Plasmid construction

Yeast expression plasmids contained either the complete (GALL) or crippled (GALS) Gal1/10 promoter, for galactose inducible/glucose repressible transgene expression [1-2], and cassette containing a centromere sequence and autonomously replicating sequence for stable copy number control [3]. The following plasmids have been previously described: pGALL-(*LEU2*), pADH-(*TRP1*)-P35 [4] ; Caspase-3-lacZ [5] ; pGALL-(*TRP1*)-MCS, pGALL-(*TRP1*)-MCS-Bcl-2 [6]; pGALL-(*LEU2*)-caspase-1 [7]; pGALL-(*LEU2*)-caspase-8 [8], pEF-FLAG-Bcl-x_L [9], pEF-FLAG-crmA^{T291R} [10]. Other plasmids were generated as outlined below. All non-viral genes were human and the inserts in all constructs were verified by sequencing. Phosphorylated oligonucleotides 1345 and 1346 were annealed, then ligated into pGALL-(*LEU2*) which had been cut with BamHI and phosphatased, to produce pGALL-(*LEU2*)-FLAG. Human Bax was amplified using primers 905 and 1577, cut with BglII and XbaI and ligated into pGALL-(*LEU2*)-FLAG cut with BamHI and XbaI. The coding region of Bcl-x_L was initially amplified using primers 626 and 627, cut with BglII and XbaI and cloned into F-pIRESneo [9], then excised using XbaI (blunted with Klenow) and EcoRI and ligated into pADH(*TRP1*), then excised using EcoRI/XhoI and ligated into pGALL(TRP1)-MCS to produce pGALL-(*TRP1*)-MCS-Bcl-xL. The Bcl-w coding region was amplified with primers 842 and 843, cut with EcoRI and NotI and cloned into pGALL-(*TRP1*)-MCS to yield pGALL-(*TRP1*)-MCS-Bcl-w. Custom DNAs for human Mcl-1 and A1 were ordered from Genscript (Piscataway, New Jersey, USA). The coding regions were excised with EcoRI and either PstI (Mcl-1) or NotI (A1) and ligated into pGALL-(*TRP1*)-MCS to produce pGALL-(*TRP1*)-MCS-Mcl-1 and pGALL-(*TRP1*)-MCS-A1. The truncated GALS promoter and terminator [2] were excised using KpnI and BamHI and cloned into pGALL-(*TRP1*) to yield pGALS-(*TRP1).* E1B19K was amplified from a plasmid donated by Paul Ekert using

primers 1003 and 1004, cut with EcoRI and NotI and cloned into pGALL(*TRP1*)-MCS to give pGALL-(*TRP1*)-E1B19K. The insert was then released by cutting with BamHI and SphI and ligated into pGALS-(*TRP1*) to give pGALS-(*TRP1*)-E1B19K. The DPV022 and SPPV14 coding regions were removed from the pGALL(*TRP1*) plasmids [11-12] using BamHI and SphI and ligated into pGALS-(*TRP1*) to produce pGALS-(*TRP1*)-DPV022 and pGALS-(*TRP1*)-SPPV14. The CrmA gene was amplified from pEF-Puro-CrmA [13] with primers 310 and 311, then cut with EcoRI and NotI and cloned into pGALL-(*HIS3*) [2] to yield pGALL-(*HIS3*)-CrmA. HA-Puma was excised from HA-Puma-pEF [14] (provided by Paul Ekert) with BglII and XbaI and ligated into pGALL-(*URA3*) [5] which had been cut with BamHI and XbaI to produce pGALL-(*URA3*)-HA-Puma. GFP^{S65T}-nostop-pGALL(*TRP1*)-MCS was made by amplifying GFP^{S65T} from p416 MET25 GFP^{S65T} (a kind gift from Trevor Lithgow) using primers 1321 and 1548, cutting the product with BglII and SacII, then ligating it into pGALL-(*TRP1*)-MCS digested with BamHI and SacII. Genes encoding pro-survival Bcl-2 relatives were subcloned out of pGALL(*TRP1*)-MCS vectors into this plasmid as EcoRI/XhoI (Bcl-x_L, Bcl-w), EcoRI/ PstI (Mcl-1) or EcoRI/NotI (A1) fragments. Oligonucleotides 1591 and 1592 were annealed and ligated into EcoRI/XbaI-digested pMAL-c2X (New England Biolabs, Ipswich, Massachusetts, USA) to introduce His_6 and FLAG tags downstream of MBP. The gene encoding this fusion product was amplified using primers 1599 and 1600 and ligated into pGALL(URA)-2µ, which was created by replacing the PvuI/PvuI fragment containing the CEN6/ARSH4 cassette of pGALL(*URA3*) with the corresponding 2µ ori cassette from pRS426 [15]. FLAG-pEF was made by ligating annealed oligonucleotides 1541 and 1542 into pEF-puro [13] cut with BamHI and XbaI. BamHI/XbaI inserts of Bcl-2, Mcl-1, Bcl-w and A1 (partial digest products were used where

necessary) were ligated into FLAG-pEF cut with BamHI and XbaI, to express FLAG-tagged proteins in mammalian cells.

The following oligonucleotides were used for cloning:

310: 5’GGAATTCAGATCTATGGATATCTTCAGGGAAATCGC3’

311: 5’GCACTAGTGCGGCCGCTTAATTAGTTGTTGGAGAGCAATATC3’

626 : 5’GCAGATCTATGTCTCAGAGCAACCGGG3’

627: 5’GCTCTAGACTATTTCCGACTGAAGAGTG3’

842: 5’GGAATTCATGGCGACCCCAGCCTCGGC3’

843: 5’CCCCGCGGCCGCTCACTTGCTAGCAAAAAAGGC3’

905: 5’GCAGATCTATGGACGGGTCCGGGGAGCAG3’

1003: 5’GGAATTCATGGAGGCTTGGGAGTGTTTG3’

1004: 5’GAATGCGGCCGCTCATTCCCGAGGGTCCAGGC3’

1321: 5’GCGAGATCTATGGGTAAAGGAGAAGAACTTTTC3’

1345: 5’GATCATGGACTACAAGGACGACGATGACAAGG3’

1346: 5’GATCCCTTGTCATCGTCGTCCTTGTAGTCCAT3’

1541: 5’GATCACCATGGACTACAAGGACGACGATGACAAGGGATCCGCAAGCTT GCT3’

1542: 5’CTAGAGCAAGCTTGCGGATCCCTTGTCATCGTCGTCCTTGTAGTCCATGGT3’

1548: 5’GCTGCACCGCGGAATTCGGATCCTTTGTATAGTTCATCCATGCCATG3 ‘

1577: 5’CTTTATTATTTTTATTTTATTGAGAGGGTGG3’

1591: 5’AATTCATGGACTACAAGGACGACGATGACAAGGGATCCCATCATCATCA TCATCACTAAT3’

1592: 5’CTAGATTAGTGATGATGATGATGATGGGATCCCTTGTCATCGTCGTCCTTG

TAGTCCATG3’

1599: 5’GTCGGATCCATGAAAATCGAAGAAGGTAAAC3’

1600: 5’ TTAAGTTGGGTAACGCCAG3’

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