Published online 20 February 2007

Nucleic Acids Research, 2007, Vol. 35, No. 6 ***e43***
doi:10.1093/nar/gkm067

Vectors for co-expression of an unrestricted number of proteins

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Received November 10, 2006; Revised January 17, 2007; Accepted January 23, 2007

ABSTRACT

A vector system is presented that allows generation of *E. coli* co-expression clones by a standardized, robust cloning procedure. The number of co-expressed proteins is not limited. Five 'pQLink' vectors for expression of His-tag and GST-tag fusion proteins as well as untagged proteins and for cloning by restriction enzymes or Gateway cloning were generated. The vectors allow proteins to be expressed individually; to achieve co-expression, two pQLink plasmids are combined by ligation-independent cloning. pQLink co-expression plasmids can accept an unrestricted number of genes. As an example, the co-expression of a heterotetrameric human transport protein particle (TRAPP) complex from a single plasmid, its isolation and analysis of its stoichiometry are shown. pQLink clones can be used directly for pull-down experiments if the proteins are expressed with different tags. We demonstrate pull-down experiments of human valosin-containing protein (VCP) with fragments of the autocrine motility factor receptor (AMFR). The cloning method avoids PCR or gel isolation of restriction fragments, and a single resistance marker and origin of replication are used, allowing over-expression of rare tRNAs from a second plasmid. It is expected that applications are not restricted to bacteria, but could include co-expression in other hosts such as Bacluovirus/ insect cells.

INTRODUCTION

Co-expression of subunits is an important technique in the study of protein complexes. Proteins that form tight complexes with partners may not be soluble when over-expressed alone (1). Proteins that form stable homodimers may only form heterodimers upon co-expression, but not by mixing the purified complex partners (2,3). The Protein Structure Factory has established high throughput protein expression procedures for human proteins in *E. coli* (4,5). To achieve co-expression in a systematic way we have created a new vector system described here.

Co-expression is often achieved with two or more plasmids, each carrying the gene of one subunit and a different selection marker (6). The plasmids should also have different compatible replicons, although co-expression with plasmids that have the same replicon has been demonstrated (7). Instead of multiple vectors, several genes can be inserted into the same vector. This requires a vector with more than one multiple cloning site and the use of different sets of restriction enzymes for each cloning step (8). Genes are transcribed either from individual promoters or from a single promoter, leading to a long polycistronic mRNA. Polycistronic expression has been reported to lead to lower expression of the more downstream encoded protein, which can be exploited to influence the stoichiometry of a protein complex (9). Several-fold higher expression with individual promoters compared to polycistronic transcription has been reported (10). A recent evaluation of co-expression experiments performed by the Structural Proteomics in Europe (SPINE) consortium compared examples of co-expression with various systems (11).

The pETDuet system of Novagen allows construction of a co-expression plasmid for two genes in a single PCR step. Alternative approaches to co-expression from a single vector require different sets of restriction enzymes for multiple insertions. Berger *et al.* have developed a Baculovirus co-expression system that allows iterative addition of genes to a Baculovirus vector (12). Alexandrov *et al.* have developed a vector with a 'LINK' sequence that allows fusion of two plasmids into one (13). The method allows cloning of all cDNAs of interest into one standard expression vector

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by a standardized, high-throughput procedure. Then, co-expression plasmids are generated by fusion of LINK sequences with the ligation-independent cloning method (LIC) that avoids PCR, which may introduce mutations. Two alternative rare cutting restriction enzymes are used. Inserts to be cloned are unlikely to contain both sites. The method is limited to co-expression of two proteins.

We have designed a modified version of the LINK sequence cloning technique. Vectors with two modified LINK sequences were created that allow the creation of co-expression plasmids by the established LINK cloning procedure but avoid the duplication of the vector backbone. In principle, the vectors can accept an unlimited number of genes to be co-expressed.

To demonstrate the usefulness of the method, co-expression and binding of VCP and the C-terminus of AMFR (14,15) is demonstrated. In addition, the co-expression of four subunits of the human transport protein particle (TRAPP) (16) from a single plasmid and an analysis of their stoichiometry is shown.

MATERIALS AND METHODS

Vector construction

The vector pQTEV2 was constructed from pQE-2 (Qiagen) by replacing the multiple cloning site with synthetic oligonucleotides to introduce a TEV protease site and attB Gateway recombination sites. To obtain pQLinkH, LINK sequences were inserted by PCR amplification of bp 2–379 of pQTEV2 with the primers 5′-TTTCTCGAGC TTAATTAACA ACACCATTTG TCGAGAAATC ATAAAAAATT TATT-3′ and 5′-TTTTGCTAGC CTTAATTAAC CACTCCATTT GTAACCACTC CATTTAAATG GTGTTGTTAC TTGGATTCTC ACCAATAAAA A-3′ and cloning of the product into pQTEV2 using XhoI and NheI. To construct pQLinkG, a GST coding sequence was PCR amplified from pGEX-5X-1 (GE Healthcare) with primers carrying EcoRI and PstI restriction sites. The fragment was cloned between the EcoRI and PstI sites of pQLinkH, thereby removing the His-tag. pQLinkHD and pQLinkGD were obtained from pQLinkH and pQLinkG—which contain attB sites—by Gateway BP reactions with pDONR221, which inserted the Gateway destination vector cassettes. pQLinkHD and pQLinkGD confer both ampicillin and chloramphenicol resistance and require the strain DB3·1 for propagation. The vector pQLinkN without a tag was generated from pQLinkH using QuikChange™ site-directed mutagenesis (17) with the double-stranded oligonucleotide 5′-AAAGAGGAGA AATTAACTAT GGGATCCAGT CTTCGCATGC AT-3′. The sequences of the new vectors were verified by sequencing and were submitted to GenBank (EF025686–EF025689, EF196676).

Cloning of expression clones

The *E. coli* strain SCS1 with the helper plasmid pRARE for over-expression of rare tRNAs (18) was used as a host for cloning of expression and co-expression clones, unless indicated otherwise. The VCP coding sequence (GenBank NP_009057) was cloned into pQLinkH and AMFR C-terminal regions (GenBank NP_001135) and into pQLinkG (AMFR) with BamHI and NotI. BamHI/NotI fragments of full-length cDNAs encoding four human TRAPP subunits were cloned into pQLinkH. The Bet3 (GenBank AF041432) and Mum2 (AAD44697) full-length cDNAs lack the native start and stop codons and were cloned with the BamHI site adjacent to the second codon and with a stop codon downstream of the NotI site, which encodes for three additional C-terminal alanine residues. Full-length Tpc6 (CAD61947) and synbindin (AAH10866) cDNAs were cloned with the BamHI site adjacent to the native start codon and with a TGA stop codon replacing the native stop codon. The Bet3:Tpc6 pETDuet-1 clone was described previously (2).

Cloning of co-expression clones

Co-expression plasmids were cloned according to Alexandrov *et al.* (13). To produce a co-expression plasmid from two pQLink plasmids, 0.2–0.5 μg of one plasmid was digested overnight with 5 units PacI in 10 μl at 37°C while the other was cleaved with 5 units SwaI at 25°C. GST-plasmids were digested with PacI because the GST coding region contains a SwaI site. Enzymes were inactivated at 65°C for 20 min and 5 μl DNA was treated with 1.3 units LIC qualified T4 DNA polymerase (Merck Biosciences) in 20 μl (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 μg/ml BSA, 5 mM DTT, 2.5 mM dCTP for the PacI digest and 2.5 mM dGTP for the SwaI digest). Upon incubation for 30 min at 25°C and heat inactivation at 65°C for 20 min, the two plasmids were mixed and heated to 65°C and cooled to room temperature for annealing. Two microlitres of 25 mM EDTA were added, followed by *E. coli* transformation. Transformants were tested for expected inserts by colony PCR with primers pQTEV3U, 5′-TATAAAAATA GGCGTATCAC GAGG-3′ and pQTEV3L, 5′-CCAGTGATTT TTTTCTCCAT TTT-3′ and 59°C annealing temperature. These primers are positioned upstream of the LINK1 and downstream of the LINK2 sequences, respectively.

Co-expression of AMFR and VCP and GST pull down

His-VCP:GST-AMFR co-expression plasmids were expressed in 2 l with 1 mM IPTG for 4 h at 16°C. Cells were harvested, resuspended in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM β-mercaptoethanol, 1 mM PMSF and 1 tablet/50 ml lysate of Roche complete EDTA-free protease inhibitor cocktail and lysed via incubation with 0.5 mg/ml lysozyme for 30 min on ice, followed by addition of 8 units/ml benzonase and treatment with an Avestin Emulsiflex C5 homogenizer. Insoluble material was removed by centrifugation (30 min, 75 000 g) and the lysate was loaded on a glutathione sepharose column. The column was washed with wash buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated overnight with 0.1 mg/ml TEV protease in wash buffer at 8°C. Proteins mobilized by the protease were collected and analysed by SDS-PAGE.

Co-expression and pull down with nickel chelating beads

Protein expression and purification were performed as described (19). Briefly, proteins were expressed in 1 ml cultures with 1 mM IPTG at 37°C (TRAPP proteins) or 16°C (AMFR and VCP). His-tag fusion proteins and their bound interaction partners were immobilized on Ni-NTA agarose (Qiagen), washed with 20 mM Tris-HCl, pH 8.0, 300 mM NaCl and 20 mM imidazole and then eluted with 70 µl 500 mM imidazole in wash buffer. Six-microlitre aliquots of eluate were analysed by SDS-PAGE.

BL21 (DE3) *E. coli* clones with pETDuet-1 Bet3:Tpc6 (2) and pQLinkH Bet3:pQLinkN Tpc6 plasmids were cultured in 50 ml and protein expression was induced with 1 mM IPTG at $OD_{600} \sim 0.7$ for 4 h.

Purification and characterization of TRAPP subcomplexes

TRAPP co-expression plasmids were expressed in 0.5 l overnight express™ instant TB medium (Novagen). Cells were lysed using a French Press (SLM-AMINCO) and purified using Ni-NTA agarose (Qiagen), according to the manufacturer's instructions. Gel filtration was performed on a Superdex 200 26/60 column (GE Healthcare) equilibrated with 20 mM Tris-HCl pH 7.5, 200 mM NaCl and 2 mM DTT.

RESULTS

Vector construction

We have constructed five vectors for protein expression in *E. coli* that allow preparation of co-expression constructs by a simple cloning procedure (Figure 1). The vectors contain two LINK sequences (13) that flank the expression cassette of promoter, multiple cloning site and a transcriptional terminator. LINK1 contains a PacI restriction site and LINK2 a SwaI and a PacI site (Figure 1A). The LINK sequences allow insertion of a PacI fragment of one plasmid at the SwaI site of another plasmid by LIC (Figure 1B).

The vectors pQLinkH and pQLinkG have a His-tag and a GST-tag, respectively, followed by a TEV protease site and a multiple cloning site. The vector pQLinkN is used for expression of proteins without a tag. It contains a start codon adjacent to the BamHI site and can be used to express a protein complex with only one tagged subunit for isolation. In the vectors pQLinkHD and pQLinkGD, the TEV site and the multiple cloning site of pQLinkH and pQLinkG were replaced by a cassette for Gateway recombination cloning. The vectors pQLinkH and pQLinkG contain Gateway attB sites and inserts can be shuttled by a reverse Gateway reaction into a pDONR donor vector to obtain Entry clones. Vector sequences were submitted to GenBank (EF025686–EF025689, EF196676) and vectors will be available from Addgene.

The cloning of co-expression plasmids by LIC was very robust in our hands and about 90% of transformants contained the expected insert. Correct clones are easily recognized by the large product size of colony PCRs.

Co-expression of VCP and AMFR

The GST coding sequence contains a SwaI site, which requires that pQLinkG clones are cut with PacI and not with SwaI when generating a co-expression clone. To circumvent this limitation, pQLinkG2 was generated by introducing a silent mutation that removes this site in pQLinkG. To study the interaction of VCP and AMFR, a human VCP cDNA was cloned into pQLinkH and three C-terminal fragments of the 643 amino acid coding region of AMFR were cloned into pQLinkG. These fragments comprise the Cue domain (amino acids 450–510), the downstream region up to the C-terminus (498–643) and a combination of both (455–643). Previously, the larger constructs were found to interact with VCP, but not with amino acids 450–510 (15). His-VCP:GST-AMFR co-expression clones were obtained by inserting a PacI fragment of the VCP plasmid into the LINK2 sequence of the AMFR pQLinkG plasmids. Protein expression was induced and the expression products of two His-VCP:GST-AMFR co-expression clones were coupled to a glutathione agarose column. The column was treated with TEV protease to separate AMFR and GST and elute AMFR and any attached proteins. Co-expression was successful and, as shown in Figure 2A, the longer AMFR fragment bound to His-VCP while the shorter one did not.

Reverse pull-down experiments were also performed by coupling His-VCP to nickel chelating beads. Co-purification with this matrix was generally less efficient than with glutathione agarose. Co-purified bands were observed upon Coomassie staining and anti-GST western blotting for the AMFR constructs 456–643 and 498–643 (Figure 4B). Only a weak background of co-purified GST and of the small AMFR construct was observed. These results indicate that amino acids 498–643 of AMFR, the region downstream of the Cue domain, are responsible for the interaction with VCP, as it was found by Grelle *et al.* (15).

Purification and characterization of TRAPP subcomplexes

Upon construction of a co-expression plasmid, only the outer LINK sequences reconstitute (Figure 1B). Therefore, it is possible to continuously increase the number of genes by combining plasmids that carry any number of expression cassettes. We have constructed a co-expression plasmid for four subunits of the human TRAPP complex. Bet3, Tpc6, Mum2 and synbindin were cloned into pQLinkH, and the expression of individual proteins was tested by small-scale expression and metal chelate affinity chromatography (Figure 3A). A Bet3 and Tpc6 co-expression plasmid was constructed and a several-fold increase in the yield of purified Tpc6 was achieved (Figure 3A). This result is in agreement with earlier studies that showed that the Bet3:Tpc6 heterodimer is likely to represent the physiologically relevant form of both proteins (2). Mum2 was included in the plasmid, and finally a plasmid with all four subunits, including synbindin, was constructed. The yield of purified Mum2 was also increased upon co-expression. All four subunits of the complex were successfully co-expressed and the

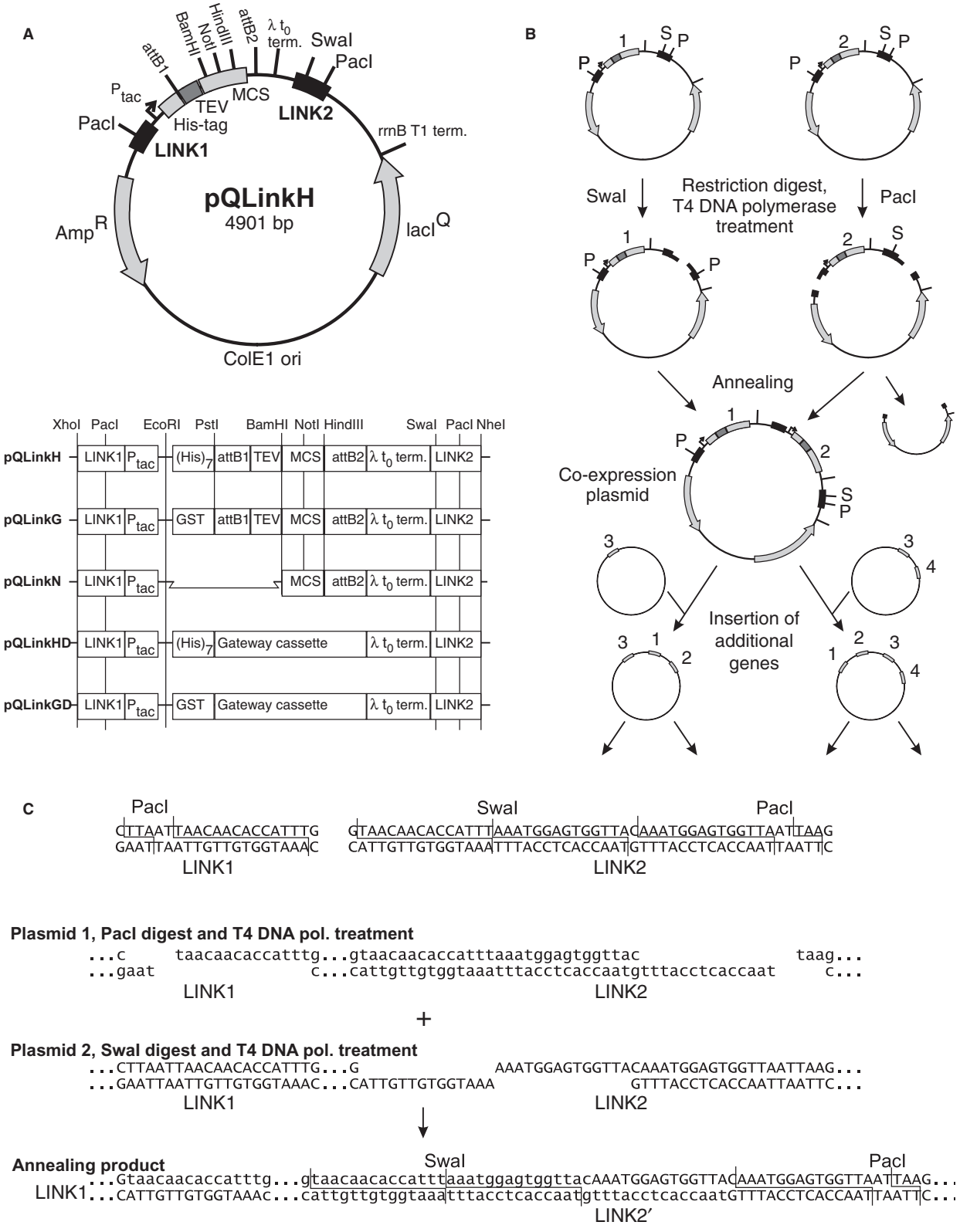

Figure 1. Map of the pQLink vectors and construction of co-expression plasmids. (**A**) Map of vector pQLinkH and genetic elements of all pQLink vectors. The LINK sequences are shown with lines indicating overhangs generated by restriction digest and T4 DNA polymerase treatment. MCS = multiple cloning site, TEV = TEV protease cleavage site, term. = transcription terminator. (**B**) Construction of a co-expression plasmid from two pQLink plasmids with two different cDNA inserts, labelled 1 and 2. The resulting plasmid can accept additional inserts, labelled 3 and 4. S = SwaI, P = PacI. (**C**) The LINK sequences and their digestion and annealing. The lines indicate overhangs generated by restriction digest and T4 DNA polymerase treatment. Two plasmids, identified by upper and lower case nucleotide codes, are digested and annealed, leading to a product with a LINK1 and a LINK2′ sequence, slightly larger than the original LINK2 sequence.

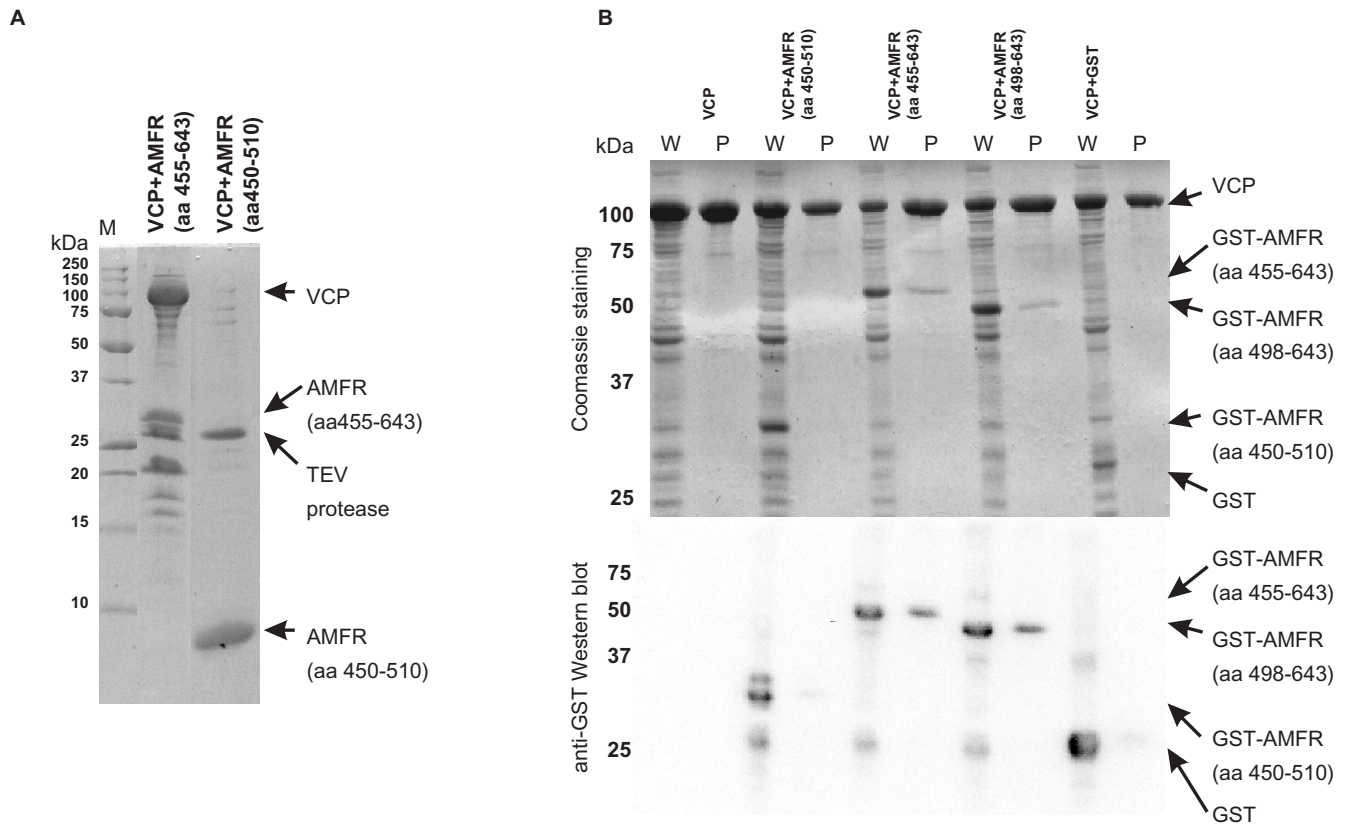

Figure 2. Co-expression of His-tagged VCP with GST-tagged AMFR fragments. SDS-PAGE and Coomassie staining of whole cells solubilized in SDS-PAGE sample buffer (W) and proteins purified under non-denaturating conditions (P) (**A**) Proteins were eluted from a glutathione agarose column by TEV protease treatment and analysed by SDS-PAGE and Coomassie staining. Degradation products of the larger AMFR construct were observed. W = whole cellular protein extract, P = purified proteins, M = molecular weight marker. (**B**) Protein products of co-expression clones of His-VCP with GST-AMFR and GST alone were purified with nickel chelating beads and separated by SDS-PAGE and visualized by Coomassie staining (upper panel) or anti-GST western blot (lower panel, reagents: anti-GST HRP conjugate, GE-Healthcare, and Western Lightning Western Blot Chemiluminescence Reagent, Perkin Elmer).

yield of purified protein was equal to or better than that for the expression of the individual proteins.

We compared the expression achieved by our system to the pETDuet co-expression system. The plasmids pETDuet-1 Bet3:Tpc6 and pQLinkH Bet3:pQLinkN Tpc6 both encode for His-tagged Bet3 and untagged Tpc6 protein. The expression clones showed good expression of both proteins, with comparable protein levels in both systems (Figure 3B). Bet3 from pQLinkH was more strongly expressed and migrated at a higher molecular weight because of the TEV cleavage site not present in pETDuet-1.

With expression constructs containing up to four subunits in hand, we used the pQLink system to study the assembly and stoichiometry of TRAPP subcomplexes. Combinations of Bet3:Tpc6, Bet3:Tpc6:Mum2 and Bet3:Tpc6:Mum2:synbindin were expressed and affinity purified using nickel chelating beads. Samples were then analysed by gel filtration to isolate the protein subcomplexes (Figure 4A). As previously described, Bet3 and Tpc6 form a heterodimer, which binds Mum2 (2). The isolated Bet3:Tpc6:Mum2 complex exhibited a molecular weight of ~90 kDa. A heterotrimer would have a molecular weight of only 65 kDa, which suggests that the observed complex contains a fourth TRAPP polypeptide. It might result from binding of a Mum2 dimer to a Bet3:Tpc6 heterodimer, since Mum2 alone forms homodimers (unpublished observation). Upon including synbindin in the co-expression system, a complex of similar size was formed, corresponding to a 85-kDa tetramer containing one molecule each of Bet3, Tpc6, Mum2 and synbindin. This tetrameric complex could be isolated and crystallized (not shown).

DISCUSSION

The vectors described here allow generation of expression clones for His-tag and GST-tag fusion proteins and untagged proteins by restriction enzyme or Gateway recombination-based methods. The vectors are capable of expressing single proteins and can be converted into co-expression plasmids by a simple, PCR-free LIC reaction that is suitable for high-throughput projects. Any combination of pQLink plasmids is possible.

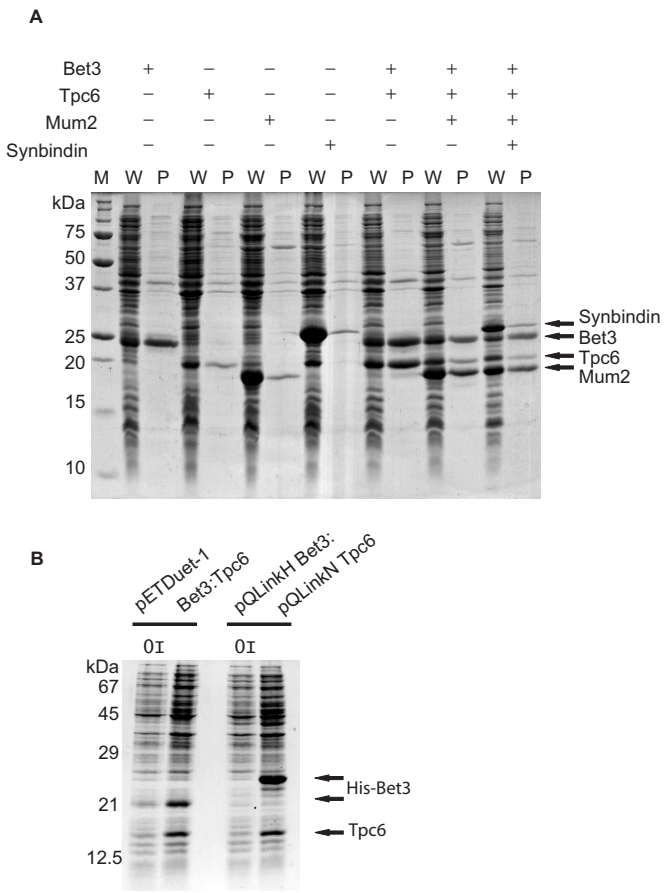

Figure 3. Co-expression of four subunits of the human TRAPP complex. (**A**) Protein expression products and nickel affinity chromatography products of seven clones expressing different subunits were analysed by SDS-PAGE and Coomassie staining. W = whole cellular protein extract, P = purified proteins, M = molecular weight marker. (**B**) Expression test with pETDuet-1 Bet3:Tpc6 and pQLinkH Bet3:pQLinkN Tpc6 in BL21 (DE3). Total cellular protein was analysed before (0) and after (I) 4-h induction.

The use of different tags or the combination of tagged and untagged complex subunits facilitates the production of homogenous complex preparations.

The presence of an internal SwaI or PacI site in the gene of interest would prevent the use of the system. However, SwaI and PacI are rarely cutting enzymes with eight base pair recognition sites, and one is free to choose either PacI or SwaI for a given gene. Therefore we expect that almost all genes of interest can be converted into co-expression clones. The comparison of yields showed that this expression system works equally well as the pETDuet system.

The specific interaction of a C-terminal region of AMFR with VCP was confirmed. Co-expression of the four TRAPP subunits Bet3, Tpc6, Mum2 and synbindin was demonstrated. Figure 3 shows that co-expression generally does not decrease the yield of individual proteins. On the contrary, all four proteins were obtained with similar or increased yield upon co-expression and affinity purification. Gel filtration revealed that the four TRAPP subunits form a heterotetramer containing one

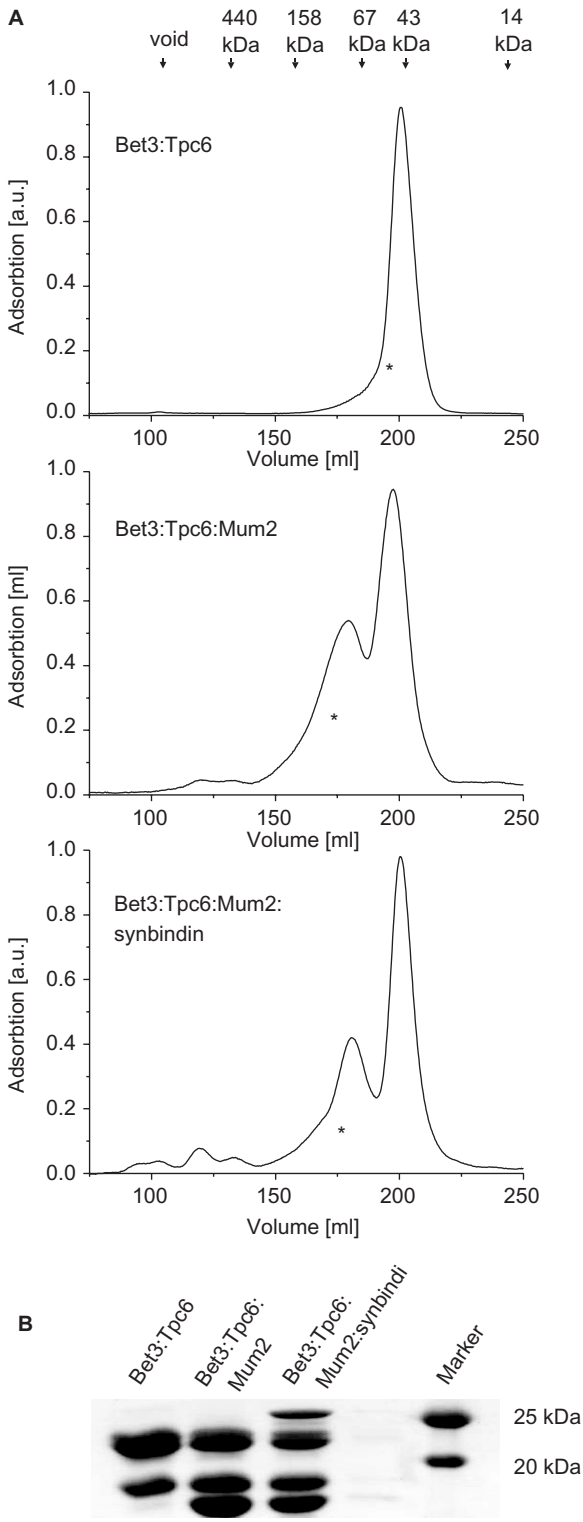

Figure 4. Analysis of TRAPP subcomplexes. (**A**) Proteins were co-expressed, purified with nickel chelating beads and loaded on a calibrated gel filtration column. (**B**) SDS-PAGE and Coomassie staining of isolated subcomplexes (marked with an asterisk in A).

molecule of each of the four TRAPP subunits. Bet3, Tpc6 and Mum2 without synbindin form a complex of similar size, which suggests that this complex is also a tetramer that might include two Mum2 molecules. These findings

agree with a recent study that identified TRAPP subcomplexes consisting of Bet3:Tpc6:Mum2 and Mum2:synbindin, respectively (20).

In addition to the co-expressions documented here, we generated co-expression constructs for 20 other pairs of human proteins using the Gateway pQLink vectors. Cloning was robust and expression levels of individual and co-expressed proteins were similar in virtually all cases (data not shown). This further demonstrates the robustness of our method.

In principle, there is no limit to the number of proteins that can be co-expressed, even though one should expect that the cloning efficiency decreases as the size of the plasmid constructs increases. It seems likely that our method can be transferred to expression host systems other than *E. coli*. For Baculovirus vectors, a co-expression system that also relies on duplication of expression cassettes including promoters and transcription terminators has been described (12). This demonstrates that duplication of promoters and terminators in a Baculovirus vector is unproblematic as has been shown by Alexandrov *et al*. and in our work with *E. coli* vectors.

ACKNOWLEDGEMENTS

We thank Janett Tischer for excellent technical assistance and Annett Boeddrich and Klaas Max for VCP and AMFR plasmids. The vector pQTEV2 was created by Asgar Ergin. This work was funded by the German Federal Ministry of Education and Research (BMBF) in the National Genome Network programme (NGFN FKZ 01GR0472), the SFB 633 of the German Research Foundation and SPINE II Complexes (LSHG-CT-2006-031220). Funding to pay the Open Access publication charge was provided by the BMBF.

Conflict of interest statement. None declared.

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