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Cloning vectors for the production of proteins in *Dictyostelium discoideum*

(Recombinant DNA; myosin; cytoskeleton; expression vector; cellular slime mold; transformation)

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SUMMARY

We constructed and tested a series of cloning vectors designed to facilitate protein production and purification in *Dictyostelium discoideum* (*Dd*). These vectors carry the origin of replication of the *Dd* high-copy-number plasmid Ddp2, expression cassettes consisting of the strong, constitutive actin (*act15*) or the inducible discoidin (*dis1y*) promoters, a translational start codon upstream from a multiple cloning site and sequences for the addition of epitope or affinity tags at the N- or C-termini of any protein. The affinity tag used corresponds to 7 (N-terminal fusion) or 8 (C-terminal fusion) His residues. The epitope tags correspond to an 11-amino-acid sequence from human c-myc, recognised by monoclonal antibody (mAb) 9E10, and the Glu-Glu-Phe sequence recognised by mAb YL1/2 to α -tubulin. Both these mAb are commercially available. The YL1/2 epitope offers a second affinity tag for the purification of proteins under native conditions. The functional competence of the vectors was tested by determining their ability to promote the expression of various *Dd* myosin constructs. High synthesis levels were obtained for each vector; up to 1 mg of homogenous, functional protein per g of cells was obtained after purification of the recombinant products.

INTRODUCTION

The cellular slime mold *Dictyostelium discoideum* (*Dd*) has many features that make it an attractive eukaryotic host for the production of recombinant (re-) proteins. *Dd* is biochemically well characterised and can be grown readily in the laboratory on a large scale. Our work is mostly focused on the characterisation of cytoskeletal proteins like myosin. Functional myosin motor fragments

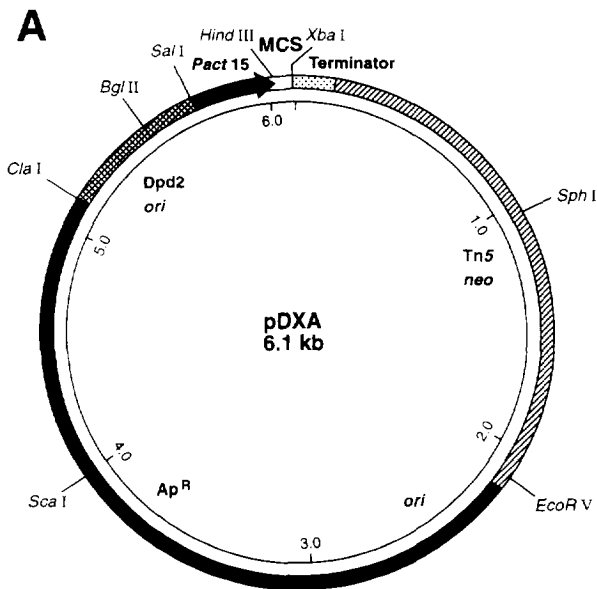
can be produced and purified in milligram quantities using *Dd* (Manstein et al., 1989) and detailed kinetic (Ritchie et al., 1993) and structural studies (Schröder et al., 1993) have been carried out using myosin motor domains produced in *Dd*. The new vectors were designed to improve production of re-proteins in *Dd* for in vitro and in vivo studies. Introduction of the Glu-Glu-Phe epitope tag (Stammers et al., 1991) or poly-His tag (Janknecht et al., 1991) facilitates the rapid and efficient

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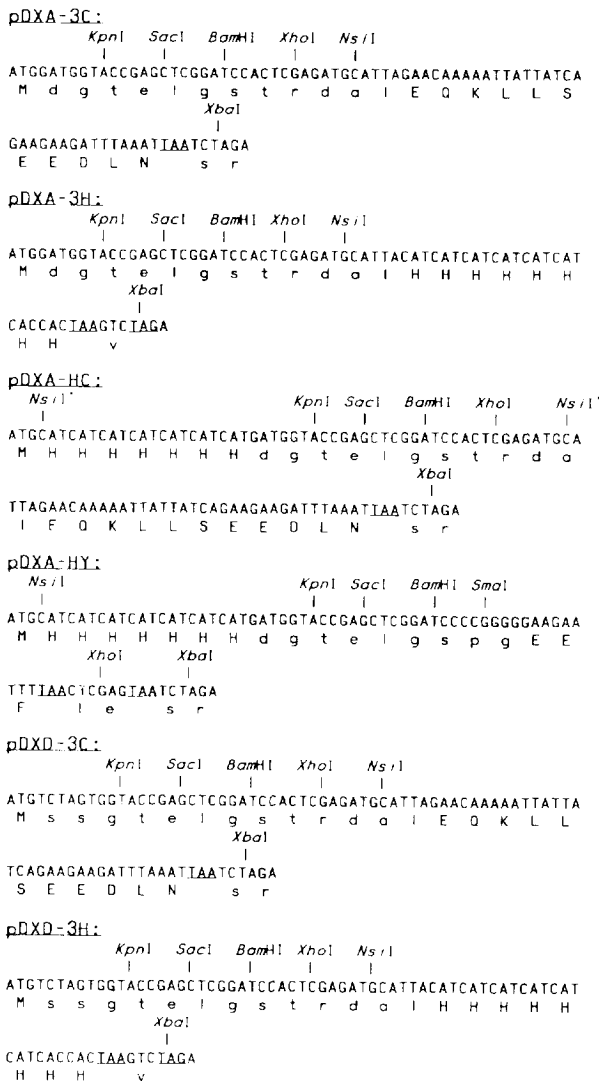
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Abbreviations: aa, amino acid(s); Ab, antibody(ies); *act*, gene encoding actin; Ap, ampicillin; bp, base pair(s); DCD, catalytic domain of *Dd*

myosin; *Dd*, *Dictyostelium discoideum*; Ddp2, *Dd* plasmid 2; *dis*, gene encoding discoidin; *E.*, *Escherichia*; kb, kilobase(s) or 1000 bp; mAb, monoclonal Ab; MCS, multiple cloning site(s); MHC, myosin heavy chain; *mhcA*, gene encoding MHC; *neo*, neomycin-resistance gene; nt, nucleotide(s); NTA, nitrilotriacetic acid; ORF, open reading frame; oligo, oligodeoxyribonucleotide; *ori*, origin of DNA replication; *P*, promoter; PAGE, polyacrylamide-gel electrophoresis; Pn, penicillin; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; ^R, resistance-resistant; re-, recombinant; SDS, sodium dodecyl sulfate; Sm, streptomycin; tag (affinity or His tag), His₇- (N-terminal) or His₈- (C-terminal) protein tag.



B



purification of overproduced protein and the Glu-Glu-Phe and *c-myc* epitope tags can be used for the analysis of protein function and fate within the cell.

EXPERIMENTAL AND DISCUSSION

(a) Plasmid construction

The vectors (Fig. 1) were constructed by replacing the phage f1 *ori* of pB10TP2 (Early and Williams, 1987) with a *Dd ori* from plasmid Ddp2 (Leiting et al., 1990; Chang et al., 1990) and insertion of expression cassettes consisting of the strong, constitutive *act15* promoter (pDXA series) or the inducible *disI_y* promoter (pDXD series)

Fig. 1. Structure of the vectors. (A) Schematic map of pDXA. Maps of the pDXD vectors are identical, except for the *P* sequence. (B) Sequences of the MCS of each of the vectors. In addition to the restriction enzymes indicated, the MCS are cut by the neoschizomers *Asp718* (*KpnI*), *Ecl136II* (*SacI*) and *PspAI* (*SmaI*). For aa, capital letters indicate tag sequences and start Met, stop codons are underlined. **Methods:** pB11 was constructed by replacing the filamentous phage f1 *ori* of pB10TP2 with the Ddp2 *ori*. pB10TP2 (Early and Williams, 1987) was first digested with *BglII*, the overhanging ends were filled in with *PolIk*, and then digested with *ClaI*. The 592-bp fragment that can act as a *Dd ori* was taken from plasmid pNDeI (Leiting and Noegel, 1988) and inserted as *XbaI* (blunt)-*ClaI* fragment. Plasmid pB12 was generated by inserting the *Pact15* into pB11 adjacent to the Ddp2 *ori*. Digestion of BS18/*HindIII* (Firtel and Chapman, 1990) with *XbaI*+*HindIII* liberated the *Pact15*. The *P* fragment was filled in with *PolIk* and ligated into pB11, digested with *NsiI*+*SalI* and treated with T4 DNA polymerase to create blunt ends. pB12 was next digested with *PspAI* and filled in with *PolIk* to destroy a unique *PspAI*/*SmaI* site. The resulting plasmid was called pB13. Sequences for a start codon, a MCS, and encoding epitope or affinity tags were inserted using oligo pairs. pB13 was digested with *XbaI*+*HindIII*, the two strands of the oligo were annealed at 60 C and then ligated without phosphorylation to ensure insertion of a single copy. pDXA-3C was created using the oligo pair A (5'-AGCTTAAAAAATGGATGGTACCGAGCTCGGATCCACTCG-AGATGCATTAGAACAAAAATTATTATCAGAAGAAGATTTAAATTAATCTAG) and B (5'-ATTAATTTAAATCTTCTCTGATAATAATTTTTGTTCTAATGCATCTCGAGTGGATCCGAGCTCGGTACC-ATCCATTTTTTAAAGCT). Other vectors of the pDXA series were generated by removing part of the pDXA-3C MCS with *HindIII*+*BamHI* or *BamHI*+*XbaI* and insertion of synthetic oligos. The sequences of the oligo pairs used for the construction of pDXA-3H, pDXA-HC, and pDXA-HY can be deduced from panel B of the figure. pDXD vectors carrying the *PdisI_y* were created by isolating the *P* fragment as *XhoI*-*XbaI* (blunt) fragment from pVEII (Blusch et al., 1992) and inserting it between the *SalI* and *Asp718* (blunt) sites of pDXA-3H in place of the *Pact15*. The resulting plasmid was recut with *KpnI* (blunt)+*BamHI* and the reading frame that is used in all other constructs was restored by the insertion of a sequence made of the synthetic oligos C (5'-TGGTACCGAGCTCGGATC) and D (5'-CGAGCTC-GGTACCA). This resulted in the generation of vector pDXD-3H (6.8 kb). pDXD-3C was created by removing the *BamHI*-*SphI* fragment from pDXD-3H and replacing it with the *BamHI*-*SphI* fragment from pDXA-3C. The nt sequences of the vectors are available from the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession Nos. X85118-X85123 and from gopher: worms.emsbio.nwu.edu:70/11/Vector Sequences.

(Clarke et al., 1987; Blusch et al., 1992; Liu et al., 1992; Blusch and Nellen, 1994), a start codon upstream from a MCS, and sequences for the addition of epitope or affinity tags at the N- or C-termini of any protein. The pB10TP2 sequences provide the *act6* Tn5 *neo^R* cassette which confers G418^R (Nellen et al., 1984), the bacterial Ap^R gene, a high-copy-number *E. coli* plasmid *ori*, and *Dd* polyadenylation and termination signals (Early and Williams, 1987). The resulting expression vectors are approx. 6.1 kb (pDXA series) or 6.8 kb (pDXD series) in size. Their MCS sequences provide restriction sites for the insertion by blunt cloning of any gene of interest in frame with the start codon and tag sequences provided. Vectors of the pDXA series allow the synthesis of proteins carrying a C-terminal *c-myc* tag (pDXA-3C), a C-terminal His-tag (pDXA-3H), an N-terminal His-tag and C-terminal *c-myc* tag (pDXA-HC) and N-terminal His-tag and C-terminal YL1/2 epitope-tag (pDXA-HY). Vectors carrying the inducible *disI* promoter can be used for the production of proteins that are fused to either a C-terminal His- (pDXD-3H) or *c-myc*-tag (pDXD-3C).

(b) Transformation of *Dd*

Autonomous replication of the expression vectors described here requires the presence of the *Ddp2 ORF* product which is known to be essential in *trans* for the replication of plasmids carrying the *cis*-acting *ori* sequence (Leiting et al., 1990; Slade et al., 1990). This can be achieved by cotransformation with plasmids bearing a copy of the *ORF* gene or by the use of the AX3-ORF⁻ cells which carry integrated copies of the *Ddp2 ORF* gene. Southern blots of transformed AX3-ORF⁻ lines are generally consistent with their extra-chromosomal replication and indicate that the expression vectors are present at low copy number (<10 copies). The observation of lower than expected copy numbers with plasmids carrying the *Ddp2 ori* is not without precedent. Rutherford and coworkers reported copy numbers in the range of 10–50 copies per cell for another *Ddp2*-based vector (Yin et al., 1994). Fig. 2 shows the results obtained with three constructs derived from pDXA-3H for the production of myosin motor fragments of 88 (pSW29), 100 (pSW29-1R) and 115 kDa (pSW29-2R). The size of these plasmids is 8.4, 8.7 and 9.0 kb, respectively. Undigested and *Xba*I-digested pSW29-DNA purified from *E. coli* served as size controls for linear plasmid (lane 1) and supercoiled monomer (lane 1') mobilities. Hybridisation of the probe to 8.4, 8.7 and 9.0-kb bands in *Xba*I digests of transformant DNA confirmed the presence of the intact plasmids (lanes 2 to 4) and the pattern of bands observed with undigested DNA samples indicates that the major plasmid form in the transformants was a supercoiled extrachromosomal monomer (lanes 2' to 4').

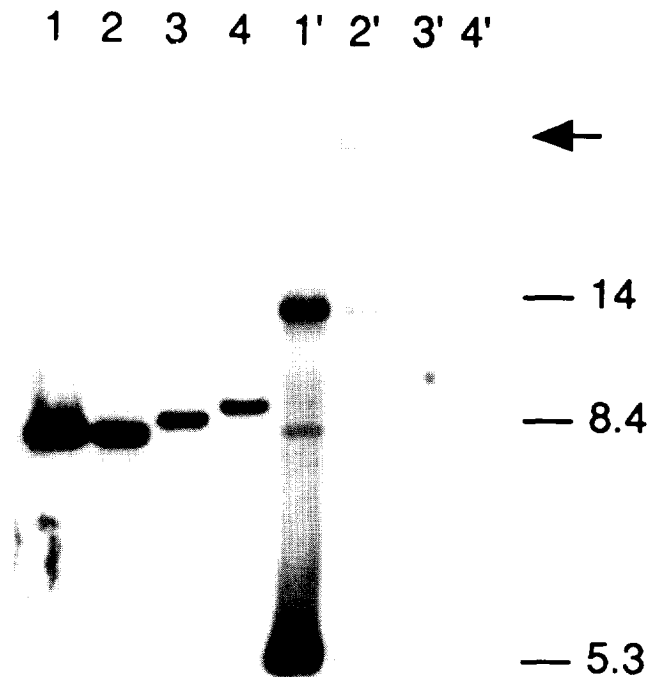


Fig. 2. Southern analysis of AX3-ORF⁻ cells transformed with pDXA-3H derived vectors. Lanes: 1, *Xba*I digested pSW29 purified from *E. coli*; 2–4, *Xba*I digested DNA from cells transformed with pSW29, pSW29-1R and pSW29-2R, respectively. 1', undigested pSW29 purified from *E. coli*; 2'–4', unrestricted DNA from cells transformed with pSW29, pSW29-1R and pSW29-2R. **Methods:** A detailed description of the AX3-ORF⁺ cells will be published elsewhere. Briefly, the AX3-ORF⁺ cells were generated starting with strain DH1 (Harwood et al., 1995) which is auxotrophic for uracil. Cells were transformed by electroporation with a plasmid carrying the *pyr5-6* gene and the *Ddp2 ORF* gene. Transformants were selected in FM medium (Gibco-BRL) and individual clones were tested for transformation competence by supertransformation with plasmids bearing *neo* and the *Ddp2 ori*. The clone giving the highest yield of transformants was used for further studies. Transformations were carried out by electroporation as described earlier (Egelhoff et al., 1991). However, DD-Broth20 containing (per litre): 20 g protease peptone (Oxoid)/7 g yeast extract (Oxoid)/8 g glucose/0.33 g Na₂HPO₄·7H₂O/0.35 g KH₂PO₄ was used instead of HL5 medium. The use of DD-Broth20 during transformation resulted in well-defined, independent foci where HL5 made up from the same ingredients failed to give individual colonies. 5 × 10⁶ cells and 10 μg of plasmid were used per transformation. Following electroporation, cells were chilled on ice for 10 min and then transferred to four petri dishes containing 10 ml DD-Broth20 (with Pn at 100 U/ml and Sm at 100 μg/ml) and allowed to recover for 18 h. G418 was added at a concentration of 20 μg/ml. Generally, 800–1500 colonies were obtained. Southern blot analysis was performed as described previously (Egelhoff et al., 1990) using a 0.8% agarose gel. Lanes were loaded either with 10 ng of plasmid DNA or 10 μg of genomic *Dd* DNA. A 0.8-kb *Nco*I-*Sph*I fragment of the G418^R cassette was used as the probe. Mobility of DNA standards is shown to the right in kb. The arrow indicates mobility of linear chromosomal DNA.

(c) Use of the vectors for the production of re-proteins in *Dd*

The vectors were tested for function by determining their ability to promote the synthesis of various *Dd* myosin constructs. Efficient protein production was obtained with all vectors. Myosin heavy chain (MHC) fragments that were fused to either a C-terminal Glu-Glu-Phe or *c-myc* epitope tag could be readily detected on immunoblots using the mAb YL1/2 and 9E10, respectively (data not shown). However, it should be noted that mAb YL1/2 cross-reacts with a *Dd* protein of approx.

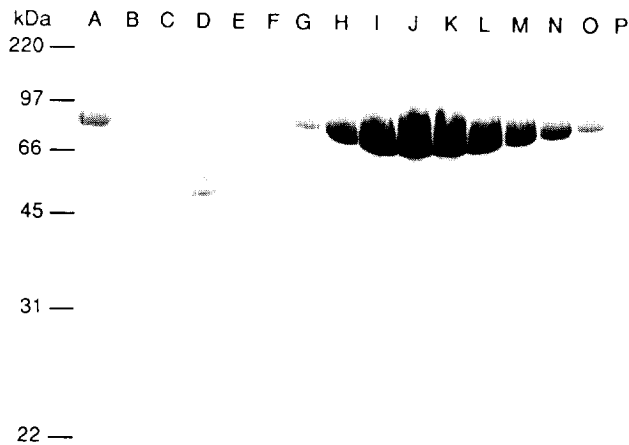


Fig. 3. Purification of a His-tagged myosin motor domain. Lanes: **A**, high-speed supernatant of Mg^{2+} -ATP extracted cytoskeleton fraction as loaded onto the Ni^{2+} -NTA column; **B** and **C**, flow-through; **D** and **E**, 50 mM imidazole wash; **F**–**P**, elution of DCD-K143Q using a gradient from 50 to 500 mM imidazole. Proteins were separated by SDS-PAGE and stained with Coomassie blue. **Methods:** Cells transformed with a pDXA-3H derivative that drives the production of DCD-K143Q were grown in 5-litre Erlenmeyer flasks, each containing 2.5 litres of DD-Broth20. Cells were shaken at 200 rpm at 21 °C. Protein was typically purified starting with 15 litres of culture medium, which yielded about 50 g of cells. Cells were harvested at a density of 8×10^6 cells per ml. High-speed supernatants of Mg^{2+} -ATP extracted cytoskeleton fractions were prepared as described by Manstein and Hunt (1995). Shortly, cells were resuspended and lysed in 300 ml L-buffer (50 mM Tris-HCl pH 8.0/2 mM EDTA/0.2 mM EGTA/1 mM DTT) containing 0.5% Triton-X100/15 µg RNaseA per ml/protease inhibitors. The lysate was incubated on ice for 1 h. Upon centrifugation ($230\,000 \times g$, 1 h), the re-protein remained in the pellet. The pellet was washed in 100 ml of HKM-buffer (50 mM HEPES pH 7.3/30 mM K-acetate/10 mM $MgSO_4/7$ mM β -mercaptoethanol/5 mM benzamidine/40 µg per ml PMSF) and centrifuged for 45 min at $230\,000 \times g$. The re-protein was released into the supernatant by extraction of the resulting pellet with 60 ml HKM-buffer containing 10 mM ATP. After centrifugation ($500\,000 \times g$, 45 min), the supernatant was loaded onto a Ni^{2+} -NTA column (1.5 × 11 cm). Next, the column was washed in LS-buffer (50 mM HEPES pH 7.3/30 mM K-acetate/3 mM benzamidine), HS-buffer (as LS-Buffer, but with 300 mM K-acetate), and LS-buffer containing 50 mM imidazole. The re-myosin was eluted using a linear gradient of LS-buffer and I-buffer (0.5 M imidazole pH 7.3/3 mM benzamidine), starting with 10% I-buffer and reaching 100% after 15 min. The flow rate was 3 ml/min and 3-ml fractions were collected.

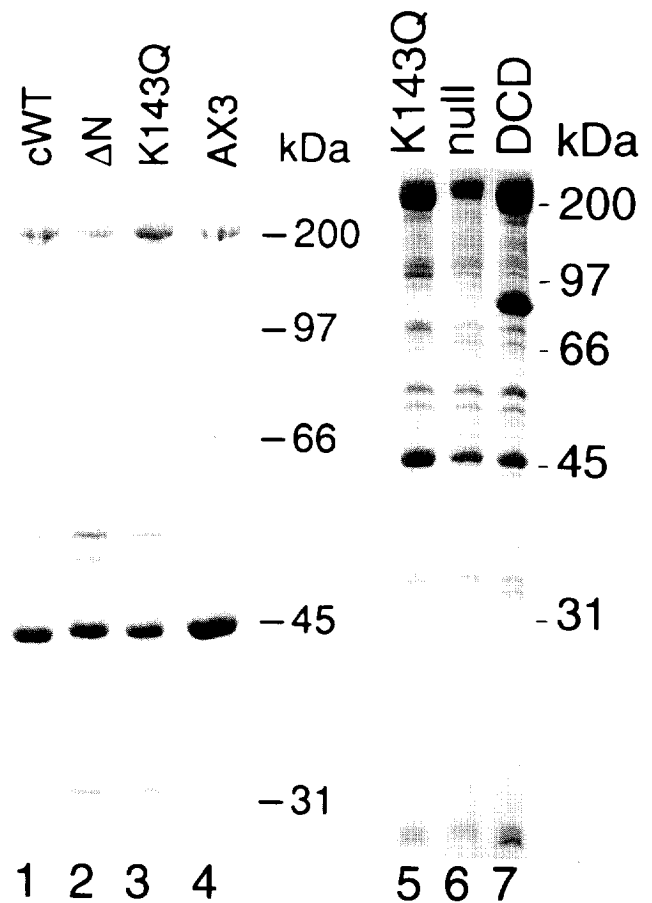


Fig. 4. Complementation of myosin 'null' cells. Lanes: **1**, transformants producing the cloned *Dd* wild-type MHC (cWT); **2**, a truncated MHC that lacks the small N-terminal subdomain consisting of 80 aa (ΔN); **3** and **5**, a MHC carrying a missense mutation (K143Q) were analysed by SDS-PAGE. AX3 cells (**4**) were used as a positive control (AX3) and HS1 cells (**6**) were used as negative control (null). For comparison, AX3-ORF⁺ cells producing DCD are also shown (**7**). The size of the *Dd* MHC is approx. 220 kDa and that of DCD is 88.2 kDa. **Methods:** The constructs were introduced into HS1 cells (Ruppel et al., 1994) by cotransformation with a plasmid that is identical to pNDe ΔI (Leiting et al., 1990) except for the lack of a G418^R cassette. Transformants were selected in DD-Broth20 supplemented with Pn, Sm and 10 µg G418/ml. Typically 400–700 colonies were obtained with each electroporation of 10^7 cells. All transformants tested produced a MHC at levels similar or higher than wild-type cells. AX3 cells produce myosin at levels of at least 0.5% of the total cell protein (Clarke and Spudich, 1974). Myosin enriched fractions were generated as described by Manstein and Hunt (1995) and analyzed by 0.1% SDS-12% PAGE. Coomassie blue was used as protein stain.

120 kDa and a protein of the same size co-elutes with the re-product from columns of immobilised YL1/2.

The purification of a mutant catalytic domain fragment of *Dd* myosin, carrying a missense mutation that converts a highly conserved basic residue (Lys¹⁴³) to a Gln (DCD-K143Q), is described in detail in Fig. 3. The protein was purified to homogeneity by Ni^{2+} -nitrilotriacetic acid (NTA) chromatography (Qiagen, Dorking, UK). The purified protein was fully functional based on actin-activated ATPase activity. The basal ATPase rate for

DCD-K143Q was 0.03 s^{-1} and in the presence of $25 \mu\text{M}$ actin the ATPase rate was 2.4 s^{-1} , an 80-fold increase. Approximately 1% of the total protein was recovered as purified DCD-K143Q, which corresponds to a yield of 5 mg per 10 g of cells. Plasmids of the pDXA series were also used to complement *mhcA* 'null' cell line HS1 (Ruppel et al., 1994) with wild-type and specifically altered myosins (Fig. 4). All transformants tested produced a MHC at levels similar or higher than AX3 cells. The uniformity of expression level displayed by individual transformants was particularly striking in these experiments. In fact, there was no detectable difference in expression level between individual transformants and the total population of transformants.

(d) Conclusions

The cloning vectors described here provide a convenient means of producing proteins in *Dd*.

(1) The MCS sequences in these vectors provide restriction sites for the insertion by blunt cloning of any gene of interest in frame with the start codon and tag sequences provided.

(2) High transformation efficiencies are obtained, both, by transforming the vectors into AX3-ORF⁻ cells or when using other cells by co-transformation with plasmids carrying the *ORF* gene.

(3) The vectors are maintained at low copy number and extrachromosomally.

(4) In axenic medium, proteins can be synthesized constitutively under the control of the *act15* promoter or under the control of the *dis1* promoter, which is known to be regulatable.

(5) Proteins are produced at high levels and can be easily detected and purified when fused to the affinity or epitope tags provided.

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