (Fig. 7.6). Two possible explanation for this difference are (i) a toxic effect of EBNA-1 on U937 cells, or (ii) a low activity of the HSV-1 thymidine kinase gene promoter, compared to the SV40 viral enhancer/promoter, in U937 cells. The possibility of EBNA-1 toxicity seems less likely, since EBNA-1 has been expressed in U937 cells when introduced using an integrating vector (T. Ley, personal communication). Should the low transfection efficiency of our episomes prove to be due to a weak promoter driving the selectable marker, we can readily configure an appropriate alternative episome.

Stable gene transfer into monocytic cells now seems feasible. Even if stable transfection proves inadequate
for the inhibition of M-CSF or M-CSFR expression in monocytic leukemia cell lines, the use of antisense oligonucleotides may allow us to achieve the transient inhibition of gene expression in these cell lines (Tortora et al., 1990). However, as I had been unable previously to achieve gene transfer into monocytic leukemia cell line, I chose to explore the effects of M-CSFR in a cells which do not normally express that receptor, the erythrocytic leukemia cell line K562 (Chapter 8).
Fig. 7.6. Episomes currently being transfected in collaboration with T. Ley. The episomes pCD8α/REP9α, pCD8β/REP9β, and M/CSFR/Billneo are schematically depicted. Herpes simplex virus promoter and termination sequences flank the neo gene in pCD8α/REP9α and pCD8α/REP9β, while simian virus 40 promoter and termination signals flank the neo gene in pM-CSFR/Billneo. B, BamHI; E, EcoRI; EBNA-1, Epstein-Barr virus nuclear antigen-1; EBV oriP, Epstein-Barr virus origin of replication; K, KpnI; neo, neomycin phosphotransferase; PA, SV40 late termination/polyadenylation sequences; pBR and pBT, ampicillin resistance gene and pBR322 origin of replication from pBR322 and pBluescript (Stratagene) respectively; RSV, Rous sarcoma virus 5' long terminal repeat; SFFV, splenic focus forming virus long terminal repeat.
CHAPTER 8:  EXPRESSION OF M-CSFR IN NON-MONOCYTIC CELL
LINES: TESTING THE HYPOTHESIS OF PHENOTYPIC IMPRINTING.

8.A. ABSTRACT.

Human M-CSF receptor was expressed in the inducible
myeloid leukemia cell line K562 through gene transfer, in
order to assess whether this receptor, when stimulated by
recombinant human M-CSF (rhM-CSF), could alter the
differentiative pathway of these cells. No significant
differences were observed in the differentiative response
of this and control transfectant cells to rhM-CSF and/or
phorbol ester, as assessed by morphology and the
expression of non-specific esterase, and expression of
the surface antigens LeuM3 (CD14) (a marker of monocytic
differentiation), and platelet glycoprotein IIIa (a
marker of megakaryocytic differentiation). The M-CSFR-
expressing K562 cells proliferated more slowly than
controls, but no M-CSF-related effect on proliferation
was evident. We conclude that the M-CSF/M-CSFR
ligand:receptor pair cannot, in and of itself imprint a
monocytic phenotype nor modulate the differentiation
program of K562 cells. Hence, in contrast to previous
murine studies, our investigations in a human cell line
suggest that the functional repertoire of these molecules is, at least in part, cell type- and lineage-restricted.

8.B. INTRODUCTION.

Gene transfer has provided a valuable experimental tool for defining the functional repertoire of ligand:receptor pairs in hematopoietic cells. The application of this technology to the molecular analysis of colony stimulating factors (CSFs) and their receptors (CSFRs) is of particular interest. CSFs are known to play coupled roles in the proliferation and differentiation of normal hematopoietic progenitors. However, the relationship between the proliferative and differentiative effects of individual CSFs, and the molecular underpinnings of their pleiotropic effects, have thus far eluded precise definition. The ability to artificially express CSFRs via gene transfer in diverse cell backgrounds, encompassing both natural and foreign cellular environments, has introduced an elegant strategy for molecularly dissecting the complexities of CSF phenomenology.

The M-CSF:M-CSFR ligand:receptor pair was originally characterized by virtue of its role in regulating the proliferation and differentiation of monocyte/macrophage
progenitors. The M-CSF receptor has a relatively restricted cellular distribution, as it is normally present only on cells of the monocytic or placental lineages. Its presence on the latter (placental) cells has suggested the possibility of a more expanded functional repertoire for the M-CSF:M-CSFR molecular pair. To define this functional repertoire, several groups have now reported the introduction of the M-CSFR coding sequence into different cell types, attempting to confer M-CSF responsiveness upon such cells:

(i) In the cases of NIH 3T3 cells (Roussel and Sherr, 1989) and Chinese hamster lung fibroblasts (Hartman et al. Growth Factors, in press), exogenously-introduced M-CSFR transduced mitogenic signals in response to rhM-CSF. In two other cases, expression of transfected M-CSFR conferred M-CSF responsiveness, with not only a proliferative response, but also phenotypic changes indicative of macrophage differentiation.

(ii) rhM-CSF transduced a mitogenic signal in murine myeloid FDC-P1 cells transfected with human M-CSFR; additionally, phenotypic changes indicative of maturation along the monocyte/macrophage lineage were evident, including increased expression of Mac-1 and RB6-8C5 antigens, loss of thy 1.2 antigen expression, and the
appearance of a large number of primary granules (Rohrschneider and Metcalf, 1989). These investigators proposed that the ability of FDC-P1 cells to differentiate in response to a signal transduced by M-CSFR might have been possible only because that cell line is already in the myeloid lineage; other more distantly related lineages might not be able to enter a monocytic differentiation pathway.

(iii) Borzillo et al. (1990) showed that murine early pre-B lymphoid cells expressing a transfected M-CSFR gene underwent phenotypic changes suggestive of macrophage differentiation when stimulated with rhM-CSF. The cells became enlarged and adherent, nuclear to cytoplasmic ratio increased, cytoplasmic vacuoles appeared, displayed butyrate esterase and Mac-1 positivity, and lost expression of the lymphocyte antigens B220 and BP-1. Since some transformed B-cell lines in culture have been reported to differentiate spontaneously into macrophage-like cells, this observation of M-CSF/M-CSFR-induced phenotypic changes in a pre B-cell line does not contradict the hypothesis that a differentiative signal provided by M-CSFR may be restricted to cells closely related to the macrophage lineage. Interestingly, these M-CSF-dependent phenotypic
changes occurred when the cells were cultured in IMDM, but not when the cells were cultured in RPMI 1640.

(iv) Kato and Sherr (1990) reported that the interleukin-3 (IL-3) dependent murine myeloid 32DCL3 cell line acquired the ability to proliferate in response to rhM-CSF after transfection with a human M-CSFR cDNA. Except for the induction of non-specific esterase activity, no other evidence of monocytic differentiation (expression of the Mac-1 antigen, increased adherence, ability to phagocytose particles, present antigens to T-cell hybridomas, or mediate antibody dependent cytotoxicity) was seen when these transfectants were cultured in the presence of rhM-CSF. One interesting feature of these transfectants, however, was that in the presence of rhMCSF, they lost their ability to differentiate along the granulocytic lineage in response to GM-CSF. This result suggests that M-CSF could act through its receptor to not only induce differentiation along the monocytic lineage, but also to block differentiation along other lineages.

All of these previous investigations were confined to murine cells. In the present study, we determine whether human M-CSFR is also capable of conferring M-CSF responsiveness and a monocytic differentiative signal to
K562 cells. K562 is a human myeloid leukemia cell line (Lozzio and Lozzio, 1975; Andersson et al., 1979) which has a bipotential capacity to differentiate along the erythrocytic (Villeval et al., 1983), and megakaryocytic (Tetteroo et al., 1984) lineages in response to specific chemical inducers. Of particular interest to us was the recent demonstration by Yu et al. (1989) that c-fes, when expressed in K562 cells through gene transfer, enables these cells to undergo changes characteristic of monocyte/macrophage differentiation in response to TPA induction. Here we determine whether M-CSFR gene transfer (plus M-CSF stimulation), like c-fes gene transfer, can confer on K562 cells the capacity for monocytic differentiation. In this way, we aim to extend the previous observations with M-CSFR gene transfer in murine cells and to shed light on the nature of "phenotypic imprinting" in human leukemic cellular backgrounds.
8.C. MATERIALS AND METHODS.

8.C.i. Plasmids.

The plasmid assembly schemes for pM-CSFR/REP7B and pCD8α/REP48 are detailed in sections 8.C.i. and 3.C.i, respectively.


Cell culture and lipofection were performed according to procedures described in sections 3.C.ii and 3.C.iii. T74 (obtained as a gift from G. Stein, CWRU) is a fibroblast cell line transfected with a human M-CSF cDNA expression construct, secreting high levels of soluble M-CSF into the culture medium.


TPA (12-0-tetradecanoyl phorbol-13-acetate; Sigma, Inc.) was suspended in ethanol at a stock concentration of 10 μg/ml. Cells were induced (at a density of 2 x 10^5/ml) with TPA at a concentration of 0.5 or 5.0 ng/ml as specified. Recombinant human M-CSF (rhM-CSF) was obtained as a gift from R. Stanley (Albert Einstein College of Medicine), and used at a concentration of 2500 units/ml (1.1 nM).
s.c.iv. Immunofluorescence and flow cytometric analysis.

Fluorescein isothiocyanate (FITC)-conjugated anti-LeuM3 (CD14) (Becton Dickinson), FITC-conjugated anti-platelet glycoprotein IIIa (DAKO), and rat anti-human CSF-1R (Oncogene Sciences) primary antibodies were used. For M-CSFR staining, FITC-conjugated rabbit anti-rat IgG (Miles) was used as the secondary antibody.

8.D. RESULTS.

To determine whether an exogenously introduced M-CSFR can confer M-CSF responsiveness, along with associated proliferative and monocytic differentiative features, to human myeloid leukemia cells, K562 (myeloid leukemia) cells were stably transfected with the episome pM-CSFR/REP7B. In pM-CSFR/REP7B, the complete cDNA coding sequence for human M-CSFR has been subcloned into our Epstein-Barr virus episomal expression vector pREP7B (Chapter 3). M-CSFR expression on hygromycin B-resistant (hyg²) pM-CSFR/REP7B K562 transfectants was documented by immunostaining using a monoclonal anti-human M-CSFR (Oncogene Sciences) primary antibody. The flow cytometric data shown in Fig. 8.1 confirms high levels of M-CSFR expression on these transfectants; no detectable M-CSFR was present on non-transfected K562 cells or
Fig. 8.1. M-CSFR expression in pM-CSFR/REP7B transfected K562 cells. K562 cells stably transfected with pM-CSFR/REP7B were analyzed by flow cytometry using a rat monoclonal anti-human CSF-1R antibody (Oncogene Sciences) plus an FITC-conjugated rabbit anti-rat IgG (Miles) secondary antibody (stippled) or using the secondary antibody alone (not shaded). Cell number (vertical axis) is plotted against log fluorescence (horizontal axis).
pCD8α/REP4B hyg² K562 transfectants expressing the irrelevant human T lymphoid glycoprotein CD8α (data not shown).

The differentiation responses of pM-CSFR/REP7B and control pCD8α/REP4B transfectants were compared. Transfectants (2 x 10⁵ cells/ml) in complete (RPMI-based medium) were incubated with 2500 units/ml rhM-CSF or no rhM-CSF; alternatively K562 transfectants maintained in IMDM medium were treated with either 10% T74 conditioned medium, 2500 units/ml rhM-CSF, 0.5 ng/ml TPA, 5.0 ng/ml TPA, or 2500 units/ml rhM-CSF plus 5.0 ng/ml TPA. Cell counts after 48 hours did not reveal any proliferative effect of rhM-CSF on the pM-CSFR/REP7B transfectants. After 48 hours, cells were cytochemically stained for nonspecific esterase. In uninduced pM-CSF/REP7B and control pCD8α/REP4B K562 transfectants, nonspecific esterase staining revealed occasional weakly positive cells with no significant differences between these cell lines in either the percentage of esterase positive cells or the intensity of staining; this esterase positivity was completely quenchable by sodium fluoride (data not shown), indicating that the nonspecific esterase activity detected was of monocytic origin (Li et al., 1973). Furthermore, rhM-CSF induction did not result in
increased nonspecific esterase positivity in either the pM-CSFR/REP7B or the pCD8α/REP4B transfectants. TPA induced equivalent, small increases for both cell lines in the percentage of esterase positive cells and the intensity of staining.

To further probe for evidence of monocytic imprinting via the M-CSF:M-CSFR pair, cells were immunostained for the monocytic marker LeuM3 (CD14). Flow cytometric analysis demonstrated that the expression of LeuM3 was induced equivalently for both the M-CSR⁺ and control transfectants. rhM-CSF did not affect the level of LeuM3 expression in either cell line (with or without TPA present).

While M-CSF does not cause M-CSFR⁺ K562 transfectants to express monocytic markers, it is still possible that it could induce differentiation along the more conventional (for K562) megakaryocytic pathway. To assess this possibility, cells were immunostained for the megakaryocytic marker pgIIIa. No evidence for pgIIIa expression following rhM-CSF treatment as obtained. Furthermore, the rhM-CSF did not affect the level of pgIIIa induced by TPA. Erythroid markers were not assessed in the present study.
**8.E. DISCUSSION.**

K562, a human chronic myeloid leukemia cell line, demonstrates phenotypic heterogeneity and differentiative multipotentiality. While the erythroid and megakaryocytic features of this cell line have been the focus of most previous studies, its monocytic characteristics have attracted less attention. The report of Lozzio et al. (1981) that these cells can spontaneously differentiate along the monocytic lineage in culture, and our finding of occasional nonspecific esterase positive K562 cells is consistent with this claim. Our further observations of an increase in the percentage and intensity of esterase positive cells, and of the monocyte-specific surface antigen LeuM3 (CD14) following TPA treatment, further substantiate the potential of this cell line for monocytic differentiation. In light of the recent demonstration that monocytic features can be imprinted on K562 cells by c-fes gene transfer, we aimed in this study to see if similar imprinting could be alternatively achieved through M-CSFR gene transfer. Previous experience with M-CSFR gene transfer has been limited to murine cells. We here report that K562 are refractory to such phenotypic imprinting via the human M-CSF:M-CSFR pair.
No morphological (increased adherence, greater nuclear to cytoplasmic ratio, or appearance of primary granules), cytochemical (nonspecific esterase) or immunochemical (LeuM3; pgIIIa) evidence for M-CSF-driven differentiation in M-CSFR\textsuperscript{+} transfectants. Moreover, no M-CSF-driven proliferative effects were noted. The phosphorylation status of the exogenously introduced M-CSFR remains to be assessed.

Our findings indicate that the ability of M-CSF:M-CSFR to impact upon cellular functions may be confined to a limited cellular repertoire. In contrast to previous findings with murine cells of both hematopoietic and non-hematopoietic lineages, we have been unable to document significant proliferative or differentiative effects upon a human myeloid leukemia cell line with an established monocytic differentiative potential. Elucidating the cellular determinants of M-CSFR function will be of considerable interest and may ultimately lead to insights into how phenotypes are designated in normal and transformed hematopoietic cells.
CHAPTER 9: A MEMBRANE-ASSOCIATED CYTOKINE CAN FUNCTION AS A CELLULAR ADHESIN.

9.A. ABSTRACT.

The precise nature of the functional repertoire of membrane-associated cytokines has remained elusive. To probe functions of membrane-associated cytokines, chimeric gene transfection technology was employed to generate a glycoinositolphospholipid-modified variant of the cytokine monocyte/macrophage colony stimulating factor (M-CSF). This peptide variant, when expressed on the surface of human bone marrow stromal cells, was shown to function as a potent cellular adhesin. The finding that cytokines tethered to cell surfaces can promote cellular adhesion argues for a multifunctional perspective on cell surface-associated cytokines and furthermore, has considerable implications in the context of cellular targeting for cell-based therapies.

9.B. INTRODUCTION.

Cellular adhesion is mediated by homotypic and heterotypic molecular interactions at membrane interfaces (Littman, 1987; Springer et al., 1987; Williams and Barclay, 1988). Heterotypic molecular pairs known to
contribute to functional interactions between hematopoietic cells include LFA-1:ICAM-1 (Marlin and Springer, 1987), CD2:LFA-3 (Selvaraj et al., 1987), CD8:class I major histocompatibility complex (MHC) protein (Norment et al. 1988), and CD4:class II MHC protein (Doyle and Strominger, 1987).

A growing number of cytokines are now known to exist in both soluble and membrane-associated forms. These include interleukin-1 (Kurt-Jones et al., 1985), interleukin-2 (Kaplan et al., 1988), and tumor necrosis factor (Kriegler et al., 1988). A membrane-bound form of monocyte/macrophage colony stimulating factor (M-CSF) was first described by Cifone and Defendi (1974). The known ligand functions such cytokines, when in their soluble forms, has prompted arduous searches for analogous ligand functions for their naturally occurring membrane-associated forms. The interpretation of such studies, however, has been complicated by the relatively low levels of molecules expressed at the cell surface and by molecular shedding problems. Stable gene transfer and protein engineering strategies offer important experimental approaches for resolving such functional issues.
We (Tykocinski et al., 1988) and others have introduced a chimeric gene transfection technology for artificially appending peptides to cellular membranes through glycoinositolphospholipid (GPL) anchors. According to this technology, a chimeric gene construct is assembled in which the coding region for the peptide is linked in-frame to the 3'-end sequence of a protein that is naturally GPL-anchored. Signals within the coding sequence of the latter protein direct a post-translational modification event whereby a GPL moiety is substituted for the hydrophobic extension peptide present in the nascent peptide. We have previously shown, for instance, that the extracellular domain of the T lymphocyte surface molecule CD8 can be linked to membranes through a GPL anchor by this process, using the 3'-end sequence of decay-accelerating factor (DAF) to direct the GPL substitution process (Tykocinski et al., 1988). Moreover, by using Epstein-Barr virus episomal expression vectors (Groger et al., 1989), high level surface expression of molecules so engineered has been achieved.

We have been intrigued by the possibility that a cytokine such as M-CSF, when cell surface-associated, may, by virtue of its affinity for cell surface-
associated receptors on other cells, serve an additional, unexpected function as a cellular adhesin. To test this hypothesis, we have generated human bone marrow stromal cell transfectants bearing an artificially engineered, membrane-binding variant of the cytokine M-CSF, employing a GPL-anchoring strategy and episome-based gene expression. We now report that M-CSF, when artificially tethered to cellular membranes, can significantly enhance adhesion to M-CSF receptor (c-fms product)-bearing cells, thereby functioning as a cellular adhesin.

9.C. MATERIALS AND METHODS.


The 1.8 kb coding region fragment (XhoI – EcoRI) of p3ACSFR1 (Wong et al., 1987) was inserted into the corresponding sites of pBluescript (Stratagene, Inc.). To generate a GPL-anchored variant of M-CSF, pM-CSF/BT was cut with NcoI, filled-in with the polIk, and subsequently cut with BamHI. The 3' AvaII (filled-in) to BamHI fragment from the DAF subclone pDF2.1/BT (Tykocinski et al., 1988) was subcloned into this vector to generate an in-frame M-CSF·DAF chimeric sequence. The KpnI – BamHI fragment of the resultant plasmid containing
the chimeric sequence was subcloned into the corresponding sites of pREP4α (Groger et al., 1989) to generate pM-CSF-DAF/REP4α (Fig. 9.1).

An M-CSF receptor EBV episomal expression construct was generated as follows: A 4.0 kb EcoRI fragment of pc-fms 102 (Coussens et al., 1986) was subcloned into the EcoRI site of pBluescript (Stratagene, Inc.) to generate pM-CSFR/BT. The 3.6 kb BamHI fragment of this subclone, containing the entire M-CSFR coding region, was subcloned in a sense orientation into the BamHI site of pREP7B to generate pM-CSFR/REP7B (Fig. 9.1).

9.c.ii. Transfection and selection procedures.

The KM-102 stromal cell line was kindly provided by K. Harigaya (Harigaya and Handa, 1985) and maintained in McCoy's 5a medium (Gibco, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (M.A. Bioproducts)/10 mM HEPES/40 μg/ml gentamycin sulfate in a humid 5% CO₂ atmosphere at 37°C. pM-CSF-DAF/REP4α was introduced into KM-102 cells by lipofection. Briefly, cells were grown to 50% confluence in six-well plates, and washed twice with PBS and once with Opti-MEM (Gibco). Cells were then incubated for 5-8 hours at 37°C with 3 ml Opti-MEM, containing 10 μg DNA and 30 μg lipofectin, before adding
Fig. 9.1. pM-CSFR/REP78 and pM-CSF-DAF/REP4a. M-CSFR and chimeric M-CSF-DAF cDNAs are shaded. The coding regions are indicated by the thicker box. EBNA-1 dl 7 (in pM-CSFR/REP78) contains a 700 bp in-frame deletion of the IR3 region of EBNA-1 (see section 1.B). B, BamHI; DAF, decay accelerating factor; EBNA-1, Epstein-Barr virus nuclear antigen-1; EBV oriP, Epstein-Barr virus origin of replication; hph, hygromycin phosphotransferase; K, KpnI; M-CSF, monocyte/macrophage colony stimulating factor; PA, SV40 late termination/polyadenylation signals; pBR, pBR322 origin of replication and ampicillin-resistance gene; RSV, Rous sarcoma virus 5' long terminal repeat;
3 ml of complete medium containing 20% FBS. Seventy-two hours post-transfection, selection for stable transfectants were generated by replacing the medium with fresh medium containing 75 μg/ml hygromycin B (Calbiochem, Inc.). Stably hyg² transfected colonies were picked at 2-3 weeks using cloning rings (Bellco, Inc.), expanded and maintained in 100 μg/ml hygromycin B.

pM-CSFR/REP7B and pM-CSF-DAF/REP4α were independently introduced into K562 cells by lipofection as well (as described in section 3.C.iii). M-CSFR expression was confirmed by indirect immunofluorescence and flow cytometry, using a rat monoclonal anti-M-CSFR primary antibody (Oncogene Sciences) and an FITC-conjugated rabbit anti-rat IgG secondary antibody (Miles ICN).

9.C.iii. Immunoprecipitation.

GPL-modified M-CSF was immunoprecipitated from pM-CSF-DAF/REP4α transfected K562 cell lysates using a rabbit anti-M-CSF polyclonal antibody (Genzyme), according to a protocol supplied by Oncogene Sciences. Briefly 10⁶ K562 cells were labeled overnight with 50 μCi ³⁵S cysteine (Amersham). 20 μl (20 μg) anti-M-CSF antibody plus 100 μl of a 50% slurry (V/V) protein A-
sepharose (Pharmacia) in PBSTDS (phosphate buffered saline with 1% Triton X-100, 0.1% sodium deoxycholate, 0.2% sodium azide, and 0.004% sodium fluoride) were added to cell lysates (lysed in ice cold PBSTDS, disrupted by repeated aspiration through a 21 gauge needle, microfuged for 30 minutes at 4°C and precleared with 100 μl 50% protein A-sepharose in PBSTDS). After a 4h incubation on a rotating device at 4°C, the immunoprecipitate was collected by centrifugation for 15' at 1500 RPM and washed 4 times in ice cold PBSTDS. The pellets were disrupted by boiling for 5' in electrophoresis sample buffer (plus or minus β-mercaptoethanol for reducing or non-reducing conditions, respectively). Samples were run on 10% SDS-polyacrylamide gels.


To quantitate the strength of cell:cell binding, we employed a modification of the method of McClay et al. (1981). Briefly, 3 x 10⁶ nontransfected or transfected KM-102 cells were placed in wells of a polyvinyl, flat-bottom 96-well plate (Dynatech Laboratories) with 0.1 ml complete medium per well, and the cells were incubated at 37°C for two days. Wells were pretreated with fetal bovine serum for two hours to promote attachment of the
KM-102 cells. The KM-102 cells were generally 60-80% confluent at the time of the cell:cell binding assay. The K562 target cells, labeled with $^{35}$S-methionine, were washed, resuspended in complete medium at $5 \times 10^5$ cells per ml, and added directly to the wells at $5 \times 10^4$ cells per well. The plates were incubated at $37^\circ C$ for 2.5 hours to allow for maximal binding. Medium was added to each well to produce a positive meniscus, and then plates were carefully sealed with adhesive plate sealers (Dynatech Labs, Inc.). The plates were inverted and centrifuged for 10 minutes at room temperature using Sorvall micro-plate carriers. A relative centrifugal force (RCF) of $900 \times g$ was used for most experiments. Post-centrifugation, still inverted plates were flash-frozen at $-80^\circ C$, and the bottoms of each well, containing the stromal cells and bound targets, were cut off and placed in scintillation vials for counting. The number of K562 cells bound per well was calculated as follows:

$$\text{CELLS BOUND/WELL} = \frac{\text{CPM BOUND TO STROMAL CELLS} \times (5 \times 10^6)}{\text{TOTAL CPM ADDED/WELL}}$$

Each group represents the mean of at least triplicate samples. Representative KM-102 stromal cell wells were harvested and counted to ensure that equivalent cell numbers were present in each well.
For antibody blocking experiments, antibodies were added directly to cell suspensions or to wells (as indicated) and incubated at room temperature for 30 minutes just prior to the addition of target cells to the wells. The final concentrations of antibodies used in the blocking studies were: rabbit anti-human M-CSF polyclonal antibody (Genzyme), 10 μg/ml; rat anti-c-fms/CSF-1 receptor (Oncogene Science, Inc.), 2 μg/ml; and mouse monoclonal anti-human transferrin receptor (Hybritech, Inc.), 8 μg/ml. Heat-inactivated normal rabbit serum (4 μl/1 x 10^5 cells) was added in each case to prevent the cross-linking of cells through Fc receptors.

9.D. RESULTS.

Our experimental strategy was predicated upon our ability to achieve high level stable expression of cell surface molecules using self-replicating EBV episomal expression vectors (Groger et al., 1989; Chapters 1-5). For these cell:cell binding studies we engineered paired cellular transfectants alternatively expressing membrane-associated M-CSF or M-CSFR. For the former, we stably transfected pM-CSF-DAF/REP4α into the SV40 large T-transformed human bone marrow stromal cell line KM-102
(Harigaya and Handa, 1985). Indirect immunofluorescence and flow cytometric analysis demonstrated high level surface expression of M-CSF on the pM-CSF-DAF/REP4α hyg² KM-102 transfectants (data not shown). No M-CSF was detectable on nontransfected KM-102 cells or KM-102 cells transfected with the irrelevant EBV episome RSVCATα/220.2 (Hauer et al., 1989). Immunoprecipitation, followed by SDS polyacrylamide gel electrophoresis under non-reducing conditions demonstrated that the M-CSF detected by indirect immunofluorescence in the pM-CSF-DAF/REP4α-transfected K562 cells is the predicted 70 kd, the predicted size of glycosylated, dimeric M-CSF (Fig. 9.2). A phosphatidylinositol-specific phospholipase C (PIPLC) cleavage experiment, with immunostaining of pM-CSF-DAF/REP4α hyg² K562 transfectants, untreated, or incubated for 1h in the presence or absence of PIPLC, demonstrates weak, but detectable, cleavage of M-CSF (Fig. 9.3), indicating that the M-CSF molecule does undergo GPL modification. Since DAF's GPL anchor was also relatively resistant in these K562 cells, it is likely that the M-CSF variant, like DAF, is largely GPL modified in these cells.

In parallel, we produced K562 transfectants stably expressing natural human M-CSFR on their surfaces. To
Fig. 9.2. Immunoprecipitation of M-CSF-DAF. Lysates from $^{35}$S cysteine-labeled K562 cells, transfected with either pM-CSF-DAF/REP4α or pRSVCATc/220.2, were immunoprecipitated using a rabbit polyclonal anti-M-CSF antibody. A protein of approximately 70 kd, present in the pM-CSF-DAF transfectants but not in the pRSVCATc/220.2 transfectants, is consistent with the predicted molecular weight of a glycosylated M-CSF dimer.
M-CSF-DAF IMMUNOPRECIPITATION
Fig. 9.3. PIPLC cleavage of DAF-tethered M-CSF. 10^6 K562 cells transfected with pM-CSF-DAF/REP4α were incubated in 0.5 ml complete medium (37°C) for 1h in the presence or absence of 1 unit PIPLC (Boehringer Mannheim). Cells were then immunostained for DAF (upper panels) using an anti-DAF monoclonal antibody, provided by E. Medof (CWRU), and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Miles) as a secondary antibody, or for cell surface-associated M-CSF (lower panels) using a rat monoclonal anti-M-CSF antibody (Oncogene Sciences) and fluorescein isothiocyanate-conjugated sheep anti-rat IgG (Miles) as a secondary antibody. Cells not treated with PIPLC are indicated by a solid line, cells treated with PIPLC are indicated by the stippled areas, and cells stained with non-specific mouse IgG are indicated by a dashed line. Cell number (vertical axis) is plotted against relative log fluorescence intensity (horizontal axis) for each figure.
PIPLC CLEAVAGE EXPERIMENT ON pM-CSF-DAF/REP4α TRANSFECTANTS

Stained for DAF

Stained for M-CSF
this end, pM-CSFR/REP7B, a human M-CSF receptor expression construct, was stably transfected into K562 cells; pRSVCATα/220.2 hyg^R K562 transfectants expressing the irrelevant enzyme chloramphenicol acetyltransferase (CAT), were used as controls.

We next used a cell:cell binding assay to assess the capacity of artificially tethered M-CSF to promote cellular adhesion to M-CSFR-bearing cells. Specifically, we determined whether the pM-CSF·DAF/REP4α KM-102 transfectants would bind preferentially to M-CSFR^+ cellular targets. Cell:cell binding between KM-102 cells and M-CSFR^+ K562 targets cells was enhanced approximately three-fold when M-CSF was present on the surface of the KM-102 cells (Fig. 9.4). This is specific for M-CSF expression, since KM-102 transfectants expressing high levels of CD8 on their surface (M. Weber and M. Tykocinski, unpublished observations) or an antisense IL-6 construct (pα-IL-6/REP5.1), demonstrated no enhanced binding to M-CSFR^+ targets. Moreover, no significant augmentation of adhesion was evident when nontransfected K562 cells, or K562 cells transfected with pRSVCATα/220.2, were used as cellular targets. Overall, K562 transfectants appear to be slightly more adhesive than nontransfected cells, with increased binding to
Fig. 9.4. Increased cell:cell binding mediated by M-CSF-DAF:M-CSFR. The binding of K562 cells (nontransfected, pM-CSFR/REP78 transfected, or pRSVcATα/220.2 transfected) to KM-102 stromal cells transfected with pM-CSF-DAF/REP4α is depicted as a function of K562 cells bound/well. Antibodies used in blocking studies to determine the specificity of the M-CSF-DAF:M-CSFR interaction are indicated. Anti-M-CSF, polyclonal anti-monocyte/macrophage colony stimulating factor antibody; anti-M-CSFR, monoclonal anti-monocyte/macrophage colony stimulating factor receptor antibody; anti-TFR, monoclonal anti-transferrin receptor antibody; NRS, normal rabbit serum.
the former.

To definitively establish that it is the membrane-associated M-CSF, and not secondarily expressed surface molecules on the pM-CSF-DAF/REP4α K562 transfectants, that is specifically responsible for the enhanced adhesion, we performed antibody blocking analyses. Prior incubation of M-CSFR+ K562 target cells with antibodies directed against the M-CSFR or alternatively, of surface M-CSFR+ KM-102 cells with polyclonal anti-M-CSF antibodies, each partially inhibited this specific cellular interaction (Fig. 9.2). By contrast, the simultaneous addition of these two antibodies, directed against both members of the ligand:receptor pair, completely blocked the specific binding. Normal rabbit serum, which did not inhibit binding, was included in all experiments to prevent the cross-linking of cells through Fc receptors expressed on the K562 cells (R. Looney, U. of Rochester, personal communication). The antibody-mediated inhibition that was observed was specific for the M-CSF:M-CSFR pair, since antibodies against the human transferrin receptor (hTFR), known to be expressed on K562 cells had no blocking effect.
9.E. DISCUSSION.

M-CSF has been known for some time to occur in a cell surface-associated form, but the full extent of the functional repertoire of this natural variant remains unclear. Various published reports have suggested roles for cell surface-associated M-CSF as a biosynthetic intermediate and as a cytokine, with activities paralleling those of its more intensively studies soluble counterpart. In this report, we provide evidence of an additional possible role for this natural M-CSF variant. Employing stable gene transfer and protein engineering technologies, along with our specific ability to express an artificially GPL-modified, membrane-binding M-CSF variant on cell surfaces, we have demonstrated that cell surface-associated M-CSF can function as a cellular adhesin, augmenting specific cellular binding.

In these investigations, we have introduced the principle that ligands classically associated (in their soluble forms) with signal transduction functions can also subserve cellular adhesion functions in their membrane-associated forms. Clearly, this may be applicable to other members of the growing set of naturally occurring membrane-associated cytokines. Cell:cell binding assays, conventionally used in the
literature for establishing adhesin function for other cell surface molecules, were used in these experiments, with high levels of ligand and receptor expressed on the respective cells. While the significance of these experimental observations for natural surface ligands at physiological concentrations in their native cellular setting remains to be determined, it is tempting to speculate that such cytokine:cytokine receptor interactions may play a significant role in the functional bridging of hematopoietic cells.

The membrane-binding form of M-CSF used in these studies was a GPL-modified genetically-engineered derivative of M-CSF, produced by chimeric gene transfection technology. One notable property of GPL-modified proteins is that, by virtue of their amphophilic properties, they readily incorporate into cell membranes in the presence of low, non-lytic concentrations of non-ionic detergents such as NP-40 (Medof et al., 1984). Hence, cells can be readily coated with GPL-modified proteins, bypassing the requirement for gene transfection into the cell whose surface is being molecularly engineered. In turn, coating cells (or liposomes) with GPL-modified cytokines offers a potential route for altering their adhesion, and perhaps even homing,
properties. Hence, the cytokine tethering technology that is introduced in this work has considerable pharmaceutical implications, from the standpoint of configuring cell- or liposome-based drug delivery systems.

In this study, we have used human bone marrow stromal cells as transfection targets. Recently, our group reported success of stable gene transfer into, and antisense RNA-mediated gene inhibition in, such cells (Weber, M., Gerson, S., and Tykocinski, M., manuscript submitted). We here provide another novel application for this ability to genetically engineer stromal cells. The ability to manipulate the adhesive properties of these cells offers the prospect of exploiting these cells in manipulating cellular interactions in the bone marrow compartment. In a sense this represents a first step towards the goal of what might be termed "stromal cytotherapy." In this case, we have used stromal cells as vehicles for delivering a cytokine/adhesin.

In a recent study, we have shown that a known cellular receptor/adhesin can unexpectedly function as a membrane-associated ligand. Specifically, CD8α, which has been extensively characterized with respect to its accessory (receptor/adhesin) molecular function, also
functions as an inhibitory ligand, mediating suppressive effects on specific T cell targets (Kaplan et al., 1989). The current report describes an inverse situation in which a molecule classically considered in terms of its ligand function can serve as an adhesin. These findings support a growing multifunctional perspective of cell surface molecules, suggesting that a broader view of the functional capacities of such molecules, encompassing ligand, receptor, enzymatic, and adhesin functions, is warranted.
LITERATURE CITED.


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