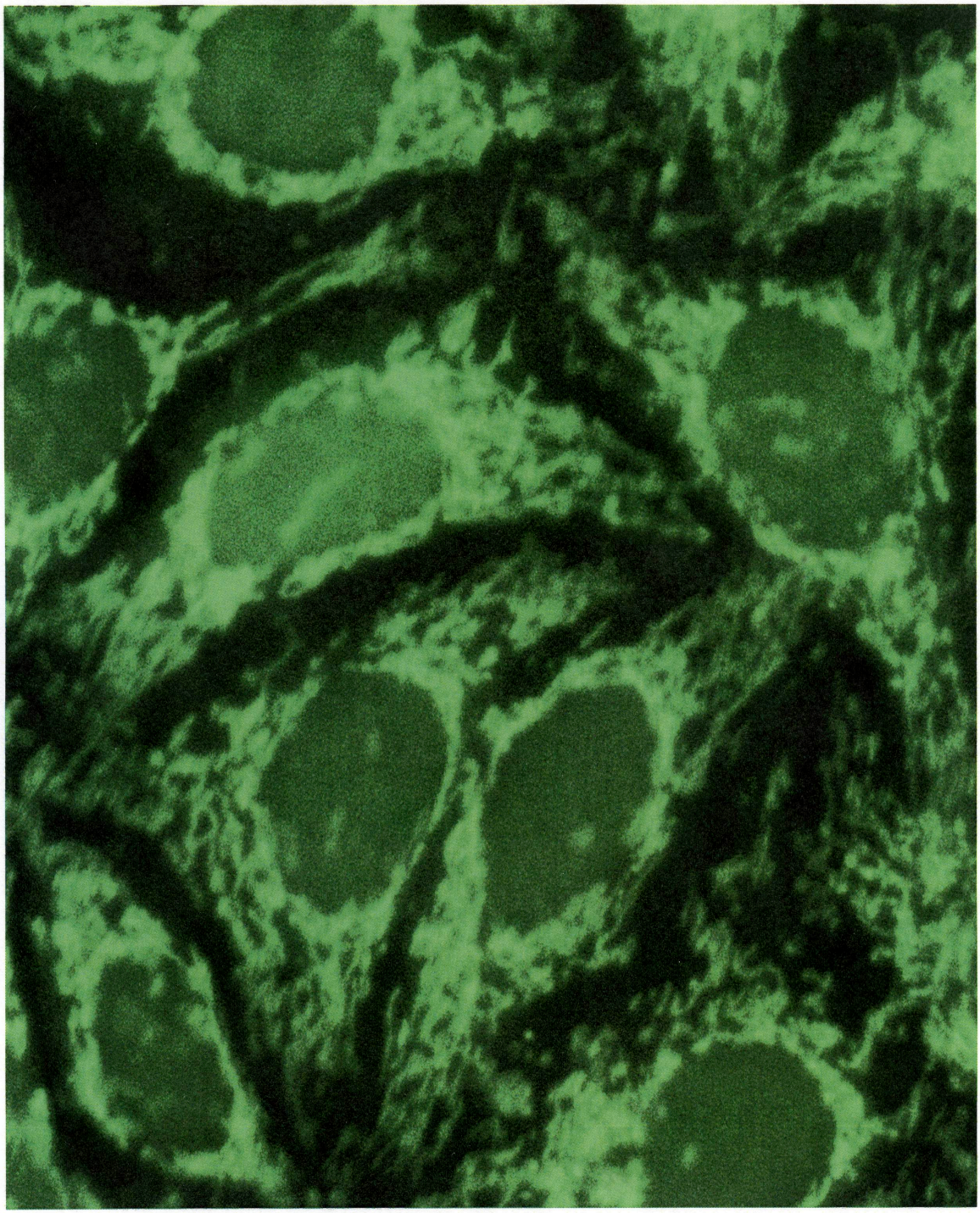
Cytochrome C-GFP



Materials Available:

GFP-cytochrome C Human Cytochrome C is cloned into the pEGFP-C1 plasmid (Clontech) resulting in the GFP being on the amino terminal of Cyto C. This plasmid was used in the reference below. PF195 / Addgene 41181

Cytochrome C-GFP We have since made another construct that has the GFP on the c-terminal of cyto C. Mouse Cytochrome C is cloned into the pEGFP-N1 plasmid (Clontech) resulting in the GFP being on the carboxy terminal of Cyto C. PF1521 / Addgene 41182

pBABE-Cytochrome C-GFP Cytochrome C-GFP was cloned into the retroviral vector pBabe-puro and is regulated by the LTR promoter. PF1552 Addgene 41183

pBABE-Cytochrome C-GFP Cytochrome C-GFP was cloned into the retroviral vector pBabe in place of the puro cassette such that cytochrome C-GFP is regulated by the SV40 promoter. PF1527 / Addgene 41184

We routinely grow the pBabe constructs in STBL2 or similar bacteria at 30 degrees since these are retroviral constructs. We find this reduces recombination.

Reference

Goldstein, J.C., Waterhouse, N.J., Juin, P., Evan, G.I., and Green, D.R. The co-ordinate release of cytochrome c during apoptosis is rapid, complete, and kinetically invariant. Nature Cell Biol, 2, 156-162, 2000.

Cloning Strategies

PF195 Addgene 41181

GFP-cytochrome C (kanamycin resistance). The Cc can be cut out of pEGFP-C1 (Clontech, PaloAlto, CA) as a 330bp EcoRI/BamHI fragment. The PF195 was used for the NCB paper referenced below. Be aware of the difficult time we had in generating a stably transfected cell line, not only expression level but also localization. We assume this is due to the GFP tag being on the N-terminal (in spite of the name Cyto C-GFP in the NCB paper).

PF1521 Addgene 41182

Cytochrome C-GFP (kanamycin resistance) We have since made another construct that has the GFP on the c-terminal. We also call this CYTOG. This is in the pEGFP-N1 vector. Mouse cytochrome c was amplified with the sense primer 5'-ACGTGTCGACCTAATATGGGTGATGTTGAAAAAGG and anti-sense primer 5'-ACAGATCTTTCTCATTAGTAGCCTTTTTAAG. The fragment was cloned into the pIB/V5-His Topo vector (Invitrogen, Carlsbad, CA) and then digested with SalI and BglII. This SalI/BglII fragment was ligated into pEGFP-N1 (Clontech, PaloAlto, CA) at the XhoI and BamHI sites.

PF1552 Addgene 41183

pBABE-CYTOG (ampicillin resistance, CYTOG under the LTR promotor). Cytochrome c-GFP (PF1521 above) was amplified with the sense primer 5'-GAGCTGGTTTAGTGAAC and antisense 5'-AGTAGAATTCCTACAAATGTGGTATG and cloned into pYes2.1/V5-His Topo (Invitrogen, Carlsbad, CA). This construct was verified by sequencing and digested with BglII and EcoRI. The resulting fragment was cloned into pBabe-puro at the BamHI and EcoRI sites in the multiple cloning region.

PF1527 Addgene 41184

pBABE-CYTOG (ampicillin resistance, CYTOG under the SV40 promotor). Cytochrome c -GFP (in PF1521 above) was amplified with the sense primer 5'-ACGTAAGCTTGCCACCATGGGTGATGTTGAAAAAGGCAAG and the anti-sense primer 5'AATCGATTTACTTGTACAGC and cloned into pcDNA3.1/V5-His Topo vector (Invitrogen, Carlsbad, CA). Cytochrome c-GFP was cut out with HindIII and ClaI and ligated into pBABE in place of the puro resistance cassette, which had been removed by a HindIII and ClaI co-digestion.

Reference

Goldstein, J.C., Waterhouse, N.J., Juin, P., Evan, G.I., and Green, D.R. The co-ordinate release of cytochrome c during apoptosis is rapid, complete, and kinetically invariant. Nature Cell Biol, 2, 156-162, 2000.

GFP-Human Cytochrome C

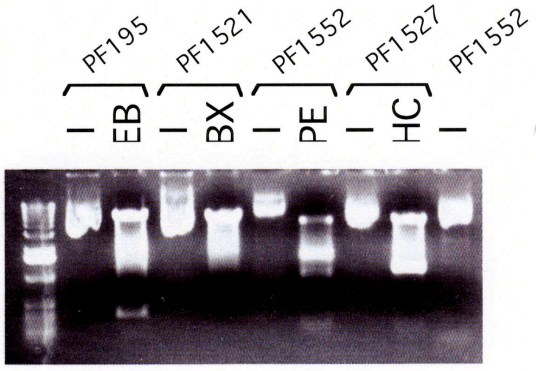
ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGAC
GTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAG
CTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACC
TACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAG
ATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAG
GCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGAC
GAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTAT
GCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGAC
GTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTG
AACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCAC
CTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTCC
AGATCTCGAGCTCAAGCTTCGAATTCAATGGGTGATGTTGAGAAAGGCAAGAAGATTTTT
AAGTGTTCCCAGTGCCACACCGTTGAAAAGGGAGGCAAGCACAAGACTGGGCCAAATCTC
CTCTTTGGGCGGAAGACAGGTCAGGCCCCTGGATACTCTTACACAGCCGCCAATAAGAAC
ATCATCTGGGGAGAGGATACACTGATGGAGTATTTGGAGAATCCCAAGAAGTACATCCCT
AAAATGATCTTTGTCGGCATTAAGAAGAAGGAAGAAAGGGCAGACTTAATAGCTTATCTC
GCTACTAATGAGTAA

Mouse Cytochrome C-GFP

ATGGGTGATGTTGAAAAAGGCAAGAAGATTTTTGTTCAGAAGTGTGCCCAGTGCCACAC
TGTGGAAAAGGGAGGCAAGCATAAGACTGGACCAAATCTCCACGGTCTGTTCGGGCGGA
AGACAGGCCAGGCTGCTGGATTCTCTTACACAGATGCCAACAAGAACAAAGGCATCACC
TGGGGAGAGGATACCCTGATGGAGTATTTGGAGAATCCCAAAAAGTACATCCCTGGAAC
AAAAATGATCTTCGCTGGAATTAAGAAGAAGGGAGAAAGGGCAGACCTAATAGCTTATC
TTAAAAAGGCTACTAATGAGAAAGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAG
GAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCA
CAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGA
AGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTG
ACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTT
CAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACG
GCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATC
GAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTA
CAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGG
TGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTAC
CAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAG
CACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGG
AGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGG

Digest 1 μg plasmid for 2 hours, 37°
Run on 1.2% agarose/TAE gel

PF195	GFP-Cytochrome C	EcoRI/BamHI
PF1521	Cytocrome C-GFP	BglII/XbaI
PF1552	(LTR)Cytocrome C-GFP	PstI/EcoRI
PF1527	(SV40)Cytocrome C-GFP	HindIII/ClaI



Transfection/Transduction of Cells

Keep in mind the great effort it took to generate the first GFP-Cytochrome C cells, not only expression levels but proper localization. This was probably due to the GFP being on the N-terminal of Cytochrome C and the fact the cloning of a stable cell line was done with circular plasmid. The circular plasmid no doubt increased transfection efficiency but the GFP-Cytochrome C coding sequence was probably disrupted upon integration into the host genome.

The retroviral constructs now allow the stable transduction of Cytochrome C-GFP into many actively dividing cell types. Still, your transduced cells will need to be sorted for expression levels and selected for proper localization. You may want to occasionally sort your cells for bright green.

The retroviral constructs have LTRs which seem to be sensitive to undesired recombination, even in RecA- bacteria. We routinely use STBL2 or an equivalent strain of bacteria to grow the retroviral constructs at 30 degrees. This seems to work very well.

Microscopy

The Hela cytochrome c-GFP cells can be viewed by confocal or fluorescent microscopy. We have successfully imaged these cells using the following microscopes

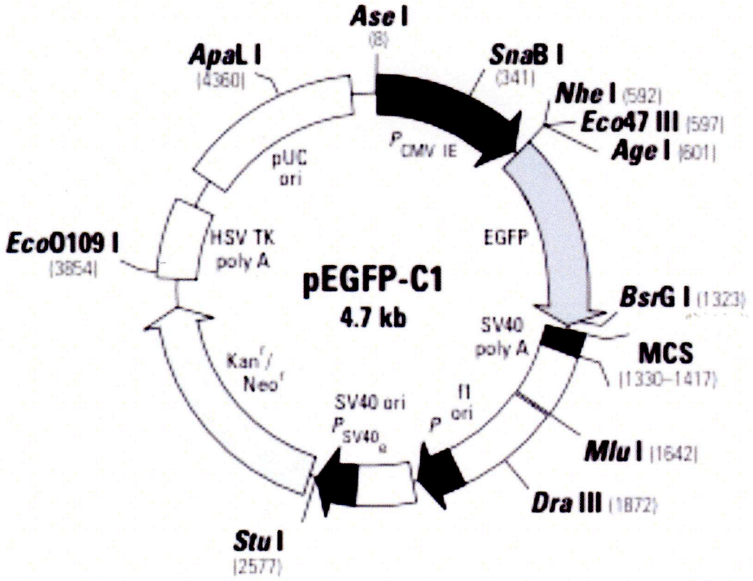
1) Nikon Eclipse TE 300 microscope and a Biorad 1024 confocal microscope. Cytochrome c-GFP is excited using a 488 nm line from an AR/Kr laser attenuated at 91%

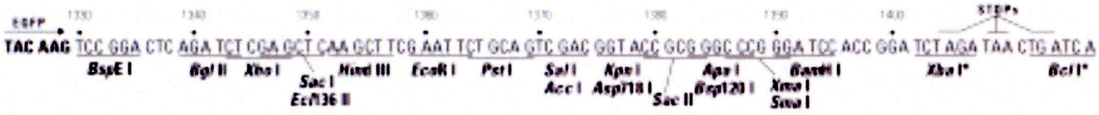
2) Zeiss Axiovert 200 microscope and a spinning disc confocal (intelligent imaging innovations) Cytochrome c-GFP was imaged using the GFP filter at 50% laser power and 500ms exposure time

3) Marianas fluorescent deconvolution microscope using the GFP filter of a mercury lamp

pEGFP-C1 Vector Information PT3028-5

GenBank Accession #: U55763 Catalog #6084-1





Restriction Map and Multiple Cloning Site (MCS) of pEGFP-C1. All restriction sites shown are unique. The *Xba* I and *Bcl* I sites (*) are methylated in the DNA provided by BD Biosciences Clontech. If you wish to digest the vector with these enzymes, you will need to transform the vector into a *dam*⁻ host and make fresh DNA.

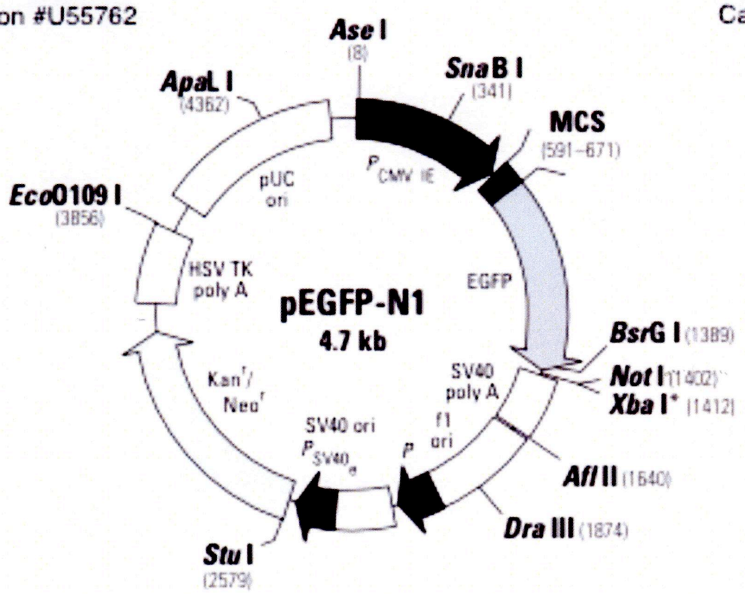
Description

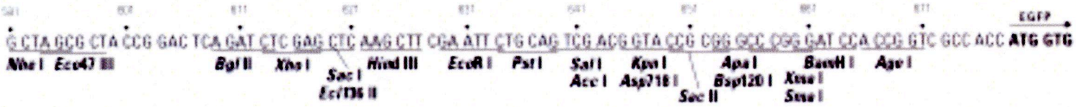
pEGFP-C1 encodes a red-shifted variant of wild-type GFP (1–3) which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nm; emission maximum = 507 nm.) pEGFP-C1 encodes the GFPmut1 variant (4) which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences (5). Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site (6) to further increase the translation efficiency in eukaryotic cells. The MCS in pEGFP-C1 is between the EGFP coding sequences and the SV40 poly A. Genes cloned into the MCS will be expressed as fusions to the C-terminus of EGFP if they are in the same reading frame as EGFP and there are no intervening stop codons. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3' end of the EGFP mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T-antigen. A neomycin resistance cassette (Neo^r), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette expresses kanamycin resistance in *E. coli*. The pEGFP-C1 backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

(PR29971; published 03 October 2002)

pEGFP-N1 Vector Information PT3027-5

GenBank Accession #U55762 Catalog #6085-1





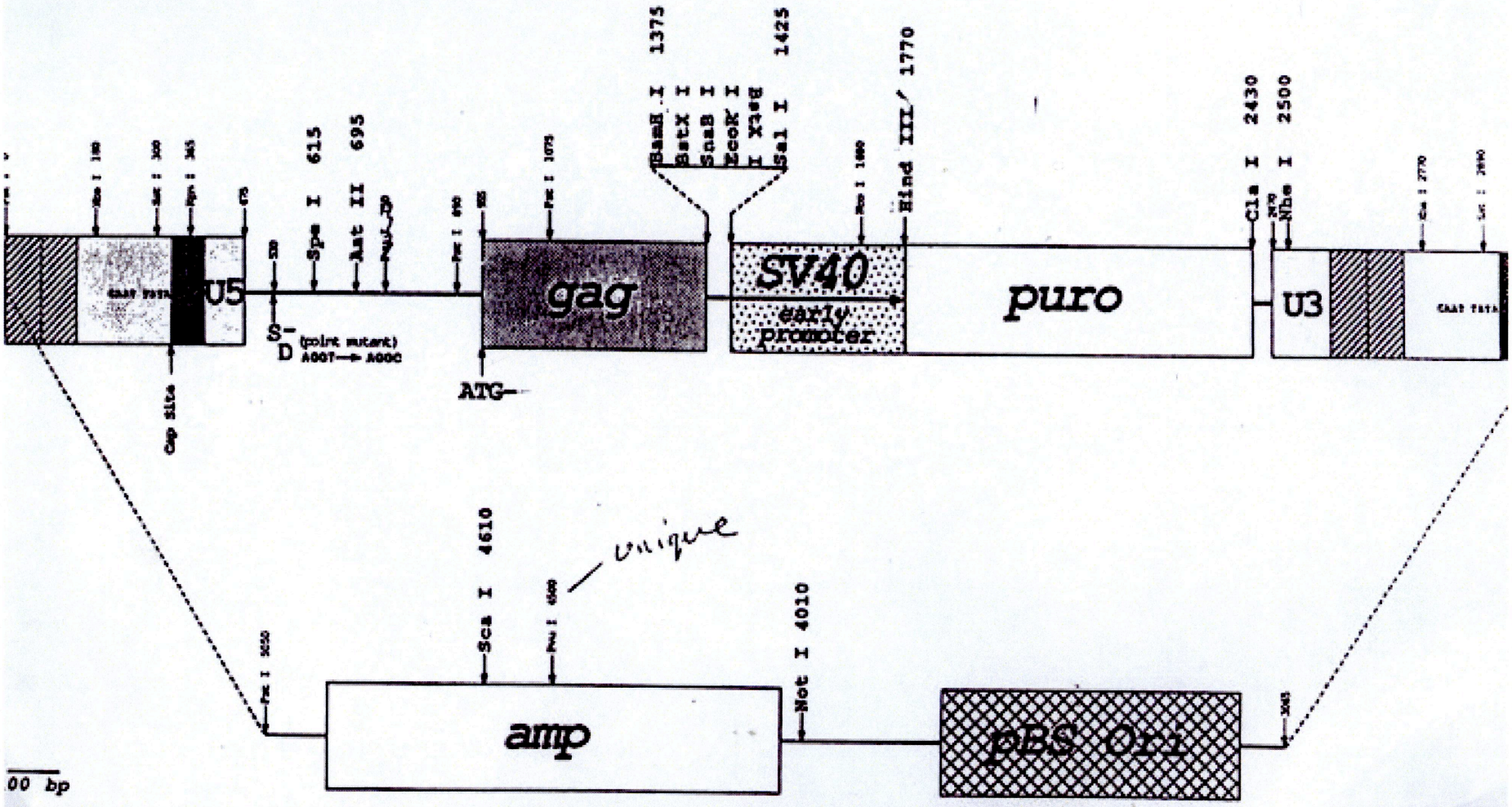
Restriction Map and Multiple Cloning Site (MCS) of pEGFP-N1 Vector. All restriction sites shown are unique. The *Not* I site follows the EGFP stop codon. The *Xba* I site (*) is methylated in the DNA provided by BD Biosciences Clontech. If you wish to digest the vector with this enzyme, you will need to transform the vector into a *dam*$^+$ and make fresh DNA.

Description

pEGFP-N1 encodes a red-shifted variant of wild-type GFP (1–3) which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nm; emission maximum = 507 nm.) pEGFP-N1 encodes the GFPmut1 variant (4) which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences (5). Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site (6) to further increase the translation efficiency in eukaryotic cells. The MCS in pEGFP-N1 is between the immediate early promoter of CMV ($P_{CMV\ IE}$) and the EGFP coding sequences. Genes cloned into the MCS will be expressed as fusions to the N-terminus of EGFP if they are in the same reading frame as EGFP and there are no intervening stop codons. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3' end of the EGFP mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T antigen. A neomycin-resistance cassette (Neo^r), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette expresses kanamycin resistance in *E. coli*. The pEGFP-N1 backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

(PR29972; published 03 October 2002)

pBabe Puro (5.1 kb)



Bold Print: unique restriction sites

Fine Print: multiple restriction sites

Select for constructs derived from this vector in Ampicillin (100µg/ml)

Select cells infected in culture in 1.0-2.5 µg/ml Puromycin