An improved dual-expression concept, generating high-quality antibodies for proteomics research

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A novel, improved dual bacterial-expression system, designed for large-scale generation of high-quality polyclonal antibody preparations intended for proteomics research, is presented. The concept involves parallel expression of cDNA-encoded proteins, as a fusion with two different tags in two separate vector systems. Both systems enable convenient blotting procedures for expression screening on crude bacterial cell cultures and single-step affinity purification under denaturing conditions. One of the fusion proteins is used to elicit antibodies, and the second fusion protein is used in an immobilized form as an affinity ligand to enrich antibodies with selective reactivity to the cDNA-encoded part, common for the two fusion proteins. To evaluate the system, four cDNA clones from putative nuclear proteins from the non-biting midge *Chironomus tentans* were expressed. Antibodies to these cDNA-encoded proteins were generated, enriched and used in blotting and immunofluorescence procedures to determine expression patterns for the native proteins corresponding to the cDNAs. The four antibody preparations showed specific reactivity to the corresponding recombinant cDNA-encoded proteins, and three of the four antibodies gave specific staining in Western-blot analysis of nuclear cell extracts. Furthermore, two of the antibody preparations gave specific staining in immunofluorescence analysis of *C. tentans* cells. We conclude that the dual-vector concept presented offers a highly stringent strategy for the generation of monospecific polyclonal antibodies, which are useful in proteomics research.

Introduction

In the post-genomic era, when increasing amounts of gene sequence information are becoming available, the next goal is to determine the function of all existing genes and their gene products. The most commonly used approach in proteomics research has been two-dimensional SDS/PAGE in conjunction with MS [1], a principle that has been refined by improvements such as two-dimensional liquid chromatography followed by MS/MS (tandem MS) [2] and various techniques for isotope labelling [e.g. ICAT (isotope-coded affinity tag)] [3]. These techniques are powerful in comparative studies of protein profiling. Excellent methods exist for the analysis of networks of protein interactions, such as two-hybrid systems [4,5] and large-scale pull-out experiments [6–8]. Nevertheless, none of these methods are optimal for systematic gene-by-gene characterization of the proteome.

Antibodies have been used for decades in various fields of research to study proteins in a case-by-case manner, but what is new in the proteomics era is the requirement of high throughput and high stringency in the generation and validation of the antibodies [9]. Monospecific antibody preparations have been demonstrated to be useful as affinity reagents for annotating functional information about proteins in a number of ways: (i) for immunolocalization at the cellular and subcellular levels [9], (ii) for probing the size and relative abundance of proteins with unknown function directly in preparations from different cells or tissues, (iii) for using antibodies in affinity-capture efforts of the native proteins from cells or tissues for direct characterization and (iv) for spotting antibodies on to arrays, which might become powerful future tools for profiling protein expressions [10,11].

A concept that has been utilized both in immunology and later in proteomics research for the generation of high-quality preparations of polyclonal antibodies is the

Key words: affinity blotting, affinity purification, dual expression, expression pattern, immunolocalization, proteomics.

Abbreviations used: ABP, albumin-binding protein; BCCP, biotin carboxyl carrier protein; HSA, human serum albumin; IMAC, immobilized-metal-ion affinity chromatography; MCS, multiple cloning site; PAAP, Protein A–alkaline phosphatase; SPA, *Staphylococcus aureus* Protein A; TSB, tryptic soy broth.

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dual-expression principle. The parallel expression of a gene in two expression systems to generate two fusion proteins, which have the target protein in common, has been used previously for certain applications [12–15]. Hey et al. [15] have used one fusion protein for immunization and the second for boosting to avoid carrier-mediated suppression, whereas others have used one fusion protein to elicit an immune response and the other fusion protein for monitoring the antibody responses [12–14] and for purification of target-specific antibodies [12,14].

When setting up such parallel expression systems suitable for functional analysis of cDNA-encoded proteins, robustness in the production is of utmost importance. Intracellular production makes it possible to express proteins that cannot be secreted through hydrophobic membranes [16]. It is also important to choose a tightly regulated promoter system, such as the phage T7 promoter [17], which makes it possible to produce proteins that normally would be deleterious to the host cell [18]. When selecting the affinity system for the products from the expression vectors, it is necessary to choose an affinity tag with a high affinity for a specific ligand. This affinity tag should allow affinity purification in buffers containing chaotropic agents such as guanidinium chloride or urea, used for solubilization of proteins precipitated into inclusion bodies. Furthermore, the affinity tags should be as resistant as possible to proteolysis.

In a previous study [19], we presented a concept employing two expression vectors, where a cDNA was expressed in *Escherichia coli* in parallel as a fusion to a staphylococcal Protein A (ZZ) tag [20] and to an ABP (albumin-binding protein) tag (residues 146–266) from streptococcal Protein G [21]. The ZZ-fusion protein was purified by affinity chromatography on IgG columns and used for immunization. The ABP-fusion protein was affinity-purified on HSA (human serum albumin) columns and used in an immobilized form for affinity enrichment of antibodies specific for the cDNA-encoded protein from the generated antiserum [19]. Since it was found later that the ABP tag had immunopotentiating carrier properties [22,23], we decided to include this tag in the fusion proteins to be used for the immunizations. However, the dual system mentioned above could not be reversed, since the ZZ-fusion protein would bind all IgG antibodies through its IgG Fc binding capacity [20,24] if used as a fusion protein for affinity capture.

Subsequently we presented a second concept employing a single vector system where the cDNA was expressed with an N-terminal His_6–ABP tag [25]. The His_6 tag allowed for IMAC (immobilized metal-ion affinity chromatography) purification of the produced proteins under denaturing conditions, resulting in recovery of both soluble and insoluble proteins, and the ABP tag was included for the immunostimulating effect. The same fusion protein was used to enrich antibodies, which, however, also resulted in the co-enrichment of antibodies specific to the ABP tag. In Western-blot analysis, validation blots, i.e. quality testing of the reactivity of enriched antibodies to the fusion proteins used for immunization would be inconclusive from the single-vector approach, since the ABP portion was present in all fusion proteins [25]. This was found to be a significant drawback, since such quality testing was found to be highly informative before using the antibodies in functional annotation studies.

Consequently, an improved dual-expression system was devised and evaluated [26]. The concept involves parallel expression of cDNA-encoded proteins, as fusion with two different tags, namely His_6–ABP and His_6 respectively. The system was found to yield high-quality antibodies, but still had two major drawbacks [26]. First, in any high-throughput expression system, it is convenient to have an efficient strategy for screening of expressions directly on the cultivations, before purification. Such a system was available for the His_6–ABP gene fusion system, but was not reproducibly robust for the His_6 system, probably due to the poor quality of His_6-reactive commercial reagents. Secondly, when expressing a mammalian protein, or a portion thereof, as fused only to a His_6 tag, the solubility of the expressed fusion protein is typically quite poor and a solubilizing fusion tag would be advantageous.

Recently, a novel gene fusion system was described, taking advantage of an engineered SPA (*Staphylococcus aureus* Protein A)-binding affinity tag, Z_{SPA-1}, enabling efficient recovery by affinity purification on Protein A-based chromatography media [27]. The 58-amino-acid Z_{SPA-1} was selected from a library constructed by combinatorial mutagenesis of a protein domain from SPA [28]. In the present study, a new dual-expression system is presented taking advantage of the novel Protein A-binding affinity tag Z_{SPA-1}. Thus gene fragments are expressed as fused to His_6–ABP and His_6–Z_{SPA-1} respectively. The novel dual-expression system is evaluated for expression and generation of high-quality antibodies to four putative nuclear proteins of currently unknown function.

Materials and methods

Construction of expression vectors

The cDNA clones were isolated from *Chironomus tentans* (non-biting midge) cDNA libraries, and four subfragments encoding portions (approx. 900 bp) of the putative nuclear proteins Y9, H7c, p47 and Y38 were PCR-amplified using specific primers and ligated into pAff8c [25] and pAff11c [27] using the restriction enzymes *Asc*I and *Not*I, according to a solid-phase subcloning procedure described previously [25].

The PCR-amplified and *Asc*I–*Not*I-restricted cDNA fragments were separated by electrophoresis on low-melting-point agarose gels and purified on agarose spin columns (Sigma, St. Louis, MO, U.S.A.) before ligation to the linearized plasmid vectors. Correct insertions were confirmed by MegaBACE sequencing (Amersham Biosciences, Uppsala, Sweden).

Expression and purification of fusion proteins

The expression vectors, based on pAff8c [25] and pAff11c [27], encoding portions of the nuclear proteins Y9, H7c, p47 and Y38, were transformed to the *E. coli* strain BL21 (DE3)pLysS (Novagen, Madison, WI, U.S.A.). For protein production, cells were grown overnight at 37 °C in 30 g/l TSB (tryptic soy broth; Difco, Detroit, MI, U.S.A.) containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol. From each clone, 5 ml of overnight culture was used to inoculate 500 ml of TSB + yeast extract, supplemented with antibiotics as described above. Cultures were grown at 37 °C to an absorbance between 0.8 and 1.0. Protein expression was then induced by the addition of isopropyl β-D-thiogalactoside (Apollo Scientific Ltd., Whaley Bridge, Stockport, U.K.) to a final concentration of 1 mM and the cultures were incubated for another 4 h before they were harvested by centrifugation at 2000 ***g*** for 10 min. Samples were taken at harvest for expression screening. Cell pellets were resuspended in 40 ml of lysis buffer [50 mM NaH_2PO_4/10 mM Tris/HCl/100 mM NaCl/6 M guanidinium chloride, pH 8.0] and sonicated (High Intensity Ultrasonic Processors, 750 W model; Sonics and Materials, Newtown, CT, U.S.A.) before adding 10 mM 2-mercaptoethanol and incubating on a stirrer for 2 h at room temperature (20 °C). Protein solutions were clarified by centrifugation at 12000 ***g*** for 10 min and the supernatants were filtered using a 0.45 μm-pore-size filter (Satorius AG, 37070 Goettingen, Germany). To purify the His_6-tagged proteins, columns were prepared with 2.5 ml of TALON metal (Co^{2+}) affinity resin (ClonTech Laboratories, Palo Alto, CA, U.S.A.) for IMAC. The IMAC procedure was performed under denaturing conditions as follows. Protein solutions were applied to columns, which were previously equilibrated with lysis buffer, and the unbound material was washed out with lysis buffer before elution in 1 ml fractions using an elution buffer (8 M urea/100 mM NaCl/100 mM acetic acid/50 mM NaH_2PO_4, pH 5.0). Protein-containing fractions were determined according to the absorbance at 280 nm. They were pooled, diluted with PBS (1.9 mM NaH_2PO_4/154 mM NaCl, pH 7.25) to 2 M urea concentration and stored at − 20 °C. Purified proteins were analysed in terms of size, purity and relative concentrations by separation on homogeneous SDS/20% polyacrylamide gels under reducing conditions and Coomassie Blue staining in the Phast system (Amersham Biosciences).

Immunization and antibody affinity enrichment

As described previously [25], two New Zealand rabbits per antigen were immunized with the pAff8c products (the cDNA products fused to His_6–ABP) by AgriSera AB (Vännäs, Sweden). Antibodies specific for the four cDNA clones were enriched from the generated antisera by affinity purification using the corresponding pAff11c products (the cDNAs fused to His_6–Z_{SPA-1}) as ligands. Briefly, the IMAC-purified pAff11-fusion proteins were subjected to SDS/PAGE and electroblotted on to a nitrocellulose membrane (Novex, Invitrogen, Carlsbad, CA, U.S.A.) according to the supplier's instructions. The membranes were blocked with milk proteins (Semper, Stockholm, Sweden) and incubated with the corresponding antisera for 2 h, followed by thorough washing. Bound antibodies were eluted with elution buffer [0.2 M glycine/1 mM EGTA (Sigma), pH 2.8] and neutralized with Tris and PBS buffer to approx. pH 8.0 [29].

Western-blot analysis

Whole-cell extracts from samples collected at harvest and purified fusion proteins were separated on Tris/glycine SDS/PAGE (10–20% gel; Novex) and electroblotted on to nitrocellulose membranes (Novex). All the membranes were blocked in 5% (w/v) milk solution for 1 h. For the pAff8c products, HSA was biotinylated with D-biotinoyl-ε-aminohexanoic acid *N*-hydroxysuccinide ester (Boehringer Mannheim) according to the supplier's instructions and the membrane was incubated with the biotinylated HSA to a final concentration of 0.03 μM for 1 h at room temperature. The membranes were washed in PBS with 0.05% Tween 20 (PBST) before incubation with streptavidin–alkaline phosphatase conjugate (Boehringer) to a final concentration of 0.5 unit/ml for 1 h at room temperature. After washing with PBST, the blot was developed with 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside/Nitro Blue Tetrazolium tablets (Sigma) according to the manufacturer's instructions.

For the pAff11c products, the membrane was incubated with 4 μg/ml PAAP (Protein A–alkaline phosphatase)-fusion protein for 30 min according to a blotting procedure described previously [27]. The PAAP-fusion protein consists of a five-domain native Protein A fused in frame to alkaline phosphatase and is encoded by the vector pACEP32 (S. Hober, unpublished work). It was produced by cultivation of RR1ΔM15 cells harbouring the plasmid in 100 ml of TSB + yeast extract, supplemented with ampicillin (200 μg/ml). Affinity purification of the PAAP-fusion protein was performed on IgG–Sepharose (Amersham Biosciences) essentially as described by Ståhl et al. [24]. The membrane was washed with PBST and developed as described above.

The blotting procedure using the affinity-enriched antibodies was performed in a similar way. However, in this

blotting, the membrane was cut into strips to incubate each antibody preparation with its corresponding antigen separately. The monospecific antibodies were diluted in the ratio 1:100 in PBS and the second antibody (goat anti-rabbit IgG–alkaline phosphatase; Sigma) was diluted in the ratio 1:10 000.

Western-blot analysis of *C. tentans* proteins

Proteins from either total nuclear or ribonucleoprotein I preparations were extracted from *C. tentans* tissue culture cells essentially as described by Wurtz et al. [30]. The proteins were separated by SDS/PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, U.S.A.) using a semi-dry electophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Membranes were blocked with 10% (w/v) non-fat dry milk in PBS and probed with affinity-enriched antibodies diluted in PBS containing 1% milk and 0.05% Tween 20. The secondary antibodies were conjugated with either alkaline phosphatase or horseradish peroxidase. Alkaline phosphatase conjugates were detected using 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside/Nitro Blue Tetrazolium. Horseradish peroxidase conjugates were detected by chemiluminescence (Amersham Biosciences).

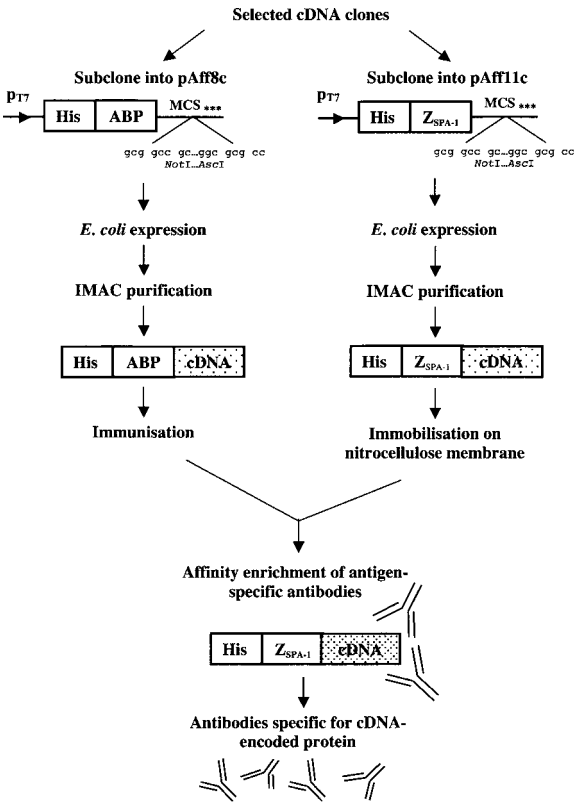
Indirect immunofluorescence

C. tentans tissue-culture cells and larval salivary glands were fixed with 4% (w/v) paraformaldehyde for 15 min at room temperature and permeabilized with either 0.5% Triton X-100 or 0.2% SDS for 10 min at room temperature. After washing three times with PBS, the cells were blocked with 3% (w/v) BSA in PBS and probed with the affinity-enriched antibodies diluted in PBS containing 1% BSA. An FITC-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) was used in the second step of the immunostaining. The cells were mounted in VectaShield (Vector Laboratories, Burlingame, CA, U.S.A.) and analysed in either an Axioplan II epifluorescence microscope (Carl Zeiss, Thornwood, NY, U.S.A.) or an LSM510 confocal microscope (Carl Zeiss).

Results and discussion

The general concept

On the basis of previously described expression systems, designed for high-throughput generation of antibodies monospecific for cDNA-encoded proteins [19,25,26], a novel, improved dual-expression concept is presented (Scheme 1). Selected cDNA clones would be subcloned, according to a PCR-based solid-phase cloning strategy



Scheme 1 The basic concept

A flow chart of the basic concept including the expression cassettes of the expression vectors is depicted schematically. The cDNA fragments are subcloned into the two expression vectors, the constructs are transformed to *E. coli* cells and expressed intracellularly. The vectors are designed for intracellular expression in *E. coli*, under control of the tightly regulated T7 promoter [17]. The dual-affinity tag of pAff8c consists of a His_6 sequence fused in frame to the ABP derived from the streptococcal Protein G [21], followed by an MCS (multiple cloning site) for insertion of the gene fragments. The expression vector pAff11c encodes a dual-affinity tag, His_6–Z_{SPA-1}, followed by the same MCS. The Z_{SPA-1} domain is a 58-amino-acid affibody [33] created by combinatorial protein engineering of an SPA domain [28]. The Z_{SPA-1} affibody shows specific binding to SPA, with the dissociation constant K_D in the micromolar range [28]. The binding of the Z_{SPA-1} affibody to its parental structure was shown to involve the Fc binding site of SPA [28]. Thus the inclusion of the Z_{SPA-1} affibody as part of a His_6–Z_{SPA-1} dual-affinity tag allows for affinity purification of expressed fusion proteins to be performed either with IMAC using the His_6 tag or on Protein A–Sepharose using the Z_{SPA-1} tag [27]. Subcloning into the vectors could typically be performed using PCR, allowing the primers to introduce in frame *Not*I and *Asc*I restriction sites respectively after PCR amplification. These two restriction endonucleases were selected since they both have recognition sequences of eight nucleotides (containing CG dinucleotides), making their recognition sequences very infrequent in most genes. This minimizes the risk of cutting into a cDNA sequence even if the complete sequence is not determined. The MCSs are followed by stop codons in three frames (***). The vectors also encode the lac repressor responsible for tight repression of transcription before induction with isopropyl β-D-thiogalactoside, and the gene conferring resistance to kanamycin. The encoded fusion proteins are purified by IMAC under denaturing conditions. The affinity-purified His_6–ABP-fusion proteins are used to generate antibodies in rabbits, whereas the His_6–Z_{SPA-1}-fusion proteins are immobilized and used for affinity enrichment of antibodies with selective reactivity to the cDNA-encoded portion of the fusion proteins. The cDNA-specific antisera can be used in various functional analyses of proteins, by blotting methods and in immunolocalization studies at both cellular and subcellular levels.

Table 1 Clones evaluated in the new dual-expression concept

Abbreviations: ORF, open reading frame; RRM, RNA recognition motif.

Clone	Size of ORF (bp)	Insert (bp)	Functional annotation/known motif	Molecular mass (kDa)	
				His_6-ABP fusion	His-Z_{SPA-1} fusion
Y9	1608	819	Unknown/Zn finger, PDZ domain[a]	49.2	42.5
H7c	2025	918	RNA helicase/DExD/H box	53.0	46.3
p47	1263	939	RNA helicase/DExD/H box	54.2	47.5
Y38	1581[b]	876	Putative RNA binding/RRM	51.0	44.3

[a] Protein–protein interaction domain (post-synaptic density protein 95/*Drosophila* disks large/zona occludens-1 domain).
[b] Truncated at the 5′-end.

described previously [25,26], into pAff8c [25] and pAff11c [27] respectively. Fusion proteins from both vectors, having T7-regulated expression [17], are produced intracellularly in parallel in *E. coli*. The fusion proteins are purified by IMAC using the common His_6 tag under denaturing conditions, thus allowing recovery of both soluble and insoluble gene products. The Aff8c-fusion proteins, containing the N-terminal His_6–ABP tag, are used for immunization of rabbits [25,26], whereas the Aff11c fusions, containing the His_6–Z_{SPA-1} tag [27], are immobilized and used for affinity enrichment of antibodies from the generated sera. The ABP is included in the pAff8c vector, since previous studies have shown that this protein domain has immunopotentiating properties [22,23]. The ABP and Z_{SPA-1} tags have the useful property that they increase the overall solubility of the fusion proteins. This should improve expression and renaturation properties, and allow expression screening on bacterial cell lysates using readily available reagents. This dual-expression procedure ensures that only antibodies reactive to the cDNA-encoded part of the fusion protein are enriched. The antibodies could then be used for several different functional analyses, including various protein-localization studies at both cellular and subcellular levels.

Nuclear proteins from *C. tentans*

To investigate the performance of the novel dual-expression system, four cDNA clones encoding putative nuclear proteins from the dipteran *C. tentans* (Table 1) were selected for evaluation in terms of the expression and generation of affinity-purified rabbit antibodies that would be useful for further functional annotation studies. The cDNA clones were isolated from *C. tentans* cDNA libraries on the basis of either the presence of RNA-interacting motifs or interaction with known nuclear proteins. Highly specific antibodies are important tools for detailed analysis of the subcellular location and function of these proteins. Moreover, obtaining such antibodies is considered a challenge, since nuclear proteins are often difficult to express with good yields in bacterial systems.

Expression analysis and purification of the fusion proteins

Gene fragments from the four selected cDNA clones, denoted Y9, H7c, p47 and Y38 (Table 1), were subcloned into the expression vectors pAff8c [25] and pAff11c [27] (Scheme 1). This generated in-frame fusions with the dual-affinity tags His_6–ABP and His_6–Z_{SPA-1} respectively. The fusion proteins, containing portions of cDNA-encoded proteins, were expressed intracellularly in *E. coli* and expression screening using convenient affinity-blotting procedures (not requiring specific immunoglobulins) was performed on bacterial cell lysates at the time of cell harvest (Figure 1). After SDS/PAGE of the cell cultures from the pAff8c expression (Figure 1A), only the His_6–ABP–H7c (Figure 1A, lane 2) was clearly visible. However, when applying an affinity-blotting procedure described previously [25], taking advantage of the ABP tag, biotinylated HSA that binds to ABP and subsequent staining of the expressed fusion proteins using conjugated streptavidin–alkaline phosphatase, protein bands of the expected mass were readily detected in whole-cell lysates (Figure 1B). BCCP (biotin carboxyl carrier protein) [31], the only protein in *E. coli* that is biotinylated, was also visible in the blot (Figure 1B, arrow). Thus the staining of BCCP could be used as an internal standard to ensure that protein from similar amounts of cells is loaded in each well. When loading the whole-cell lysates from the pAff11c expression, SDS/PAGE revealed only bands corresponding to His_6–Z_{SPA-1}–H7c and His_6–Z_{SPA-1}–p47 (Figure 1C, lanes 2 and 3). However, a convenient single-step affinity-blotting procedure, using a PAAP-fusion protein followed by staining with an alkaline phosphatase substrate [27], clearly visualized bands of the expected mass in all cultivations (Figure 1D). Affinity-blotting procedures similar to the ones described, based on general reagents, are obviously of significant value for expression

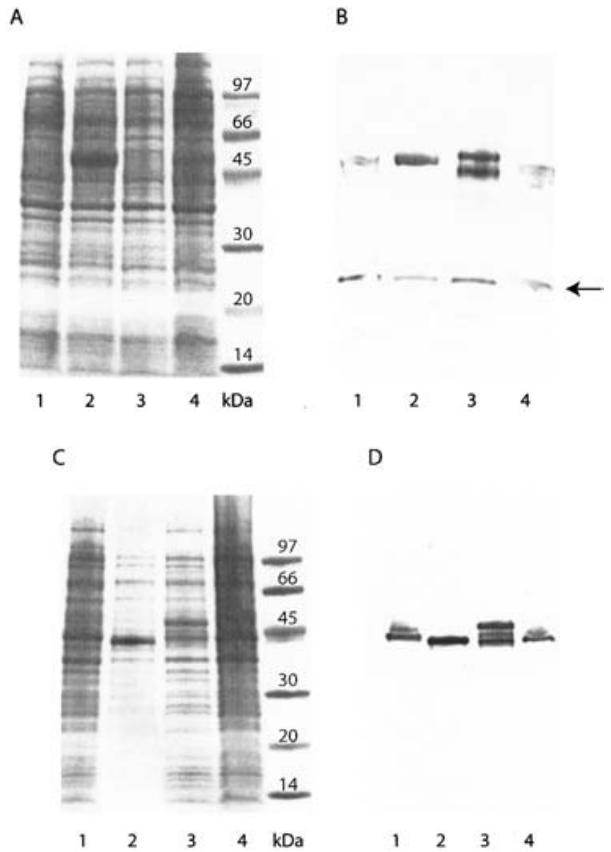


Figure 1 Expression analyses

(**A**) Whole-cell lysates from the pAff8c (His_6–ABP) cultures, taken at harvest and separated by SDS/PAGE. (**B**) pAff8c cell lysates blotted on to a nitrocellulose membrane (after separation by SDS/PAGE) and incubated first with biotinylated HSA and then with streptavidin-conjugated alkaline phosphatase. BCCP [31] (the only biotinylated protein in *E. coli*) can be detected in all lanes (arrow). (**C**) Whole-cell lysates from the pAff1c (His_6–Z_{SPA-1}) cultures, taken at harvest and separated by SDS/PAGE. (**D**) pAff1c cell lysates blotted on to a nitrocellulose membrane (after separation by SDS/PAGE) and incubated with a PAAP-fusion protein. Both blots were developed by adding phosphatase substrate. Protein lysates loaded: lane 1, Y9; lane 2, H7c; lane 3, p47; lane 4, Y38.

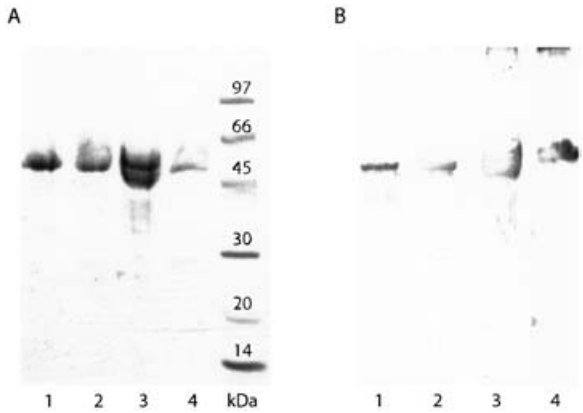


Figure 2 SDS/PAGE of affinity-purified Aff8c-fusion proteins and Western-blot analysis to validate reactivity to the corresponding recombinant cDNA-encoded proteins

(**A**) SDS/PAGE of the fusion proteins Aff8c–Y9 (lane 1), Aff8c–H7c (lane 2), Aff8c–p47 (lane 3) and Aff8c–Y38 (lane 4) respectively, after purification by IMAC. (**B**) The fusion proteins Aff8c–Y9 (lane 1), Aff8c–H7c (lane 2), Aff8c–p47 (lane 3) and Aff8c–Y38 (lane 4) respectively were separated by SDS/PAGE and electroblotted on to a nitrocellulose membrane. Each lane of the nitrocellulose membrane was allowed to react with the antibody preparation (affinity-enriched on the corresponding Aff1c-fusion proteins) and subsequently stained using a secondary enzyme-conjugated antibody.

screening of cell cultures, when large numbers of samples need to be processed in parallel.

Affinity enrichment and analysis of the purified antibodies

The fusion proteins from the pAff8c and pAff1c cultivations were purified by IMAC [32] under denaturing conditions. SDS/PAGE of the four affinity-purified His_6–ABP-fusion proteins demonstrated a high degree of purity (Figure 2A), with only His_6–ABP–p47 showing double bands, possibly indicating a slight proteolytic degradation (Figure 2A, lane 3). However, this degradation seems to occur during cultivation, since double bands were also detected in the corresponding cell culture at harvest (Figure 1B, lane 3). The observed stability of the fusion proteins with very little proteolytic degradation and the observed high degree of purity indicate that the dual-expression and recovery concept should be suitable for expression of cDNA-encoded proteins.

The affinity-purified His_6–ABP-fusion proteins were subsequently used for immunization of rabbits, and antibodies highly specific for the cDNA-encoded protein were enriched from the antisera using the corresponding His_6–Z_{SPA-1}-fusion proteins, immobilized on nitrocellulose membrane strips as affinity matrix. One of the advantages of the concept of dual expression is that the affinity-enriched antibodies can be tested by Western-blot analysis for their reactivity to the corresponding fusion proteins. In these 'validation blots', which could not be performed using the previous single-vector concept [25], it was found that the antibody preparations indeed stained the corresponding affinity-purified pAff8c-encoded His_6–ABP-fusion proteins (Figure 2B). It could, of course, be speculated that the reactivity could be partly directed to the His_6 part of the fusion proteins, which is present both in the fusion proteins used for immunization and the ones used for the affinity enrichment. However, this is unlikely, since previous dual-expression concepts, having His_6 tags in common, have not demonstrated any such generation of cross-reacting His_6-specific antibodies [26].

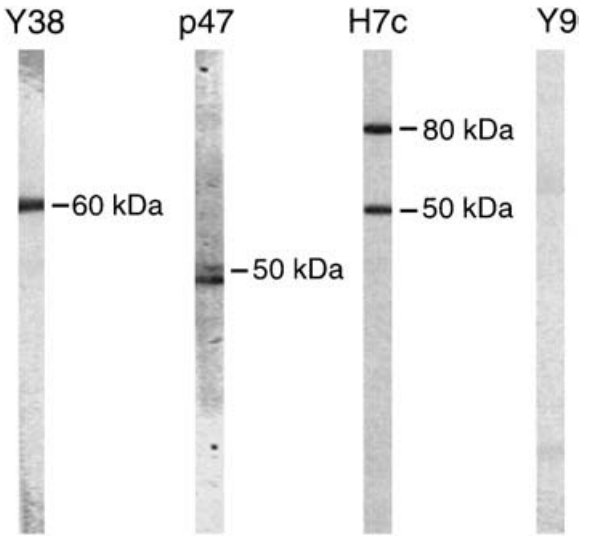


Figure 3 Western-blot analysis of affinity-enriched antibody preparations against *C. tentans* proteins

Nuclear proteins from *C. tentans* were separated by SDS/PAGE and blotted on to PVDF membranes. The membranes were cut into strips and each strip was incubated with one of the affinity-enriched antibody preparations. Enzyme-conjugated secondary antibodies were used to visualize the immunoreactivity of each antibody. The relative molecular masses of the immunoreactive bands are indicated.

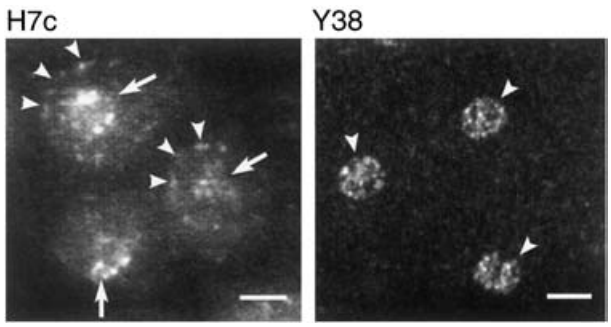


Figure 4 Immunofluorescence analysis of affinity-enriched antibodies against H7c and Y38 proteins

C. tentans tissue-culture cells (left panel) were stained with the anti-H7c antibody. Duct cells from larval salivary gland (right panel) were immunostained with the anti-Y38 antibody. In both cases, the immunostaining was visualized using an FITC-conjugated secondary antibody. Three cells are shown in each image, and the edges of the nuclei are demarcated by arrowheads. The anti-H7c antibody shows a predominant staining in the nucleolus (indicated by arrows) and in the nuclear periphery (arrowheads), whereas the anti-Y38 antibody gives a speckled staining all through the nucleus, excluding the nucleoli. Scale bars, 1 and 3 μm for H7c and Y38 respectively.

Western-blot and immunofluorescence analyses

To test the antibody preparations in a more relevant biological setting, affinity-enriched antibody preparations were used to analyse protein extracts from *C. tentans* diploid cells by Western-blot analysis (Figure 3). Three of the antibody preparations, from rabbits injected with H7c-, p47- and Y38-fusion proteins, all selectively reacted with unique protein bands. The antibodies against Y38 and p47 recognized single proteins of approx. 60 and 50 kDa respectively, in agreement with the relative molecular masses predicted from the cDNA sequences (Table 1). Two distinct bands at 50 and 80 kDa were detected by the anti-H7c antibody. The 80 kDa band approximately corresponds to the expected mass of the H7c protein. The significance of the 50 kDa band is currently unknown. The raw sera from rabbits immunized with the Y9 recombinant protein gave reactivity for multiple bands (results not shown), but the affinity-enriched Y9 preparations failed to give any significant signal (Figure 3). In summary, for three of the four proteins, the affinity-enriched antibodies gave a far more stringent pattern when compared with the corresponding untreated raw antiserum, thus demonstrating the strength of the dual-expression concepts.

The reactivity of the affinity-purified antibodies with their corresponding protein antigens was tested *in situ*. Two of the three antibody preparations gave informative staining patterns. This type of high-resolution staining represents an important aspect of the usefulness of the antibodies, because it allows an investigation of the subcellular distribution of the proteins recognized by the antibodies. Furthermore, cytological staining complements Western-blot analysis, since experimental conditions for the immunochemical reaction are not necessarily identical. *C. tentans* diploid epithelial cells or duct cells from the *C. tentans* salivary glands were fixed and stained with the H7c and Y38 antibodies respectively. Figure 4 shows that the H7c antibody preferentially stained the nucleolus, and also the nuclear periphery, in a dot-like pattern. The Y38 antibody specifically stained the nucleus in a speckled manner, excluding the nucleolus. These results demonstrate that the steady-state locations of both H7c and Y38 proteins are nuclear, but each protein has a characteristic intranuclear distribution. In summary, our results show the usefulness of the obtained affinity-purified antibodies for further functional studies of these proteins.

Concluding remarks

A dual-expression system, aimed for production of cDNA-encoded proteins to be used for immunization and subsequent stringent affinity enrichment of monospecific polyclonal antibodies, has been described. We have presented improvements to a previous dual-expression system [26] by the introduction of a novel Protein A-binding affinity tag, Z_{SPA-1}, in the second vector. A cDNA would thus be expressed both as a His_6–ABP fusion and a His_6–Z_{SPA-1} fusion, in parallel. The inclusion of Z_{SPA-1} in the

second vector offers two immediate advantages. First, it allows expression screening directly on bacterial cultures through a convenient one-step blotting procedure using a PAAP-fusion protein. This is of significant importance in high-throughput proteomics research, where only the clones giving successful expression are processed. Secondly, solubilizing protein domains of bacterial origin have been demonstrated to improve expression and to simplify recovery of proteins with low solubility [16,24], which is not uncommon when expressing eukaryotic cDNAs in bacteria. In addition, if needed, the fusion proteins could be efficiently affinity-purified on commercially available Protein A affinity-chromatography media, which was previously shown to give a very high degree of purity [27].

To evaluate the novel dual-expression system, cDNA clones encoding putative nuclear proteins from the dipteran *C. tentans* were selected for expression and generation of high-quality rabbit antibodies, which should prove useful for further functional annotation studies. These proteins were considered to be a challenge, since nuclear proteins are often difficult to express with good yields in bacterial systems. Initially, five clones were selected for expression, but one clone did not yield any detectable amount of protein, and was thus excluded. The reason for this is unknown. Nevertheless, four clones were expressed with good yields, and were conveniently identified by affinity-blotting procedures in whole-cell lysates. Both the His_6–ABP- and the His_6–Z_{SPA-1}-fusion proteins could be efficiently purified by IMAC to yield pure proteins, suitable for immunization of rabbits or as ligands for affinity enrichment of antibodies respectively. Rabbit antibodies were generated, enriched and used in blotting and immunofluorescence procedures to determine expression patterns for the native proteins corresponding to the cDNAs. The four antibody preparations showed specific reactivity to the corresponding recombinant cDNA-encoded proteins in validation blots, and three of the four antibody preparations gave specific staining in Western-blot analysis of nuclear cell extracts. Furthermore, two of the antibody preparations gave specific staining in immunofluorescence analysis of *C. tentans* cultured cells and larval salivary glands respectively. Taken together, we conclude that the presented dual-vector concept offers a highly stringent strategy for the generation of monospecific polyclonal antibodies that are useful in proteomics research.

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