Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 307 (2003) 74–79
BBRC
www.elsevier.com/locate/ybbrc

Production of human skeletal α-actin proteins by the baculovirus expression system

P. Anthony Akkari,[a,b,1] Kristen J. Nowak,[a,b,1,2] Kaye Beckman,[a,b] Kendall R. Walker,[a,b,c] Fred Schachat,[d] and Nigel G. Laing[a,b,*]

[a] *Centre for Neuromuscular and Neurological Disorders, University of Western Australia, Australian Neuromuscular Research Institute, 4th Floor, 'A' Block, QEII Medical Centre, Nedlands, Western Australia 6009, Australia*
[b] *Centre for Medical Research, West Australian Institute for Medical Research, QEII Medical Centre, Nedlands, Western Australia 6009, Australia*
[c] *School of Veterinary and Biomedical Sciences, Murdoch University, South Street Murdoch, Western Australia 6150, Australia*
[d] *Department of Cell Biology, Division of Physiology, Duke University Medical School, Durham, NC 27710, USA*

Received 23 May 2003

Abstract

Mutations within the human skeletal muscle α-actin gene cause three different skeletal muscle diseases. Functional studies of the mutant proteins are necessary to better understand the pathogenesis of these diseases, however, no satisfactory system for the expression of mutant muscle actin proteins has been available. We investigated the baculovirus expression vector system (BEVS) for the abundant production of both normal and mutant skeletal muscle α-actin. We show that non-mutated actin produced in the BEVS behaves similarly to native actin, as shown by DNase I affinity purification, Western blotting, and consecutive cycles of polymerisation and depolymerisation. Additionally, we demonstrate the production of mutant actin proteins in the BEVS, without detriment to the insect cells in which they are expressed. The BEVS therefore is the method of choice for studying mutant actin proteins causing human diseases.

Keywords: Skeletal actin; Baculovirus expression; Nemaline myopathy; Disease; Intranuclear myopathy

Humans, like all higher mammals, have at least six different actin isoforms, all expressed from separate genes [1,2]. Mutations leading to human disease have been identified in three of the six human actin genes. Mutations in cardiac muscle α-actin, *ACTC*, cause dilated cardiomyopathy [3] and hypertrophic cardiomyopathy [4,5]. Mutations in the β-actin gene, *ACTB*, have been associated with neutrophil dysfunction, recurrent infections, and mental retardation [6], and types of cancers [7,8]. Mutations in the skeletal muscle α-actin gene, *ACTA1*, have been shown to cause the skeletal muscle diseases nemaline myopathy, intranuclear rod myopathy, and actin myopathy [9–12]. Nemaline myopathy (OMIM #161800, #256030) is classified as a congenital muscle disease. It is characterised by muscle weakness and the presence of nemaline bodies (rods) in the skeletal muscles of affected individuals [13]. Intranuclear rod myopathy is also distinguished by the presence of rods, either confined solely to the cell nuclei or in conjunction with sarcoplasmic nemaline rods (e.g. [14,15]). Actin myopathy, or congenital myopathy with excess thin filaments (OMIM #102610), is characterised by large areas of skeletal muscle devoid of sarcomeres filled with a homogeneous material revealed to be masses of randomly orientated thin filaments [16,17].

We now know of around 70 different, mostly missense, mutations within *ACTA1* [18]. To elucidate how the mutations in *ACTA1* lead to skeletal muscle disease and the abnormal aggregates, functional studies with the expressed mutant proteins need to be performed. As most patients with skeletal α-actin-related muscle disease die at less than one year of age, studies with mutant skeletal

* Corresponding author. Fax: +61-8-9346-3487.
E-mail address: nlaing@cyllene.uwa.edu.au (N.G. Laing).
[1] These authors contributed equally to the work described.
[2] Present address: Department of Human Anatomy and Genetics, University of Oxford, South Parks Road, Oxford, OX1 3QX, UK.

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)01133-1

muscle α-actin protein extracted from patient muscle biopsies are difficult to achieve. Therefore, production of mutant recombinant actin proteins is required.

Regardless of multiple reports of the expression of recombinant actins in a variety of systems (e.g. [19,20]), no approach has been described for the successful expression and purification of mutant mammalian muscle actins. Despite abundant expression in *Escherichia coli*, recombinant actin proteins form insoluble inclusion bodies [21]. In yeast, mutant actins can be produced from episomal vectors to allow continued expression of the sole wild-type yeast actin protein essential for cell viability [22,23]. Nevertheless, in spite of 87–90% identity at the amino-acid level, significant differences between wild-type yeast actin and muscle actins have been identified, leading to caution in extrapolation from studies performed with yeast actins [24]. Production of mutant actins in *Drosophila* is accomplished by mutating the *Act88F* gene, the only actin isoform expressed in the indirect flight muscle of the flies [25]. Examination of flight muscle morphology is possible (e.g. [26,27]), but only a small amount of pure recombinant actin is recovered from dissected insect flight muscles [28]. Mutant human actin proteins have been produced by in vitro transcription and translation, but only minimal actin is synthesised (picogram amounts) (e.g. [29,30]).

Thus the expression of recombinant mammalian muscle actins has proved problematical in the past. The baculovirus expression vector system (BEVS) has been ascertained to perform the post-translational modifications seen in eukaryotes [31], and we have previously used this system to produce normal and nemaline myopathy-causing mutant slow α-tropomyosin proteins [32]. We consequently investigated the production of skeletal muscle α-actin protein in this system. Our preliminary studies indicated that normal and mutant skeletal α-actin proteins could indeed be successfully expressed using this method [33]. Here we report more detailed investigations of this expression system, including the confirmation of native-like activity of the recombinant non-mutated protein. Recently, Sano et al. [34] described the use of a 21 bp leader sequence derived from the 5′ untranslated sequence of the lobster tropomyosin gene for enhanced protein expression of exogenous genes in baculovirus-infected insect cells. One of the proteins that these authors used as an example for their system was skeletal muscle α-actin. We demonstrate here that high levels of expression of skeletal muscle α-actin can be achieved by the BEVS without the leader sequence.

Materials and methods

Creation of expression constructs. A variety of constructs were made using the BacPak8 transfer vector (Clontech) and the coding sequence of *ACTA1* which was amplified from cDNA transcribed from unaffected human muscle RNA. All PCR products were generated using a high fidelity DNA polymerase (Platinum *Taq* HiFi, Life Technologies). Primers for PCR amplification usually contained a restriction enzyme site to facilitate directional cloning (restriction sites are underlined in the primer sequences below). Standard cloning techniques were used to insert PCR fragments into similarly digested vector DNA [35].

The following constructs were made:

(1) *ACTA1*/BacPak8. To clone *ACTA1* without any tags into the transfer vector BacPak8 (Clontech), the primers *ACTA*1Forward *XhoI* (5′-GATCTCGAGATGTGCGACGAAGACGAG) and *ACTA*1Reverse *EcoRI* (5′-TAGGAGAATTCCTAGAAGCATTTGCGGTG GACG) were used.

(2) N′His*ACTA1*/BacPak8. The construct pcDNA3.1 HisA/*ACTA1* was made by digesting the pcDNA3.1HisA vector (Invitrogen) with *Kpn*I and creating blunt ends before inserting a similarly blunt-ended *ACTA1* sequence. The *ACTA1* insert was synthesised using the primers *ACTA*1 Forward (−2) (5′-GACGAAGACGAGACCACCG CCC) and *ACTA*1Reverse (5′-CTAGAAGCATTTGCGGTGGACG) that produced an *ACTA1* PCR fragment without the first two amino acids. This construct was used as a template for PCR with the primers His*ACTA*1 Forward *XhoI* (5′-GATCTCGAGATGGGGGGTTCTCA T) and *ACTA*1Reverse *EcoRI* which was inserted into BackPak8 to create the final construct which included a N′-terminal histidine-tag, an Anti-Xpress antibody epitope, and an enterokinase (EK) cleavage site preceding the *ACTA1* coding sequence.

(3) *ACTA1*/EGFP/BacPak8. The primers *ACTA*1/EGFP/Forward *XhoI* (5′-GATCTCGAGATGTGCGACGA AGACGAG) and *ACTA*1/EGFP/Reverse *EcoRI* (5′-GCAGAATTCGGAAGCAT TTGCGGTGGACG) were used to amplify the *ACTA1* coding sequence to insert into pEGFP-N1 (Clontech). A positive clone was treated with *Xho*I and *Not*I in a partial digest to excise the fusion cDNA sequence of *ACTA1*/EGFP which included a linker sequence consisting of 51 nucleotides (resulting in 17 amino acids) from the multiple cloning sequence. This fragment was cloned into BacPak8 DNA to create an *ACTA1* construct with a C′-terminal EGFP tag.

(4) *Mutant constructs*. Mutant *ACTA1* constructs were created using site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene) with the appropriately designed oligonucleotides and the *ACTA1*/BacPak8 or *ACTA1*/EGFP/BacPak8 parental constructs. The mutations introduced were G15R, L94P, M132V, I136M, V163L, G182D, E259V, and D286G, all mutations identified in human patients [9–11].

Cell culture. Sf9 insect cells were maintained in suspension in Erlenmeyer flasks on an orbital shaker (120 rpm) at 28 °C with serum free Sf9 SFM II medium (JR Biosciences). The cells were routinely subcultured every 3–4 days to maintain the cells in logometric phase (about 1×10^6 cells/mL) and were replaced with frozen stocks approximately every 3 months.

Transfections/protein production. To produce recombinant viruses by homologous recombination, the actin-BacPak8 transfer plasmids were co-transfected into Sf9 cells with linearised BacPak6 viral DNA (*Bsu36 I* digested) using Bacfectin as per the manufacturer's instructions (Clontech). In the case of the EGFP/skeletal α-actin constructs, successful co-transfections were quickly determined by observing green fluorescent Sf9 cells using fluorescent microscopy (Olympus, Japan). Primary virus was collected 72 h post-transfection and was used to isolate pure plaques that were subsequently amplified. Viral titres were calculated using the Rapid Titre Kit (Clontech). Subsequent viral infections were performed by inoculating Sf9 cell cultures during exponential growth with a calculated volume of recombinant viral solution to give a multiplicity of infection of 5 plaque-forming units per cell. Cells were collected at 3 days post-infection by low speed centrifugation at 6000*g* for 5 min.

Recombinant protein analysis. Cells were assayed for recombinant protein production by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis using a specific monoclonal antibody which only recognises mammalian skeletal and cardiac muscle

actins (Sigma, Clone 5C5, 1:10,000 dilution) and a rabbit anti-mouse HRP secondary antibody (1:10,000, Sigma). For *ACTA1*/EGFP fusion proteins an HRP-conjugated peptide antibody (Living Colours, Clontech) was utilised at a 1:2500 dilution. The Anti-Xpress antibody (Invitrogen, 15,000 dilution) was used with the recombinant actin proteins fused to an N′ histidine-tag. All signals were detected by ECL-Plus (AmershamPharmaciaBiotech).

DNase I affinity chromatography. Pelleted infected Sf9 cells were suspended in G-buffer (2 mM Tris buffer, pH 7.5; 0.1 mM ATP and 0.1 mM $MgCl_2$) including protease inhibitor cocktail for insect cells (Sigma), homogenised with a hand-held homogeniser, and then centrifuged at 35,000*g* for 30 min. The supernatant was loaded onto a DNase I column which was constructed and utilised as previously described [36].

Histidine–resin purification. Purification of the recombinant His/skeletal α-actin protein was performed with the Xpress Purification Kit (Invitrogen) following the manufacturer's instructions for native protein purification. An elution buffer containing 400 mM imidazole was required to elute the purified recombinant protein off the column.

Polymerisation/depolymerisation analysis. Challenged Sf9 cells were lysed with Rigor buffer (20 mM Tris buffer, pH 8.0, 100 mM NaCl, 2 mM EGTA, and 2 mM $MgCl_2$, with 14 mM DTT, and protease inhibitor cocktail (Sigma) added just prior to use) using a hand-held homogeniser. Polymerisation and depolymerisation was conducted essentially as outlined in [37]. A low speed centrifugation (6000*g* for 20 min) removed any cellular debris, after which the supernatant was dialysed against 20 L of G buffer for 27 h with five 4 L changes at 1, 2, 3, 21, and 27 h. The dialysate (containing soluble G-actin) was concentrated and then centrifuged at 300,000*g* for 2.5 h. The soluble G-actin in the supernatant was converted to F-actin by the addition of a 1:10 volume of 10× F buffer (100 mM Tris buffer, pH 7.5, 10 mM $MgCl_2$, 1 M NaCl, 2 mM DTT, and 1 mM ATP). The conversion of G-actin to F-actin was performed on ice for 2 h. The sample was centrifuged at 450,000*g* for 1 h to pellet the insoluble F-actin. After carefully washing the pellet with G buffer to remove any salts that were left on the sides of the tube, the actin was solubilised in the G buffer at 4 °C overnight. The actin was finally converted back to F-actin with the addition of a 1:10 volume of 10× F buffer and stored at 4 °C.

Protein quantification. The yield of enriched recombinant actin achieved after two rounds of polymerization and depolymerisation was estimated by SDS–PAGE electrophoresis, Western blotting, and a Coomassie dye based protein assay [38].

Results and discussion

Production of recombinant actin proteins

Sf9 cells were harvested and analysed for production of the recombinant protein by SDS–PAGE and Western blotting. After infection with normal or mutant skeletal α-actin virus, Sf9 cells produced a recombinant protein that exhibited a molecular weight indistinguishable from that of rabbit skeletal muscle actin (Fig. 1A). A pre-stained protein marker (LifeTechnologies) indicated that these proteins were between 40 and 50 kDa, consistent with the size of monomeric actin, 43 kDa [20]. The tagged recombinant actins also migrated to an appropriate position for their estimated sizes. The His/skeletal α-actin protein appeared slightly larger than normal actin (with a calculated addition of approximately 3.5 kDa) and the EGFP/skeletal α-actin proteins migrated to approximately the same position as the 70 kDa marker, which correlates with the calculated size of this fusion protein, 70 kDa (43 kDa for skeletal α-actin + 27 kDa for EGFP) (Fig. 1A). It was clear that the recombinant actins were in each case one of the most abundant proteins in the infected Sf9 cells (Fig. 1A).

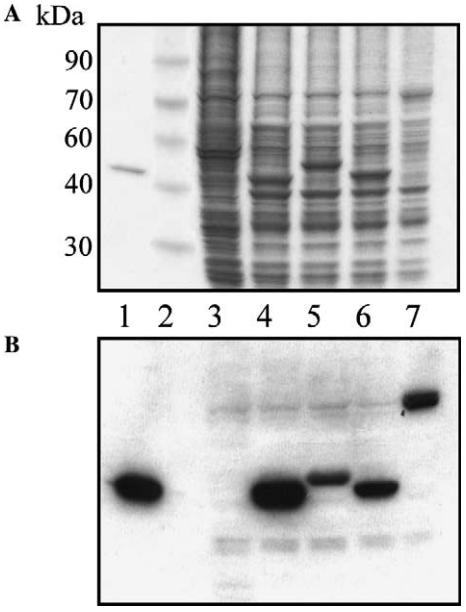

Fig. 1. Analysis of total protein from 2 μl of pelleted Sf9 cells after initial co-transfections with various *ACTA1* constructs. (A) SDS–polyacrylamide gel, (B) Western blot, using a sarcomeric muscle actin specific monoclonal antibody of the gel shown in (A). Lane 1: rabbit muscle actin, lane 2: marker, lane 3: non-transfected Sf9 cells, lane 4: non-mutated *ACTA1*, lane 5: His-*ACTA1*, lane 6: G15R-*ACTA1*, and lane 7: V163L-*ACTA1*-EGFP.

All recombinant skeletal α-actin proteins, but not the endogenous Sf9 actins, were recognized by the specific sarcomeric muscle actin antibody (Fig. 1B). One important characteristic of a viable actin expression system is the ability to produce mutant actin proteins in abundance [19]. We were able to show that eight different mutant skeletal α-actin proteins were successfully produced using the BEVS. These mutant proteins were expressed as untagged actin and as a fusion protein with EGFP with no noticeable detriment to the viability of the insect cells when compared to infected cells expressing wild-type actin. The His-tagged actin and the G15R actin showed reduced staining in comparison with the quantity of recombinant protein present as observed by Coomassie staining, suggesting that the His-tag and the G15R mutation interfere with the antibody's epitope. The His/skeletal α-actin fusion proteins reacted with the Anti-Xpress antibody, and similarly the EGFP fusion proteins were detected by the anti-GFP antibody.

DNase I affinity chromatography

DNase I affinity chromatography has been used extensively to purify actins of many origins away from total cellular proteins due to the strong and selective 1:1 interaction between DNase I and globular actin (G-actin) [20]. After chromatography of the total Sf9 cellular proteins, the major protein eluted off the column was a protein of the correct size for actin (Fig. 2). As the native insect actins were also expected to bind to the column, a Western blot was performed to confirm that recombinant skeletal α-actin protein had been purified by this method (data not shown). Additionally it is evident that the predominant band in the total protein sample, shown to represent recombinant actin, is not present in the flow-through of the column. The fact that the recombinant protein binds to the DNase I column, along with its ability to polymerise (see below), indicates that skeletal α-actin protein produced in Sf9 cells is properly folded.

Polymerisation studies and purification

Perhaps the most crucial function of actins is their ability to polymerise into filamentous F-actin [23]. We have demonstrated that recombinant non-mutant skeletal α-actin produced by the BEVS does polymerise when exposed to the appropriate high salt conditions (Fig. 3), indicating native-like function. Additionally, a significant proportion of the recombinant actin is retained when taken through successive rounds of polymerisation and depolymerisation, in a similar fashion to wild-type actin. We estimate that approximately 2 mg actin protein/L of challenged Sf9 cells remains after two rounds of polymerisation and depolymerisation.

Purification of the His-tagged recombinant proteins

It is possible that the position of a mutated amino acid in actin will prevent interaction with either DNase I and/or actin itself, rendering the purification methods already outlined ineffective. Therefore we investigated the possibility of using another purification method, namely utilisation of a histidine tag. This procedure has been successful with yeast actin where an N′-terminal histidine-tag was used to separate mutant actins from native yeast actins [39]. These researchers found no major difference between native and histidine-tagged yeast actin [39]. Our histidine-tag was also N′-terminal, and in addition we included an enterokinase (EK) site so that the histidine tag can be removed in the future if it is found to interfere with the function of actin. The EK site was engineered to be in a position so that the EK enzyme will cut flush at the N′-terminus of actin protein, leaving no additional amino acids derived from the expression construct. For all actins except those from fungi, either the initiator methionine or both the first two amino acids of the immature actin protein are acetylated and subsequently cleaved [20]. Therefore the first two amino acids of actin were not included in the expression construct so that after EK cleavage the resulting protein would be similar to mature human native skeletal muscle α-actin. Standard purification techniques for histidine-tagged proteins (Invitrogen) proved successful in purifying the recombinant His/skeletal α-actin protein to greater than 90% purity (data not shown), providing an alternative purification approach for actin mutants.

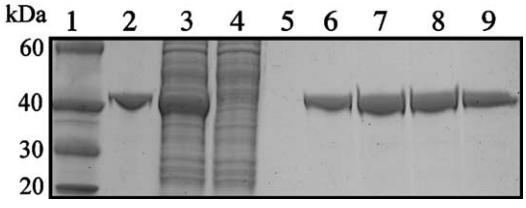

Fig. 2. Coomassie stained SDS–polyacrylamide gel showing DNase I affinity purification of recombinant actin. Lane 1: marker, lane 2: purified rabbit skeletal muscle actin, lane 3: Sf9 total proteins after 3 days of infection with non-mutated *ACTA1* virus, lane 4: flow-through after the total Sf9 proteins had been applied to the DNase I column, lane 5: final wash of the column, and lanes 6–9: successive elution fractions from the column.

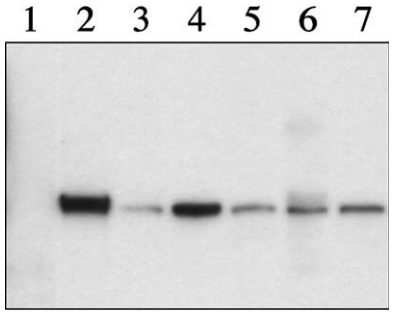

Fig. 3. Western blot showing polymerisation of non-mutated recombinant actin using a specific sarcomeric muscle actin specific antibody. Lane 1: total protein from unchallenged Sf9 cells, lane 2: total protein from challenged Sf9 cells, lanes 3 and 4: the supernatant and pellet, respectively, after a 9000 rpm spin of the proteins from the challenged cells, lanes 5 and 6: the supernatant and pellet, respectively, after a 50,000 rpm spin, lane 7: the pellet after the addition of polymerisation salts and a 70,000 rpm spin.

Mutations that lead to a variety of diseases have now been identified in three of the six human actin genes. Studies of *ACTA1* mutations have demonstrated that they cause a variety of congenital myopathies that mostly result in early death of affected individuals [9–12]. Presently no cure or effective treatment is available for these diseases and only inferences as to how and why these *ACTA1* mutations cause muscle disease can be made. Perhaps a form of treatment for affected individuals could be developed if the disease processes were

better understood. Some of the identified *ACTA1* mutations are in known functional sites for proteins that interact with actin, but other amino acid substitutions occur at codons that have not been designated with a particular function to date [18]. In addition, it has been shown that long-range communication within the actin protein takes place, meaning that a mutation away from a known binding site can still affect the binding at that site [40]. Finally, even if a mutation is situated within a known binding site, it is difficult to predict the full effect of the mutation. Perhaps such a mutation allows another protein to bind rather than the usual binding protein, causing a gain of function. Such questions may be answered once investigated by detailed functional protein–protein interaction studies. We have demonstrated that the BEVS shows promise as a method for expressing large quantities of functional wild-type skeletal α-actin, and in addition is a suitable system for expressing mutant skeletal α-actin proteins.

Acknowledgments

N.G.L., P.A.A., K.B., and K.J.N. are supported by the Australian National Health and Medical Research Council on Grants 139039, 254544 (N.G.L., P.A.A., and K.B.) and a C.J. Martin Fellowship ID #212086 (K.J.N.). K.R.W. is funded by an Australian Postgraduate Research Award. F.S. is supported by the American National Institutes of Health (RO1 EY11377-06). We thank Prof. John Sparrow for his valuable advice.

References

[1] J. Vandekerckhove, K. Weber, At least six different actins are expressed in a higher mammal: an analysis based on the amino acid sequence of the amino-terminal tryptic peptide, J. Mol. Biol. 126 (1978) 783–802.

[2] J.N. Engel, P.W. Gunning, L. Kedes, Isolation and characterization of human actin genes, Proc. Natl. Acad. Sci. USA 78 (1981) 4674–4678.

[3] T.M. Olson, V.V. Michels, S.N. Thibodeau, Y.S. Tai, M.T. Keating, Actin mutations in dilated cardiomyopathy, a heritable form of heart failure, Science 280 (1998) 750–752.

[4] J. Mogensen, I. Klausen, A.K. Pederson, H. Egeblad, P. Bross, T.A. Kruse, N. Gregersen, P.S. Hansen, U. Baandrup, A.D. Borglum, α-Cardiac actin is a novel disease gene in familial hypertrophic cardiomyopathy, J. Clin. Invest. 103 (1999) R39–R43.

[5] T.M. Olson, T.P. Doan, N.Y. Kishimoto, F.G. Whitby, M.J. Ackerman, L. Fananapazir, Inherited and de novo mutations in the cardiac actin gene cause hypertrophic cardiomyopathy, J. Mol. Cell. Cardiol. 32 (2000) 1687–1694.

[6] H. Nunoi, T. Yamazaki, H. Tsuchiya, S. Kato, H.L. Malech, I. Matsuda, S. Kanegasaki, A heterozygous mutation of β-actin associated with neutrophil dysfunction and recurrent infection, Proc. Natl. Acad. Sci. USA 96 (1999) 8693–8698.

[7] J. Leavitt, D. Goldman, C. Merril, T. Kakunaga, Actin mutations in a human fibroblast model for carcinogenesis, Clin. Chem. 28 (1982) 850–860.

[8] T. Kakunaga, J. Leavitt, H. Hamada, A mutation in actin associated with neoplastic transformation, Fed. Proc. 43 (1984) 2275–2279.

[9] K.J. Nowak, D. Wattanasirichaigoon, H.H. Goebel, M. Wilce, K. Pelin, K. Donner, R.L. Jacob, C. Hubner, K. Oexle, J.R. Anderson, C.M. Verity, K.N. North, S.T. Iannaccone, C.R. Muller, P. Nurnberg, F. Muntoni, C. Sewry, I. Hughes, R. Sutphen, A.G. Lacson, K.J. Swoboda, J. Vigneron, C. Wallgren-Pettersson, A.H. Beggs, N.G. Laing, Mutations in the skeletal muscle α-actin gene in patients with actin myopathy and nemaline myopathy, Nat. Genet. 23 (1999) 208–212.

[10] H. Jungbluth, C.A. Sewry, S.C. Brown, K.J. Nowak, N.G. Laing, C. Wallgren-Pettersson, K. Pelin, A.Y. Manzur, E. Mercuri, V. Dubowitz, F. Muntoni, Mild phenotype of nemaline myopathy with sleep hypoventilation due to a mutation in the skeletal muscle α-actin (*ACTA1*) gene, Neuromuscul. Disord. 11 (2001) 35–40.

[11] B. Ilkovski, S.T. Cooper, K.J. Nowak, M.M. Ryan, N. Yang, C. Schnell, H.J. Durling, L.G. Roddick, I. Wilkinson, A.J. Kornberg, K.J. Collins, G. Wallace, P. Gunning, E.C. Hardeman, N.G. Laing, K.N. North, Nemaline myopathy caused by mutations in the muscle α-skeletal-actin gene, Am. J. Hum. Genet. 68 (2001) 1333–1343.

[12] H. Buxmann, R. Schlosser, W. Schlote, A. Sewell, K.J. Nowak, N.G. Laing, V. Loewenich, Congenital nemaline myopathy due to *ACTA1*-gene mutation and carnitine insufficiency: a case report, Neuropediatrics 32 (2001) 267–270.

[13] K.N. North, N.G. Laing, C. Wallgren-Pettersson, Nemaline myopathy: current concepts, J. Med. Genet. 34 (1997) 705–713.

[14] H.H. Goebel, I. Warlo, Nemaline myopathy with intranuclear rods—intranuclear rod myopathy, Neuromuscul. Disord. 7 (1997) 13–19.

[15] H.H. Goebel, A. Piirsoo, I. Warlo, O. Schofer, S. Kehr, M. Gaude, Infantile intranuclear rod myopathy, J. Child Neurol. 12 (1995) 22–30.

[16] A. Bornemann, M.B. Peterson, H. Schmalbruch, Fatal congenital myopathy with actin filament deposits, Acta Neuropathol. 92 (1997) 104–108.

[17] H.H. Goebel, J.R. Anderson, C. Hubner, K. Oexle, I. Warlo, Congenital myopathy with excess of thin myofilaments, Neuromuscul. Disord. 7 (1997) 160–168.

[18] J.C. Sparrow, K.J. Nowak, H.J. Durling, A.H. Beggs, C. Wallgren-Pettersson, N. Romero, I. Nonaka, N.G. Laing Muscle disease caused by mutations in the skeletal muscle α-actin gene (*ACTA1*), Neuromuscul. Disord., in press.

[19] E.S. Hennessey, A. Harrison, D.R. Drummond, J.C. Sparrow, Mutant actin: a dead end, J. Muscle Res. Cell Motil. 13 (1992) 127–131.

[20] P. Sheterline, J. Clayton, J.C. Sparrow, (1998) Protein Profile: Actin, fourth ed., Oxford University Press, New York, 1987.

[21] S.E. Hitchcock-DeGregori, Structure–function analysis of thin filament proteins expressed in *Escherichia coli*, Cell Motil. Cytoskeleton 14 (1989) 12–20.

[22] R. Karlsson, Expression of chicken β-actin in *Saccharomyces cerevisiae*, Gene 68 (1988) 249–257.

[23] P. Aspenström, R. Karlsson, Interference with myosin subfragment-1 binding by site-directed mutagenesis of actin, Eur. J. Biochem. 200 (1991) 35–41.

[24] J.M. Buzan, C. Frieden, Yeast actin: polymerization kinetic studies of wild type and a poorly polymerizing mutant, Proc. Natl. Acad. Sci. USA 93 (1996) 91–95.

[25] E.A. Fyrberg, J.W. Mahaffey, B.J. Bond, N. Davidson, Transcripts of the six *Drosophila* actin genes accumulate in a stage- and tissue-specific manner, Cell 33 (1983) 115–123.

[26] C.C. Karlik, M.D. Coutu, E.A. Fyrberg, A nonsense mutation within the act88F actin gene disrupts myofibril formation in *Drosophila* indirect flight muscles, Cell 38 (1984) 711–719.

[27] J. Sparrow, M. Reedy, E. Ball, V. Kyrtatas, J. Molloy, J. Durston, E. Hennessey, D. White, Functional and ultrastructural effects of a missense mutation in the indirect flight muscle-specific actin gene of *Drosophila melanogaster*, J. Mol. Biol. 222 (1991) 963–982.

[28] J.E. Molloy, J.E. Burns, J.C. Sparrow, R.T. Tregear, J. Kendrick-Jones, D.C. White, Single molecule mechanics of heavy meromyosin and S-1 interacting with rabbit or *Drosophila* actins using optical tweezers, J. Biophys. 68 (1995) 298s–305s.

[29] T.L. Solomon, L.R. Solomon, L.S. Gay, P.A. Rubenstein, Studies on the role of actin's aspartic acid 3 and aspartic acid 11 using oligodeoxynucleotide-directed site-specific mutagenesis, J. Biol. Chem. 263 (1988) 19662–19669.

[30] D.R. Sheff, P.A. Rubenstein, Identification of *N*-acetylmethionine as the product released during the NH2-terminal processing of a pseudo-class I actin, J. Biol. Chem. 264 (1989) 11491–11496.

[31] C.D. Richardson (Ed.), Methods in Molecular Biology, Baculovirus Expression Protocols, Humana Press, New Jersey, 1995.

[32] P.A. Akkari, Y. Song, S. Hitchcock-DeGregori, L. Blechynden, N.G. Laing, Expression and biological activity of Baculovirus generated wild-type human slow α-tropomyosin and the Met9Arg mutant responsible for a dominant form of nemaline myopathy, Biochem. Biophys. Res. Commun. 296 (2002) 300–304.

[33] K.J. Nowak, Genetic and functional genetic investigations of neuromuscular disorders, Ph.D. Thesis, Murdoch University, Western Australia, 2002.

[34] K. Sano, K. Maeda, M. Oki, Y. Maeda, Enhancement of protein expression in insect cells by a lobster tropomyosin cDNA leader sequence, FEBS Lett. 532 (2002) 143–146.

[35] J. Sambrook, D.W. Russell, Molecular Cloning: A Laboratory Manual, third ed., Cold Spring Harbor Laboratory Press, New York, 2001.

[36] R.K. Cook, P.A. Rubenstein, The generation and isolation of mutant actins, in: K.L. Carraway, C.A.C. Carraway (Eds.), The Cytoskeleton: A Practical Approach, IRL Press at Oxford University Press, New York, 1992, pp. 99–122.

[37] J.D. Pardee, J.A. Spudich, Purification of muscle actin, Methods Enzymol. 85 (1982) 164–181.

[38] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[39] J. Buzan, J. Du, T. Karpova, C. Frieden, Histidine-tagged wild-type yeast actin: its properties and use in an approach for obtaining yeast actin mutants, Proc. Natl. Acad. Sci. USA 96 (1999) 2823–2827.

[40] D.R. Drummond, E.S. Hennessey, J.C. Sparrow, The binding of mutant actins to profilin, ATP, and DNase I, Eur. J. Biochem. 209 (1992) 171–179.