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# Expression of Recombinant Actin 5C from Drosophila in the Methylotrophyc Yeast *Pichia Pastoris*

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**Abstract**—A system of expression for the foreign actin gene in yeast cells *Pichia pastoris* has been developed. As a target protein, the Drosophila cytoplasmic actin 5C, which has 90% homology to the β-actin of higher eukaryotes, was used. In the present work, in order to develop conditions for biosynthesis of the target protein in yeast cells and a purification procedure for the recombinant protein, a GFP-actin fusion protein containing green fluorescent protein (GFP) as a fusion tag was expressed and purified. The size and survival of *P. pastoris* cells producing recombinant protein were characterized and shown to depend on the accumulation of recombinant actin. The purified fusion protein was used to obtain a polyclonal antibody necessary for testing for recombinant actin.

*Key words*: recombinant actin, green fluorescent actin, GFP-actin fusion protein, *Pichia pastoris* actin cytoskeleton.

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## INTRODUCTION

Actin is one of the major proteins of the muscle contraction system and cytoskeleton of non-muscle cells of eukaryotes (Levitskii et al., 1995; Sheterline et al., 1998; Pollard et al., 2000). At low ionic strength, actin exists in solution as a monomer (G-actin). In the presence of neutral salts it is polymerized into a double-stranded polymer (the fibrous form of actin, or F-actin).

The 3-dimensional structure of actin has been determined for actin complexes with proteins preventing polymerization: DNase I (Kabsch et al., 1990), gelsolin (McLaughlin et al., 1993; Robinson et al., 1999), and profilin (Schutt et al., 1993), as well as for ADP-actin unbound to other proteins and modified by tetramethylrhodamin-5-maleimide (Otterbein et al., 2001). Globular G-actin (42 kDa) consists of a single polypeptide chain of 375 amino acid residues forming two domains, each composed of two subdomains. The macromolecule contains nucleotide (ATP or ADP) and the tightly bound $Mg^{2+}$ cation. In studies of actin in vitro the $Mg^{2+}$ cation usually is replaced by $Ca^{2+}$ ion. The nucleotide and the bivalent ion are located in a cleft between domains. Actin is a highly conserved protein, thus homology between actin α-isoforms from skeletal muscles and the cytoplasmic β-actin of higher eukaryotes is greater than 90% (Vandekerckhove, Weber, 1978).

In the thin filaments of muscle tissue, actin is present in its fibrous form. Although under physiological conditions actin is polymerized, in the cytoplasm of eukaryotes it can be present both in a fibrous and a monomeric form. The processes of actin polymerization–depolymerization in the cell are determined by its interaction with minor regulatory proteins (Pollard et al., 1994, 2000). Due to the unique role of actin in the generation of movement in both muscle and in non-muscle cells, the processes of its polymerization and interaction with regulatory proteins are the subjects of intensive study (Levitskii et al., 1995; Sheterline et al., 1998; Pollard et al., 2000). Great attention has also been paid to actin's structure, dynamics, and structural conversions, as well as to its spectral properties and the problem of its folding (Bertazzon et al., 1990; Le Bihan, Gicquaud, 1993; Kuznetsova et al., 1999a, 1999b, 1999c; Schuller et al., 2000; Turoverov et al., 1999, 2002). Obtaining mutant recombinant forms of this protein with amino acid point mutations (Schuler et al, 2000; Doyle et al., 2001) could be a powerful tool in the performance of such studies.

The goal of the present study was the development of a highly efficient system of actin expression based on

the yeast *Pichia pastoris*. Drosophila cytoplasmic 5C actin, which is 90% homologous to the β-actin of higher eukaryotes and whose gene has been cloned and can easily be used in various gene-engineering constructions (Fyrberg et al., 1981; Verkhusha et al., 1999) was chosen as a target protein. To establish conditions for the biosynthesis of the target protein in yeast cells and procedures for purification of recombinant actin, a GFP-actin fusion protein containing the green fluorescent protein (GFP) (which was first isolated from the jellyfish *Aequorea victoria* and which has been increasingly used recently (Prasher et al., 1992; Chalfie et al., 1994; Ludin, Matus, 1998) for the visualization of gene expression and protein behavior in living cells and tissues) was expressed and purified.

## MATERIALS AND METHODS

**Construction of plasmid.** The plasmid pPIC3-GFP-actin 5C was created in the following manner. The plasmid pRmHa-3 (Bunch et al., 1988) was cut with the EcoRI and KpnI restriction enzymes, and cDNA for the GFP gene, amplified by PCR and lacking a stop-codon, was cloned into it. As a result, the pRmHa-3-GFP plasmid was obtained. A KpnI–BamHI fragment encoding 5C actin with an additional 15 nucleotides at the 5' end coding for the Gly-Thr-Ala-Ser-Ala amino acid sequence was amplified and ligated into the plasmid adjacent to the 3' end of the GFP-gene. As a result, a nucleotide sequence encoding the GFP-Gly-Thr-Ala-Ser-Ala-actin 5C fusion protein was created. Finally, the BamHI-EcoR fragment I (1900 nucleotide pairs) containing GFP–actin 5C was amplified and introduced into the pPIC3 expression vector which is appropriate for use in *P. pastoris*, to create the pPIC-3-GFP-actin 5C plasmid.

**Transformation and selection.** The *P. pastoris* GS115 strain, which is mutant for *HIS4* was transformed by plasmids pPIC-3-actin 5C and pPIC-3-GFP-actin 5C that were linearized with *Stu*I (10–20 μg per sample) in the *HIS4 region.* Transformation was performed with the aid of lithium chloride by the method described for *Saccharomyces cerevisiae* (Ito et al., 1983). Denaturated salmon sperm DNA fragments (25 μl per sample) were used at a concentration of 2 mg/ml as "helper" DNA.

$His^+$ transformants were selected on histidine-free medium. Clones carrying pPIC-3-GFP-actin 5C were studied cytologically using the fluorescence of the green fluorescent protein, as well as immunochemically using an antibody to GFP. To achieve superexpression, several transformants were cultivated until log-phase in a medium with glycerol and then transferred into methanol medium which induced synthesis of the fusion protein. Preparation of media for cultivation of *P. pastoris*, transformation and induction of the cloned protein were performed in compliance with the Invitrogen (USA) manual. To maintain induction, methanol was added every 24 h for the 3 days of cultivation. Cells for microfluorescent analysis were chosen from the medium on the first, second, and third days of growth.

**Fluorescence microscopy.** The *P. pastoris* cells expressing GFP-actin 5C were studied using an Axioskop fluorescent microscope (Carl Zeiss, Germany). Images of the cells were obtained using a VarioCam (PCO) camera analyzed with the aid of Quantum Image software and processed using the Adobe Photoshop software program.

Accumulation of reactive oxygen species (ROS) in the mitochondria was judged based on fluorescence from the ROS-dependent probe—dihydrorhodamine 123.

**Flow cytofluorimetry.** The method of flow cytofluorimetry was used to assess the GFP fluorescence of actin and to compare the profile of scattering of the transformed and initial cells of *P. pastoris.* Samples were analyzed using an ACR1400 flow cytofluorimeter (Bruker, Germany) with an HBO100 lamp as the source of exciting light. Three parameters were recorded: low-angle scattering (FS), large-angle scattering, and fluorescence in the green area (525–560 nm). Fluorescence was excited at 450–485 nm. Generally, more than 10000 cells were analyzed. For quantitative comparison of the results of various experiments, the sensitivity of each parameter was brought to the unit scale by adding calibrating fluorescent globules 1.98 μm in diameter (Bruker, Germany) to the samples. For such normalization, the globule signals were placed on an appropriate area on the scale of the corresponding parameter by changing the voltage fed into a photoelectric amplifier. Cells with a fluorescence value exceeded the fluorescence of the control cells lacking GFP protein were considered to be producing GFP protein. The arbitrary value of the cell mean fluorescence was determined as the total of the fluorescence of all analyzed fluorescent cells divided by the number of these cells.

**Purification of protein.** The cell mass was disrupted 72 h after methanol induction by stirring in the presence of glass beads (425–600 μm, Sigma, USA) in buffer *A* (2 mM Tris-HCl, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, 0.005% sodium azide, pH 8.5) in the presence of 1 mM phenylmethylsulphonylfluoride, a group of protease inhibitors (at concentrations recommended by Boehringer Mannheim, Germany), and the acetone powder of rabbit muscle actin (0.03 g powder per 1 g of cells). Then the material was centrifuged for 10 min at 10000 μg using a K-24 centrifuge, (Janetzki). Supernatant was loaded onto DNase-I-Sepharose, which was washed out after protein sorption using the initial buffer *A* followed by buffer *A* containing 10% glycerol and 10% formamide. Protein was eluted with buffer *A* containing 10% glycerol and 40% formamide. Since 40% formamide causes denaturation of actin, the eluted protein was immediately diluted 5 times with buffer *A* (Karlsson, 1988).

**Characteristics of recombinant protein.** The degree of purity of the GFP-actin 5C was determined electrophoretically in the presence and absence of SDS. Western blotting was performed as described by Verkhusha et al. (1999). Nativity of the recombinant actin and the GFP-actin 5C fusion protein was also determined using tryptophan and green chromophore intrinsic fluorescence. Fluorescent measurements were performed using a device described by Turoverov et al., (1998). The tryptophan and the green chromophore GFP fluorescence were excited at 297 and 480 nm, respectively. Fluorescence intensity was corrected for device sensitivity.

**Preparation of polyclonal antibody to the fusion protein.** For additional purification the fusion protein electrophoresis in a polyacrylamide gel without SDS was performed, and the zones of green fluorescence were cut out of the gel. The polyacrylamide gel pieces containing the target protein were homogenized with a small amount of saline and complete or incomplete Freund adjuvant was added. Rabbits were immunized with the obtained suspension subcutaneously in the back. This immunization was performed 3 times every 2 weeks. The complete adjuvant was used only for the first stage of immunization. Blood collection was performed 10 days after the last immunization. Blood was incubated for 2 h at 37°C and serum was separated by centrifugation. Immunoglobulins were separated by sedimentation in 1.75 M ammonium sulfate. After elimination of salt, the protein was lyophilized. Electrophoresis in 7.5% PAAG with SDS and electrotransfer onto nitrocellulose membranes were performed using the standard procedure.

## RESULTS AND DISCUSSION

As shown by Frankel et al. (1990) recombinant actin cannot be obtained in a bacterial system. We have also failed to obtain recombinant 5C actin and the GFP-actin 5C fusion protein in a bacterial system by means of bacterial expression vectors. The proteins were found in the insoluble fraction and were accumulated in inclusion bodies.

Therefore, we decided to use an eukaryotic system to obtain actin and its analogues. Such a possibility, although with a low yield level of 40 μg/l, was earlier shown for chicken β-actin using *Saccharomyces cerevisiae* (Karlsson, 1988). In the subsequent works of these authors the protein yield was increased 40 times. This was achieved mainly by a more effective disintegration of yeast cells and an increase in the volume of the DNase I-sepharose column (Aspenstrom et al., 1992). We decided to use the advantages of another lower eukaryote—the yeast *P. pastoris*. The peculiarities of protein processing, folding, and posttranslational modifications (glycosylation) in *P. pastoris* are more similar to those of the higher eukaryotes. *Pichia pastoris* is a methylotrophic yeast able to efficiently utilize methanol as its only source of carbon.

At the first stage, methanol metabolism in yeast cells consists in its oxidation to formaldehyde under the effects of two alcohol oxidases, AOX1 and AOX2, which are homologous to each other at the 97% level. Since the AOX1 enzymes are characterized by a low affinity to oxygen, their high total activity in cells is due to synthesis of a large amount of the enzymes. Intensified AOX synthesis was provided when methanol activated the superpowerful promoter of the *AOX*. gene. We used the pPIC3 yeast vector which was constructed such that the actin gene was controlled by the *AOX1* promoter. Therefore, the actin gene was activated in the presence of methanol as effectively as the native *AOX1* gene.

The *P. pastoris* cells were transformed by the pPIC3 vector which apart from the fused GFP and actin genes, also contained the intact histidinol dehydrogenase (*HIS4*) gene which was inactivated in the GS115 strain that we used. Since the plasmid pPIC3 is integrative, i.e., it has no yeast replication origin, $His^+$ transformants could be obtained only as a result of recombination between the plasmid and the genome of *P. pastoris*. Linearization of the vector at a unique site in *HIS4* was performed to stimulate the integration of the plasmid into the chromosomal *HIS4* gene. Thus, by selecting for $His^+$ transformants on synthetic medium lacking histidine we selected cells that recombined the Drosophila GFP-actin 5C fusion protein gene into their own genome. In a similar manner, *P. pastoris* clones were obtained that contained the wild-type actin gene of Drosophila and its mutant analogues.

Cultivation of the transformants under conditions where methanol was the only source of carbon stimulated expression of the GFP-actin 5C fusion protein and thereby controlled its induction. The GFP fluorescence allowed visualization of actin synthesis.

The form of the fluorescent structures in the *P. pastoris* cells was found to change depending on the growth stage of the culture (Fig. 1). In yeast cells at the initial stage of intensive cell division (the exponential growth phase) formed actin patches were seen, which did not always appear in the buds. At the later stages of culture growth, fluorescent cables and cross-like structures were clearly seen. Such structures are characteristic of the actin cytoskeleton in a state where actin is polymerized and forms microfilaments.

It seems probable that for the first 24 h of induction the level of the GFP-actin expression is still low. This is especially true for buds, as the budding rate at this stage of the culture growth exceeds the rate of the GFP-actin accumulation. The GFP-actin patches are unevenly distributed between the maternal and daughter cells and remain predominantly in the older, maternal, cells (Fig. 1a). However, after the first day of the culture growth on methanol a swift accumulation of the cells occurs in the culture in which the GFP-actin reaches the threshold value, i.e., the expression level sufficient for visualization of other typical structural components of

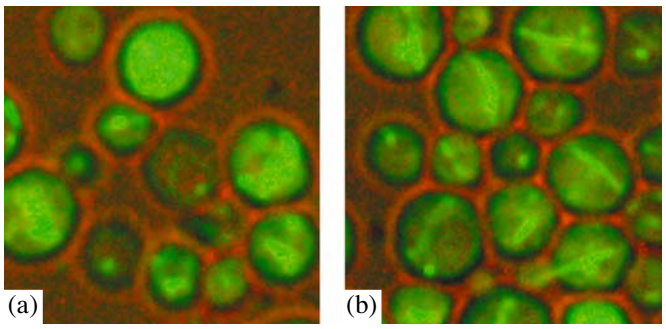


**Fig. 1.** Expression of actin in cells of yeast *P. pastoris.* Actin structures are presented in *P. pastoris* cells cultivated on the methanol-containing medium after 1 day (a) and 2–3 days (b).

the actin cytoskeleton (Fig. 1b). The older the culture, the more visualized the crossing and branching fibrillar structures.

*The ability to observe structures characteristic of the cytoskeleton in the condensed state using GFP fluorescence indicates that the foreign Drosophila actin of the GFP-actin fusion protein is copolymerizing with the yeast actin in vivo and thus is functionally active.*

Earlier it has been shown that the presence of actin monomer structures (patches) is vitally important, whereas the mutation *tmpl* which leads to the absence of traced actin cables or fibrils is not lethal (Karpova et al., 1998). Since the authors failed to find mutants with cables in the absence of patches, it was suggested that the patches were required for the assembly of cables. In *S. cerevisiae* transformants containing the actin-GFP fusion protein, only the monomer fluorescent actin was observed, while no visible cables were revealed (Doyle, Botstein, 1996). Observations of individual *D. discoideum* cells showed that filaments containing no more than 30% actin-GFP had an almost normal motility, whereas in the filaments with a higher hybrid protein level, sliding on the other molecules in the in vitro experiments was hampered (Westphal et al., 1997). The authors explain this by disturbances of the interaction of actin-GFP with myosin in the cytoskeleton structure. The hybrid protein expression in vivo was very low and it was impossible to trace its localization to some cellular structure. Comparison of our results with such literature data (Doyle, Botstein, 1996; Westphal et al., 1997) allows us to suggest that the hybrid protein N-terminal GFP construction that we made is much more functionally active than the C-terminal one.

It is to be noted that in the obtained *P. pastoris* transformants, GFP-actin is synthesized in cells regardless of the carbon source in the medium. In the absence of induction when cells are cultivated for 2 days in glycerol-containing medium, structures typical of actin also can be observed under a luminescent microscope (Fig. 2). However, in this case the GFP-actin synthesis level seems to be significantly lower than in the presence of methanol, which is indicated by the more than fourfold photoexposition (Figs. 2a–2c—700 μs, Figs. 2d–2f—3 s). Thus, it has been shown that *the recombinant actin is produced in the cell both under inducing conditions of cultivation (in the presence of*

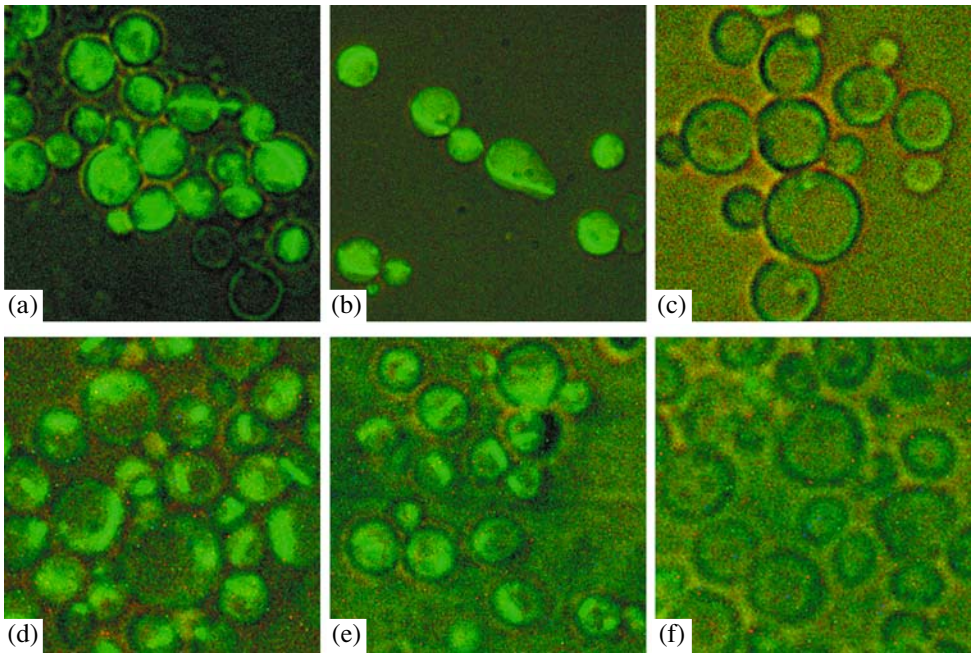


**Fig. 2.** Expression of GFP-actin in transformed *P. pastoris* cells grown on methanol (a–c) and glycerol (d–f). The initial *P. pastoris* strain (c, f) and transformants tr30 (a, d) and tr2 (b, e) are presented.

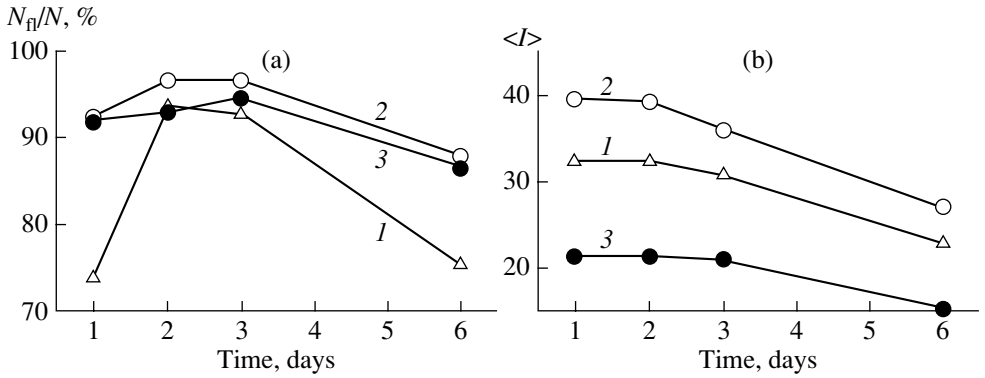


**Fig. 3.** Change of fraction of fluorescent cells (a) and the mean fluorescence intensity per one cell (b) in three independent GFP-actin-expressing transformants tr 2 (1), tr 20 (2), and tr 30 (3) after induction by methanol.

*methanol) and under non-inducing conditions (in the medium with glycerol). The difference consists only in the amount of the synthesized protein.*

By the method of cytophotometry, the dynamics of accumulation of the hybrid protein was assessed from the change in the fluorescence intensity of its GFP portion. During 6 days of cultivation of three transformants in the methanol-containing medium, the number of fluorescent cells and intensity of their fluorescence were measured (Fig. 3). In spite of the fact that the process of formation of cables begins about 24 h after the moment of the appearance of patches (i.e., monomer actin formation), it has little affect on the accumulation of fluorescent label. Based on the data presented in Fig. 3, it can be suggested that the hybrid protein concentration changes little after the first 24 h, although has a tendency to decrease which is evident after 4 days of methanol induction. As seen in Fig. 3, the fraction of fluorescent cells was approximately 100% in the tr20 and tr30 transformants after 24 hours of cultivation, while in the tr2 transformant it was 78% and rose practically to 100% as early as at the second day of cultivation with methanol. The mean intensity of the cell fluorescence (Fig. 3b) turned out to be a more variable trait that depends both on the culture age and on the transformant.

Effect of foreign actin on ROS accumulation and survival of *P. pastoris* cells

| Strain of *P. pastoris* | Percent of cells (%) containing ROS on the medium | | Relative survival on methanol |
|---|---|---|---|
| | with glycerol | with methanol | |
| tr 2 | 29 | 25 | 0.96 |
| tr 20 | 22 | 39 | 0.36 |
| tr 30 | 4 | 9 | 0.29 |
| GS 115 (initial) | 20 | 21 | 0.99 |

Note: Data of two repeats were totaled, in each of them no less than 300 cells were counted; ROS—reactive oxygen species.

One of the important characteristics of the cells producing recombinant protein is the dependence of their survival on the presence of the foreign protein. Until the present the effect of foreign actin on cell survival has not been studied. It has been shown that in *Saccharomyces* yeasts cell aging occurs by the mechanism of apoptosis whose "key" reaction is the accumulation of reactive oxygen species (ROS) in mitochondria. An active role of the actin cytoskeleton in the regulation of apoptosis in *Saccharomyces* has been shown, this role increases with cell age (Gourlay et al., 2004). By studying mutants with different SCP1 protein activities, these authors established that the level of this protein that binds F-actin and thereby restricts its dynamics is proportional to depolarization of mitochondrial membranes and ROS production. Actin is suggested to be associated with the channels of mitochondrial membranes, where it helps them to open and close. The deceleration of actin dynamics seems to result in pores or channels remaining open longer than necessary, which results in an augmentation of the release of cytochrome *c* into cytosol. Cytochrome *c* is believed to play a particular role in yeast apoptosis, as on its release into the cytosol it activates the yeast caspase YCA-1 (Karpova et al., 1998). At the same time, the increase in actin dynamics decreases the time for which the membrane pores remain open. This decreases the release of cytochrome *c* and increases the cell's lifespan.

We have demonstrated the different responses of various transformants to the presence of foreign actin under long cultivation in medium inducing its synthesis (Fig. 4). We determined the accumulation of ROS in aged (14-day-old) cells of the transformants and compared it with ROS accumulation in the initial strain. To determine ROS, dihydrorhodamin was used. The data in the table indicate that different transformants grown on glycerol and methanol have different accumulations of ROS in their mitochondria.

In the tr2 transformant, growth on methanol was not accompanied by an increase in the number of cells accumulating ROS, whereas in tr20 and tr30, the num-

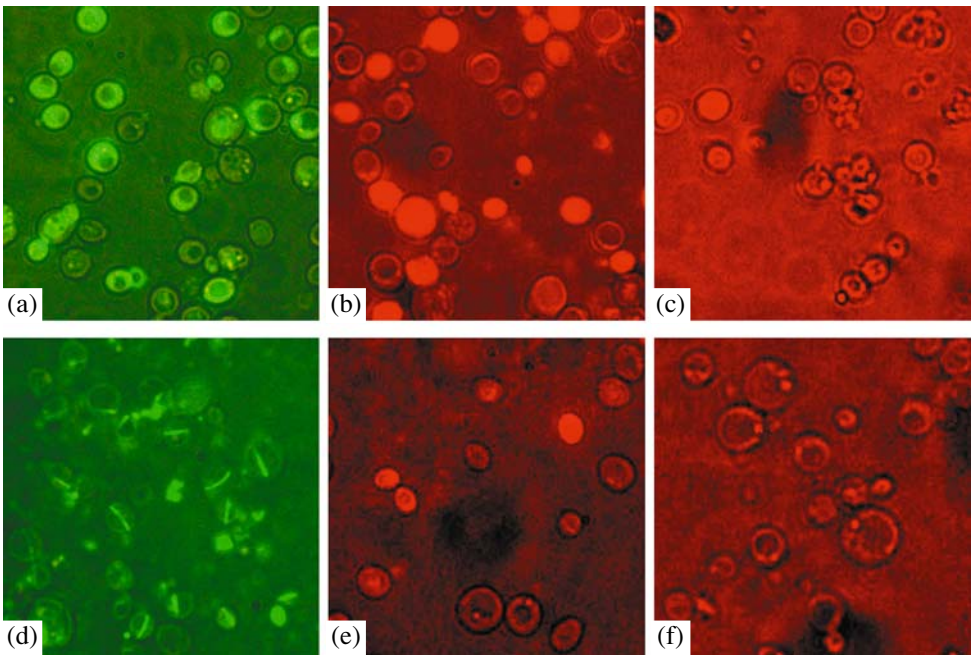


**Fig. 4.** Fluorescence of cells of two independent GFP-actin-expressing transformants: tr 20 (a, b, c) and tr 30 (d, e, f).
(a) and (d) actin-containing structures visualized by GFP fluorescence in cells grown on methanol; (b), (e) and (c), (f) fluorescence of ROS-sensitive probe dihydrorhodamine 123 in cells after long cultivation on the medium with methanol (b, e) or glycerol (c, f).

ber of the ROS-containing cells was higher on methanol than on glycerol. To find out whether the excessive ROS accumulation correlates with a decrease in survival, we investigated the comparative cloning efficiency on methanol in the following experiment.

Cells grown for 2 weeks on solid media with methanol or with glycerol were suspended in water. Their optical density peaked at a certain value ($OD_{600}$ = 1.3), then the cells were plated on a standard complete nutritional medium with glucose. Cells were plated after a

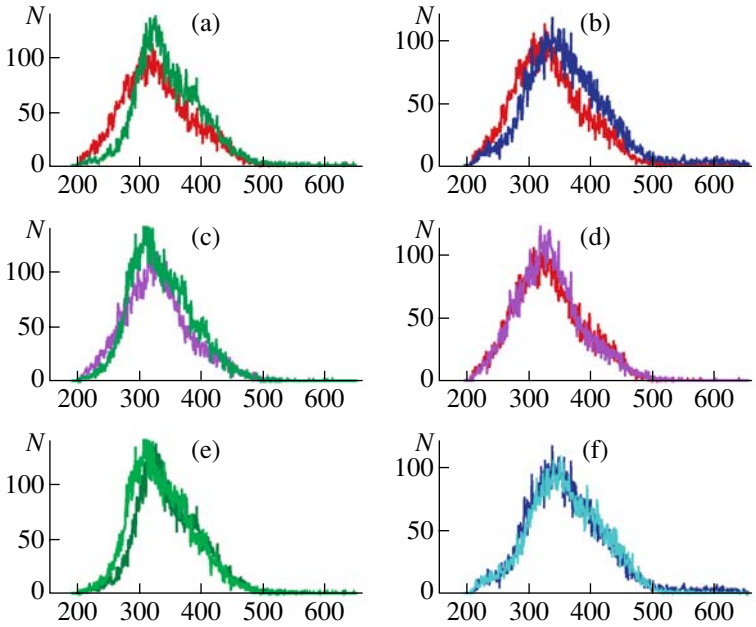


**Fig. 5.** Histograms of light scattering by initial and transformed *P. pastoris* cells containing various plasmids and grown on synthetic medium with methanol.
*P.p.*1 (*red curves on panels* (a), (b), and (d)) and *P.p.*2 (*pink curves on panels* (c) and (d)) cells of different clones of the initial *P. pastoris* culture, tr2 and tr30 (*dark- and light-green curves on panels* (a), (e) and (c), (d), respectively) transformants carrying recombinant GFP-actin; tr6 and tr7 (*blue and light-blue curves on panels* (b) and (f), respectively) transformants carrying the Drosophila recombinant actin without GFP. Here and in Figs. 6, 7, and 8, *N*—the number of cells.

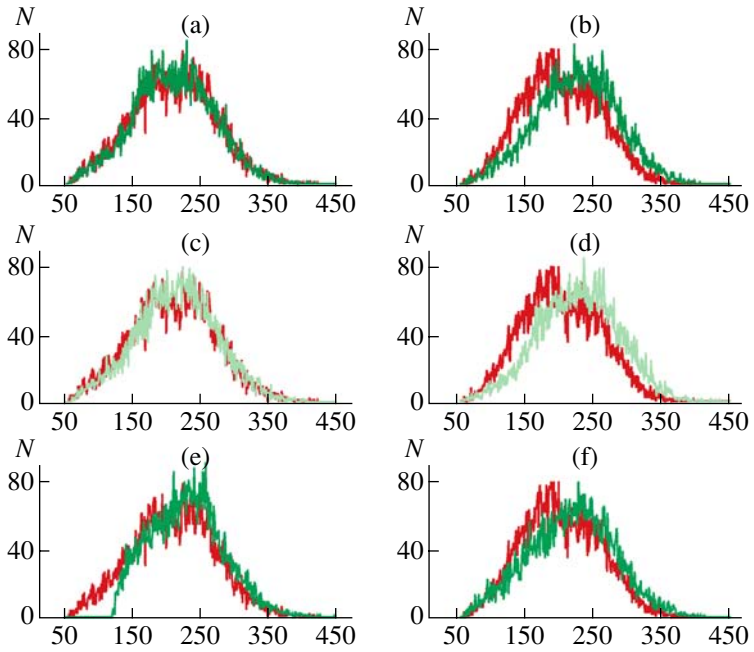


**Fig. 6.** Histograms of light scattering by cells grown on the complete nutritional medium with glycerol (a, c, and e) or methanol (b, d, and f).
*P.p.*1—*red curve*; tr2, tr20, and tr30—*dark-green, grey*, and *light-green curves*, respectively.

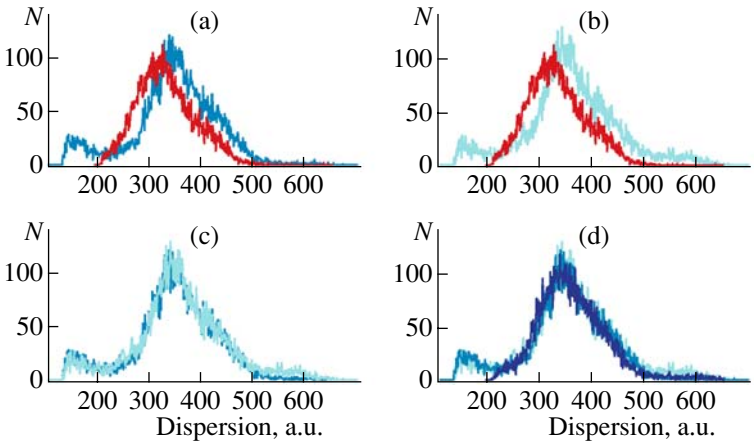


**Fig. 7.** Histograms of light scattering by cells of the initial *P. pastoris* strain and transformants carrying Drosophila non-mutant actin (tr6) and mutant actin forms (trC, trD).
(a), (b): *P.p.*1—*red curves*, trD and trC—*blue* and *light-blue curves*, respectively; (c) trD and trC (*light-blue* and *blue curves*, respectively); (d) trD, trC, and tr6 (*light-blue, blue*, and *bright-blue curves*, respectively).

$10^5$ times dilution, so that the colonies grown on Petri dishes could be easily counted. The ratio of the number of cells grown on methanol to that of cells grown on glycerol reflects the relative survival of cells on methanol.

The data presented in the table indicate that survival of different transformants on methanol is different and correlates with the data on ROS accumulation. In tr2, as in the control (i.e., in the initial strain GS115) ROS accumulation on media with methanol and glycerol differed very little and survival on both media also was equal. In tr20 and tr30, the ROS accumulation was more effective in the cells grown on methanol and their survival on methanol for 14 days decreased to about 30%.

The cause of the different response of different transformants to the presence of foreign actin remains unclear. Given that the cells of the transformants are genetically identical, these differences should be based on some epigenetic differences, for instance, on different prion status. It can be suggested that these differences are connected with the interaction of the actin

cytoskeleton with prions whose level may differ in different cells. The association of the proliferation of the prion Sup35 with the cytoskeleton was shown in *Saccharomycetes* yeast (Bailleul-Winslett et al., 1999, 2000).

The effect of the synthesis of foreign protein on the number and shape of the cells was shown by flow cytometry from the change in the profile of cell scattering. Figure 5 presents histograms of the scattering of the *P. pastoris* cells—initial and transformed, carrying the recombinant actin gene, grown in methanol-containing synthetic medium. As seen from this figure, different clones of the strain GS115 give identical histograms upon cultivation under the same conditions (Fig. 5d). Cells carrying the GFP-actin fusion protein (tr2 and tr30) or recombinant actin without fusion protein (tr6 and tr7) almost do not differ (Figs. 5e and 5f, respectively). However, as compared with non-transformed cells, all transformants are characterized by a small shift to the right (Fig. 5a–5c).

Data obtained on cells grown in the complete nutritional media differing by the source of carbon confirm that methanol is the factor that induces the above-described differences.

The tr2, tr20, and tr30 cells grown on glycerol show the same scattering as the initial *P. pastoris* strain (Figs. 6a, 6c, 6e). The same cells grown on medium containing methanol differ essentially from the initial strain (Figs. 6b, 6d, 6f). Mutations made in the recombinant actin gene called "C" (W340F, W356F), "D" (V10C, W340F, W356F), and "E" (W86F, W340F, W356F), do not affect the scattering profile (Fig. 7). They lead to a shift of scattering histograms in comparison with the *P. pastoris* strain (Figs. 7a–7b) and do not differ from cells with non-mutant recombinant actin (Figs. 7c–7d).

The ability of cells to utilize methanol has turned out to be an essential factor. All the above comparisons involved cells belonging to $Mut^+$ strains growing well on methanol-containing media. But Mut/s-mutants obtained from these strains poorly utilize methanol and therefore grow on it slowly, and their scattering histograms are markedly shifted to the right (Fig. 8). This slow budding appears to give the maternal cells time to reach a significant size.

*Thus, the differences in the forward light scattering histograms, which are observed in transformants as compared with those of the untransformed yeast cells, seem to reflect changes in the yeast cytoskeleton that are associated with intensive synthesis of foreign actin and affect the shape and size of cells. It is critical that this effect is observed on media with methanol leading to excessive production of recombinant actin, but is absent on media with glycerol.*

The fusion protein synthesized in yeast cells is sufficiently easily extracted from the disintegrated yeast biomass with a buffer solution commonly used to obtain rabbit globular actin. According to immunocytochemical analysis, the entire synthesized fusion protein passes into the solution. To purify the fusion protein, we used the ability of the natural native actin to be bound effectively with DNase I from bovine pancreas. In fact, during filtration of the yeast cell extract through a layer of DNase I-Sepharose a protein with green fluorescence bound to the sorbent. Protein is eluted with 40% formamide with subsequent dilution with initial buffer.

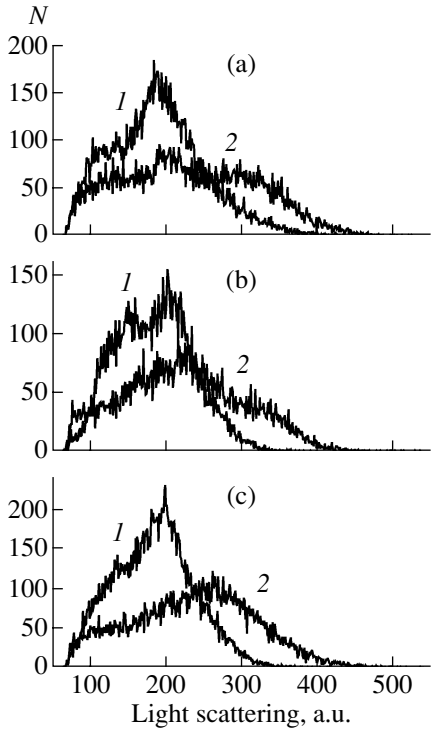


**Fig. 8.** Histograms of light scattering for transformed strains *P. pastoris* characterized by growth on the methanol-containing medium.

(a) trC, (b) trD, (c) trE; *1*—$mut^+$ strains with good growth on methanol-containing media, *2*—Mut/s-(methanol utilization slow) strains with a decreased rate of growth on methanol.

Since formamide is a denaturing agent, a special study was carried out on the effects of formamide on the structure of actin from rabbit muscles. The time dependences of the intensity of actin fluorescence were recorded. On the basis of these data kinetic curves of the changes of the parameter $A$ that characterizes the position of the fluorescence spectrum ($A = I_{320}/I_{365}$; Fig. 9) were obtained. The character of these dependences is similar to those obtained upon actin denaturation by guanidinehydrochloride (GdnHCl), with the exception that the denaturation rate is significantly lower. These results confirmed formamide to be a classic denaturant, although less powerful than urea and GdnHCl (Povarova et al., 2005). Investigation of the sucrose effect on actin has shown that the presence of 48% sucrose does not prevent, but merely decelerates the irreversible conversion of actin into the inactivated

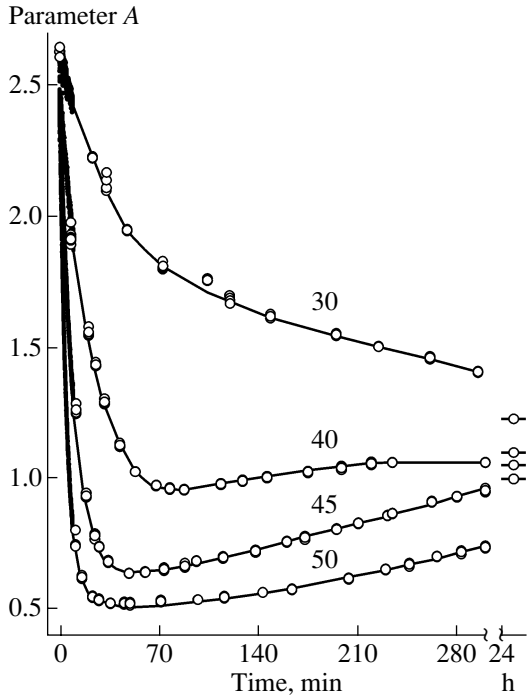


**Fig. 9.** Kinetics of denaturation of actin from rabbit muscles induced by formamide.

Time dependences of the parameter $A = I_{320}/I_{365}$ characterizing the fluorescence spectrum position are presented. The values on the curves are formamide concentration, %. Protein concentration—0.5 mg/ml; $\lambda_{exc}$ = 297 nm.

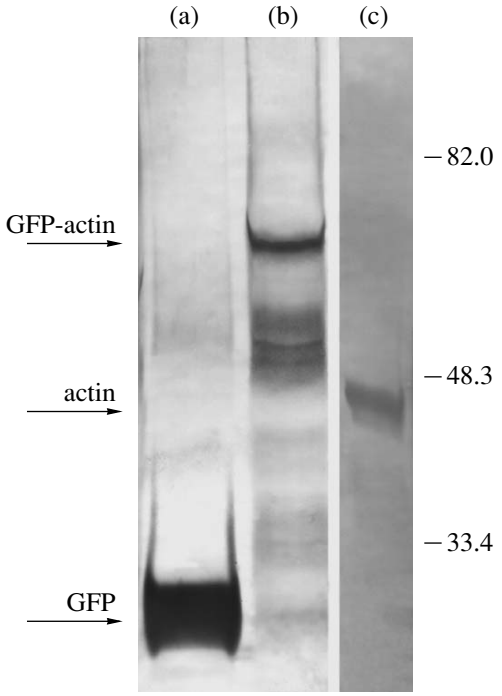


**Fig. 10.** SDS electrophoresis in polyacrylamide gel of green fluorescent protein GFP (a), GFP-actin 5C fusion protein (b), and actin from rabbit muscles (c).

state. It has also been shown that the presence of sucrose does not permit native actin to be obtained from the completely unfolded state upon a decrease in denaturant concentration.

*Thus, the column size and the elution rate are very essential factors in the production of native recombinant actin.*

According to data from fluorescence and immunochemical analysis, the eluted material contains GFP. During electrophoresis in PAAG without denaturing agents the fluorescent protein migrates more slowly than the standard GFP (Fig. 10). According to the data from electrophoresis in PAAG with SDS, this GFP-related protein has a molecular mass of 67 kDa. These results indicate that the protein obtained in this work is a fusion protein which contains an actin component (as judged from its affinity to DNase I and its fluorescent characteristics in the region of tryptophan emission) and a GFP component (as judged from immunological properties and fluorescence in the visible area). The character of the fusion protein fluorescence spectrum (Fig. 11) allows the conclusion that actin in the fusion protein has its native structure.

Polyclonal antibodies have been obtained to this protein and these will be used for further express testing of this recombinant actin and of its mutant forms synthesized in yeast.

Our results allow us to claim that we have developed a sufficiently effective system for obtaining native preparations of recombinant actins in *P. pastoris* cells. Analysis of this system in vivo has shown that the foreign actin synthesized in *P. pastoris* interacts with the

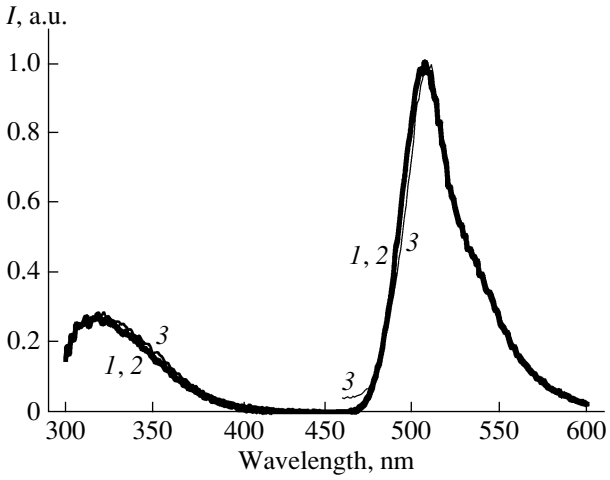


**Fig. 11.** Fluorescence spectrum of GFP-actin 5 C recombinant fusion protein.

Two fluorescence bands are characteristic of native actin and green fluorescent protein GFP. *1*, *2*, and *3*—fluorescence spectra measured at the exciting light wavelengths 280, 297, and 365 nm, respectively.

endogenous actin and affects the biological activity of these cells. Actin superproduction leads to a change in the shape and size of the cells and as well as can decrease the survival of some transformants after long cultivation on methanol.

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