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Homologous recombination in the *Dictyostelium* α-actinin gene leads to an altered mRNA and lack of the protein

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Mutation of the α-actinin gene in *Dictyostelium* has been achieved by transforming cells with the *Dictyostelium* transformation vector pDNeoII containing a 1.2 kb fragment of the α-actinin gene. Transformants deficient in α-actinin, an actin-binding protein, produced an altered mRNA that lacked the 3′ portion of the coding region. The defect in α-actinin production was not due to integration of the vector within the gene, but was apparently caused by errors produced during homologous recombination between the introduced α-actinin sequence and its complementary sequence in the coding region of the endogenous gene.

Key words: α-actinin/actin-binding proteins/*Dictyostelium*/homologous recombination

Introduction

Homologous recombination between a resident chromosomal gene and incoming sequences, which allows the specific alteration of genes in a genome, has mainly been studied in yeast and in mammalian cells. In yeast homologous recombination occurs in up to 20% of transformed cells (Orr-Weaver *et al.*, 1981), and in mammalian cells one in 10^3 cells receiving DNA undergo homologous recombination (Thomas *et al.*, 1986). When homologous recombination occurred in mammalian cells mutations were introduced at high frequency in the cognate gene (Thomas and Capecchi, 1986). These mutations resulted from an incorrect repair of a heteroduplex formed between the introduced and the chromosomal sequence.

In the course of our studies on the role of actin-binding proteins in *Dictyostelium discoideum* we attempted to abolish production of these proteins by transforming cells with antisense constructs and to modify the genes by site-directed mutagenesis using homologous recombination for introducing the mutated sequences. Actin-binding proteins are thought to interact with the actin filament system thereby regulating cell shape and motility. α-Actinin, a prominent actin-binding protein, cross-links actin filaments in a Ca^{2+}-sensitive manner. Its subunits have a mol. wt of ~97 kd and assemble in an antiparallel fashion to form a rod-like homodimer (Wallraff *et al.*, 1986). α-Actinin is encoded in *D.discoideum* by a single gene which produces a 3.0 kb mRNA (Witke *et al.*, 1986). cDNA sequencing indicated two EF-hand structures near the carboxy terminus of the polypeptide which may be the basis of the Ca^{2+}-sensitivity of α-actinin function (Noegel *et al.*, 1987). A sequence near the amino terminus proved to be highly conserved between *Dictyostelium* α-actinin and chicken fibroblast α-actinin (Baron *et al.*, 1987) and may represent the actin binding site (Noegel *et al.*, 1987).

The availability of monoclonal antibodies whose binding sites on the protein are known (Schleicher *et al.*, submitted), of cDNA clones and a simple colony blot technique for screening transformants (Wallraff *et al.*, 1986) allows us to use the α-actinin gene as a model with which to investigate mechanisms for manipulation of the genome. A *D.discoideum* mutant essentially devoid of α-actinin indicated that lack of this protein is not a lethal event (Wallraff *et al.*, 1986). *D.discoideum* offers several advantages for these studies. It is a haploid organism like yeast which can be transformed and easily cultivated. Inactivation of specific genes has been achieved in *D.discoideum* by the antisense RNA technique (Crowley *et al.*, 1985; Knecht and Loomis, 1987) and by homologous recombination (DeLozanne and Spudich, 1987). The latter mechanism led to the disruption of the myosin heavy chain gene (*mhca*). This disruption was caused by insertion of a transformation vector containing a *mhca* fragment within the 5′ flanking region of the endogenous myosin gene, which inactivated the endogenous gene, although its coding region remained intact. In the transformants only a vector-coded truncated myosin was expressed under the control of the endogenous *mhca* promoter (DeLozanne and Spudich, 1987). Mutation by introduction of homologous sequences, as shown for mammalian cells (Thomas and Capecchi, 1986), or exchange of homologous sequences between the transformation vector and the cognate gene, like the gene transplacement event in yeast (Hinnen *et al.*, 1978), has not been documented so far in *D.discoideum*.

We describe here the alteration of the α-actinin gene of *D.discoideum* leading to the absence of detectable protein. Our data indicate that this alteration is not due to insertion of the transformation vector into the resident gene but due to recombination between the introduced and the chromosomal sequences which are separated in a way that leads to a nonfunctional truncated mRNA.

Results

Construction of a transformation vector allowing transcription of inserted DNA under control of the actin 6 promoter and homologous recombination

A vector designated pDNeoII was constructed that contains the *Neo*^{r} gene of transposon TN 903 under the control of the actin 15 promoter and terminator sequences (Knecht *et al.*, 1986). The *Neo*^{r} gene renders *Dictyostelium* cells resistant to G418. DNA segments under investigation can be cloned into a multiple cloning site derived from pIC20H (Marsh *et al.*, 1984). The promoter of the actin 6 gene allows efficient transcription of the inserted DNA (Nellen *et al.*, 1984). Proper termination is ensured by a tandemly repeated actin 8 terminator (Figure 1).

A genomic 1.2 kb *Eco*RI fragment (Witke *et al.*, 1986) of the α-actinin gene of *D.discoideum* was inserted into the multiple cloning site of pDNeoII in an orientation that should lead to the production of an antisense RNA. This fragment is located near the amino terminus of the α-actinin gene and contains coding information for nearly half of the α-actinin protein. The resulting plasmid was called pDabα 1.2. The structure of the α-actinin gene and of the pDabα 1.2 vector used for transformation are shown in Figure 1.

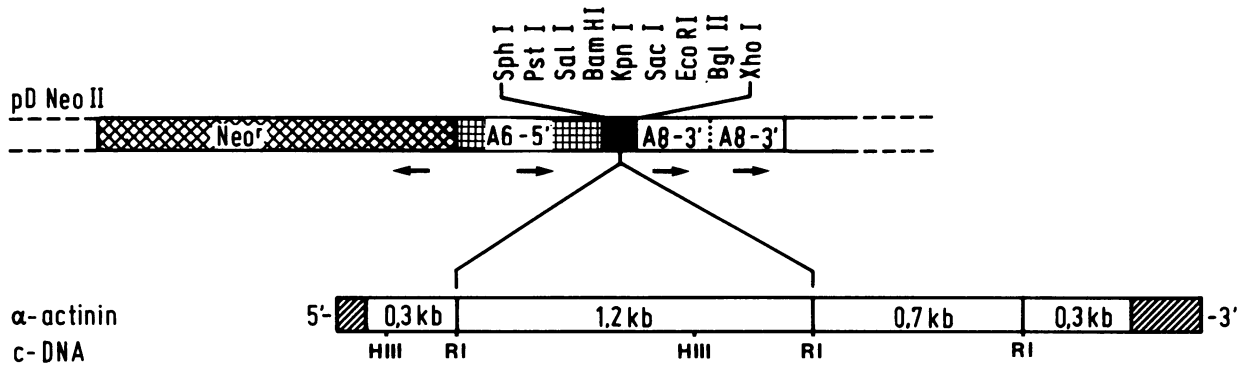

Fig. 1. Structure of the *D.discoideum* transformation vector pDNeoII (upper part) and *Eco*RI restriction map of α-actinin cDNA (lower part): pDNeoII contains the TN903 phosphotransferase gene (indicated by cross-hatching) which is under the control of the actin 15 promoter and terminator. The polylinker of plasmid pIC20H is represented by solid lines. Fragments inserted are transcribed from the actin 6 promoter (squares). Termination is ensured by two actin 8 terminators inserted as a tandem repeat. The orientation of transcription is indicated by arrows. In the lower part a full-length cDNA derived from the α-actinin gene is shown, the location of the *Eco*RI (RI) and *Hin*dIII (HIII) sites is indicated. 5′ and 3′ non-coding regions of this gene are drawn as hatched lines. The 1.2 kb internal *Eco*RI fragment is inserted into the *Eco*RI site of pDNeoII in an orientation that allows the production of α-actinin antisense RNA. The resulting transformation vector is designated as pDabα 1.2.

Isolation of α-actinin deficient transformants

Single colonies resistant to G418 were selected after transformation of *D.discoideum* strain AX2 with pDabα 1.2. These colonies were grown on a bacterial lawn and transferred to nitrocellulose. The cells were lysed on the filter by freezing and thawing and the blots probed with monoclonal antibody mAb 47-19-2 that binds to the carboxy terminal portion of α-actinin (Schleicher *et al.*, submitted). This antibody allowed us to screen for transformants that produced no α-actinin at all as well as transformants that produced truncated α-actinin polypeptides that might arise from an early stop of translation. In a first experiment five out of 48 colonies tested proved to be deficient for α-actinin (Figure 2). These colonies were further tested by immunoblot analysis with mAb 47-19-2, mAb 47-62-17 and a polyclonal antibody directed against α-actinin. mAb 47-62-17 recognizes an epitope in the middle of the polypeptide (Schleicher *et al.*, submitted) that is encoded by the 1.2 kb α-actinin insert of the pDabα 1.2 vector. The results obtained with both mAb 47-62-17 and the polyclonal antibody indicated that all four transformants lacked the entire polypeptide chain of α-actinin, rather than only a carboxy terminal portion. Transformant T-abα 3 is shown as an example in Figure 3. Probing of the immunoblot with antibodies specific for two other actin-binding proteins, severin (Figure 3) and the 120 kd gelation factor (data not shown), indicated no changes in these cytoskeletal proteins. The α-actinin-negative phenotype of the transformants proved to be stable even without continued selection for G418 resistance.

The specificity of the inactivation event was tested by transforming AX2 cells with pDNeoII and a derivative of pDNeoII carrying a DNA fragment for an actin-binding protein not related to α-actinin. None of the transformants obtained proved to be α-actinin-deficient. In comparison, transformants receiving pDNeoII carrying the 1.2 kb α-actinin fragment in either antisense or sense configuration led to the isolation of α-actinin negative clones (Table I).

Analysis of α-actinin-deficient transformants on the RNA level

Total RNA of the α-actinin-negative transformants was probed with the 1.2 kb *Eco*RI fragment for the presence of α-actinin-specific RNA. The original 3.0 kb α-actinin message could not be detected. Instead, a 1.9 kb RNA hybridized to the probe, and a minor band of ~1.6 kb was observed (Figure 4a). The 1.9 kb

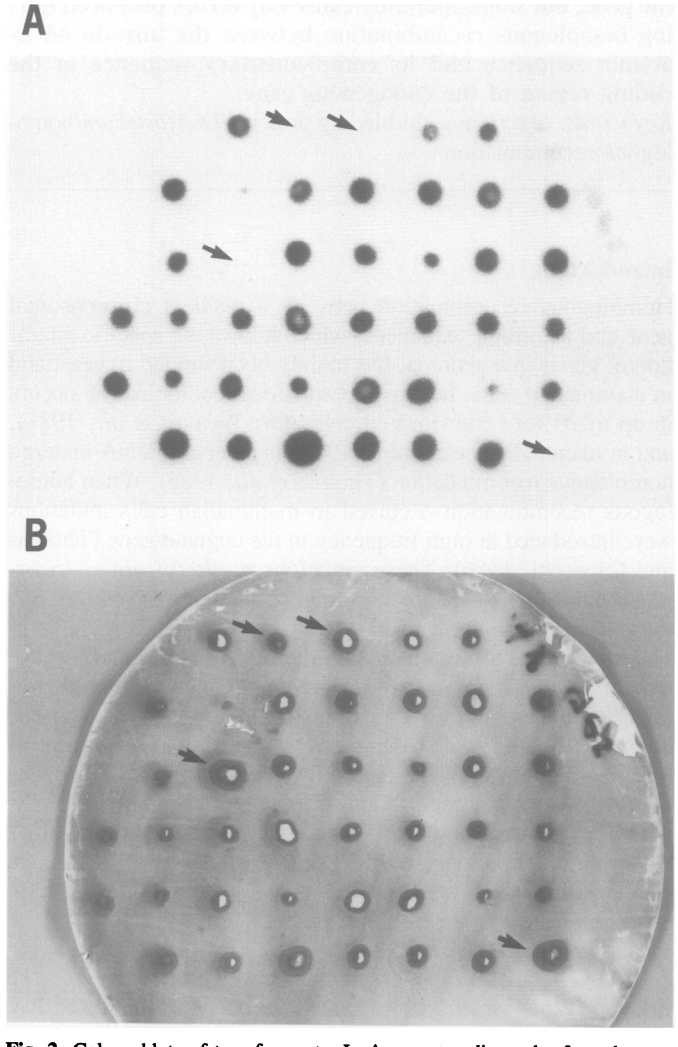

Fig. 2. Colony blots of transformants. In **A** an autoradiograph of a colony blot incubated with mAb 47-19-2 is shown, and in **B** the same filter is stained with Ponceau S to indicate the location of colonies. Arrows mark the colonies in A and B that did not react with the antibody.

RNA was further characterized by probing with cDNA-fragments that cover the complete α-actinin mRNA. The 1.9 kb RNA was recognized by a 0.3 kb *Eco*RI fragment that contained the coding

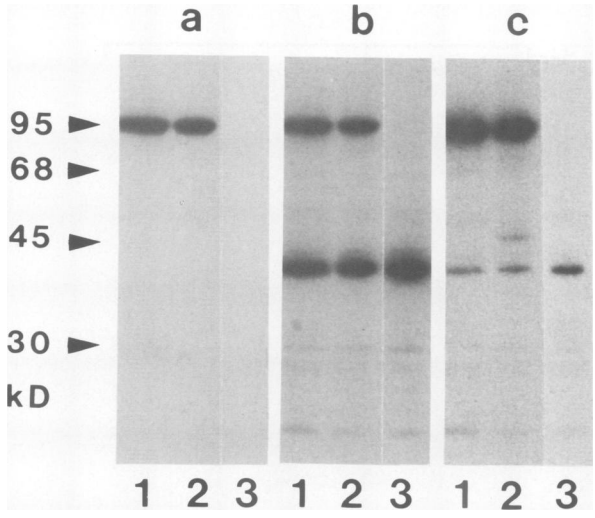

Fig. 3. Immunoblots of total cellular proteins from strain AX2 and various transformants of that strain. Equal amounts of proteins from growth phase cells of strain AX2 (**lane 1**) and of transformants T-abα 8 (**lane 2**) and T-abα 3 (**lane 3**) were separated by SDS–PAGE on a 10% acrylamide gel, transferred to nitrocellulose and incubated with α-actinin-specific mAb 47-19-2 (**a**), severin-specific mAb 101-460-2 (**b**), or α-actinin-specific mAb 47-62-17 (**c**). The same blot was successively probed with these antibodies. Labeling of α-actinin can therefore be seen in **b** and labeling of severin in **c**.

sequence for the amino terminus of α-actinin but not by fragments that were located close to the 3′-end of the α-actinin (Figure 4b). Strand-specific probes showed that this 1.9 kb RNA corresponded to the coding strand of α-actinin (Figure 4c). It was not recognized by sequences that constitute the transformation vector, such as pUC19 sequences or actin 6 sequences. The accumulation pattern of this RNA species during development differed from the pattern of actin 6 mRNA but was comparable to the accumulation of α-actinin (data not shown). Actin 6 mRNA is present at very low levels in growing cells and is induced significantly during pre-aggregative development. During post-aggregative development the actin 6 mRNA decreases (Knecht *et al.*, 1986). α-Actinin mRNA is substantially expressed in growing cells, and its amount increases slightly during early development and afterwards remains nearly constant during the following stages until it decreases shortly before fruiting body formation (Witke *et al.*, 1986). Our results suggest that the 1.9 kb RNA represents a truncated α-actinin RNA that is still under the control of its resident promoter and contains only the coding region for the amino terminal portion of the polypeptide. S1-mapping performed with RNA from T-abα 3 was in accord with this interpretation (data not shown). The 1.9 kb RNA in the transformant T-abα 3 was protected from degradation by the 1.2 kb *Eco*RI fragment but not by the neighboring 0.7 kb *Eco*RI fragment that is located near to the 3′ end of the α-actinin gene.

The minor RNA species at 1.6 kb hybridized exclusively with the 1.2 kb α-actinin fragment. A positive signal obtained with a single-stranded probe corresponding to the coding strand indicated that it was an α-actinin-specific antisense RNA (Figure 4c). The majority of transformants isolated were still positive for α-actinin. They contained the original 3.0 kb α-actinin message as shown for T-abα 8. A 1.6 kb RNA was detected also in these transformants using as a probe the 1.2 kb *Eco*RI fragment present in the transformation vector (Figure 4a). A single-stranded RNA probe specific for antisense RNA also recognized this RNA (Figure 4c). Its size and its hybridization pattern showed that it was antisense RNA transcribed from the introduced vector. The coexistence of endogenous α-actinin mRNA and antisense RNA indicates that the amount of antisense RNA made is not sufficient to inhibit α-actinin production.

Table I. α-Actinin phenotype is dependent on insert sequences

	pDNeoII	Control gene	pDabα 1.2 antisense	pDabα 1.2 sense
No. of transformants	325	311	48	27
α-actinin$^+$ transformants	325	311	43	24
α-actinin$^-$ transformants	None	None	5	3

AX2 was transformed with pDNeoII, pDNeoII containing a *D.discoideum* control gene, and pDNeoII containing α-actinin sequences either in sense or antisense orientation.

Analysis of α-actinin-deficient transformants on the DNA level

The change in the mol. wt of the α-actinin-specific RNA in the α-actinin-deficient transformants could be due to an insertion of the transformation vector into the α-actinin gene in such a way that a termination signal was created that led to a premature stop of the message. All *D.discoideum* transformation vectors described so far integrate into the chromosome as a tandem array. The copy number can vary from less than five copies to more than 200 copies (Nellen *et al.*, 1984; Knecht *et al.*, 1986). Integration into the α-actinin gene should lead to an altered hybridization pattern that can be recognized in Southern blots with different parts of the α-actinin gene as probes. Figure 5 shows the analysis of various restriction enzyme digests of genomic DNA from T-abα 3 with nick translated fragments covering the complete α-actinin cDNA. The hybridization pattern is compared with that of DNA isolated from the parent strain AX2. In none of the digests could we detect an insertion of vector sequences into the α-actinin gene nor was any other change in the hybridization pattern observed that would indicate duplication or deletion of a major portion of the α-actinin gene. A mechanism comparable to the insertional inactivation described by DeLozanne and Spudich (1987), where a fragment was introduced in addition to the normal myosin heavy chain gene, can thus be excluded for the α-actinin-negative transformants. Bands that hybridized to vector DNA did not coincide with sequences that contained the α-actinin gene. The conclusion is that pDabα 1.2 integrated elsewhere into the genome and that a transplacement mechanism, as it has been observed in yeast, is most likely responsible for the alterations of the α-actinin gene.

Discussion

We describe the isolation of stable α-actinin-negative transformants that appear to be the result of homologous recombination between an incoming DNA fragment and the resident gene without insertion of any vector sequences within or near the gene. The recombination event led to an alteration in the gene so that no normal α-actinin message was detected but an RNA species specific for sequences that code for the amino terminal part of the protein was produced. The RNA in the α-actinin-deficient transformants stops several hundred bp before a 101 bp intron located at position 2209 of the α-actinin-coding region (Witke, unpublished). Therefore alteration of intron/exon structures can be excluded as a basis of the defect. A protein resulting from the 1.9 kb RNA would have a size of ~55 kd; this is little more than half of the α-actinin subunit, and would contain the

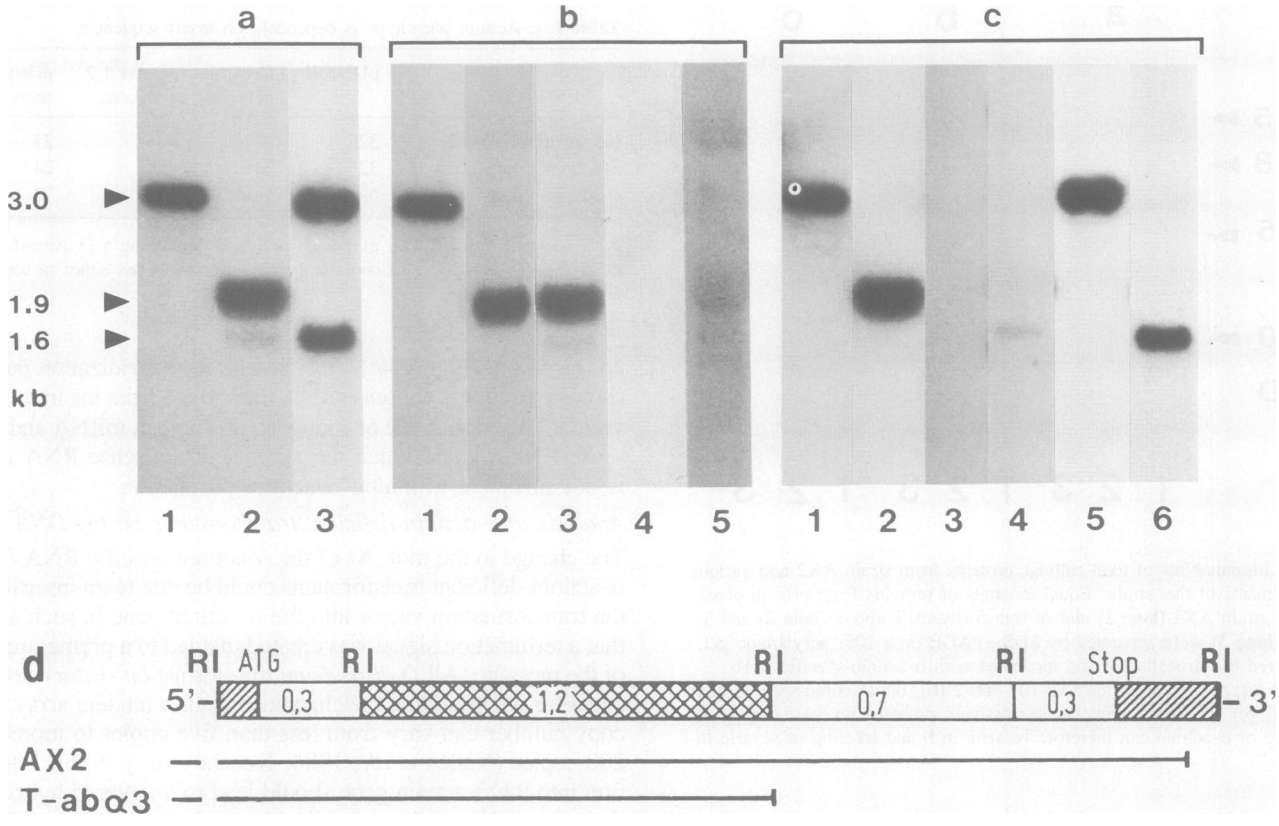

Fig. 4. Northern blot of total RNA from α-actinin-negative and -positive transformants hybridized with different double and single-stranded probes specific for α-actinin mRNA. Arrows indicate the hybridizing RNAs. Panel **a**: equal amounts of RNA from AX2 (**lane 1**) as controls, T-abα 3 (**lane 2**) and T-abα 8 (**lane 3**) were hybridized with the nick translated 1.2 kb *Eco*RI fragment. Panel **b**: hybridization pattern of T-abα 3 (**lanes 2–5**) with different *Eco*RI fragments derived from full length α-actinin cDNA *Eco*RI fragments. The 3.0 kb RNA is the original α-actinin mRNA and is recognized by all fragments used as probes (**lane 1**). **Lane 2** shows hybridization of T-abα 3 RNA with the 0.3 kb fragment coding for amino terminal sequences of the α-actinin gene, **lane 3** shows hybridization of the same RNA with the 1.2 kb fragment. No signals were obtained with the 0.7 kb fragment (**lane 4**) or the 0.3 kb fragment coding for carboxy terminal sequence (**lane 5**). Panel **c**: Hybridization patterns of AX2 (**lanes 1** and **3**) and T-abα 3 (**lanes 2** and **4**) to a single-stranded sense-specific probe (**lanes 1** and **2**) and an antisense-specific probe (**lanes 3** and **4**) are compared. With a sense-specific probe the endogenous α-actinin RNA is recognized in transformants T-abα 8 (**lane 5**). The 1.6 kb transcript is only recognized by an antisense-specific probe (**lane 6**). Both single-stranded probes are derived from the 1.2 kb *Eco*RI fragment. Panel **d**: map of the α-actinin cDNA illustrating the four *Eco*RI fragments used as hybridization probes. The region between the ATG start codon and the stop codon contains the α-actinin coding region. Untranslated regions are hatched. The 1.2 kb *Eco*RI fragment inserted into pDabα 1.2 is marked by cross-hatches. Transcripts from the genes of AX2 and T-abα 3 are represented by the lines below the α-actinin gene.

presumable actin binding site but no longer the sequences forming the EF-hand structures.

The truncated RNA did not give rise to a detectable protein. This deficiency was specific for α-actinin since other actin-binding proteins like severin and the 120 kd gelation factor remained unchanged. But we cannot yet rule out that the 1.9 kb α-actinin specific RNA of the transformants is being translated. A potential truncated polypeptide may be degraded immediately after synthesis by the ubiquitin- and ATP-dependent proteolytic pathway (Ciechanover *et al.*, 1984). Ubiquitin has been shown to be present in *D.discoideum* (Westphal *et al.*, 1986; Giorda and Ennis, 1987). In any case it is evident that the absence of α-actinin is not the consequence of an inactivation by antisense RNA. It is also clear from the size of the RNA that no complete α-actinin chain is produced in the transformants.

The inactivation event with pDabα 1.2 proved to be very specific for the α-actinin gene. No α-actinin-deficient transformants were detected when pDNeoII or a control gene were used in a transformation experiment.

The general applicability of this method is indicated by experiments in which the introduction of a transformation vector carrying sequences coding for the contact site A protein of *D.discoideum* (Noegel *et al.*, 1986) or the 120 kd gelation factor (Noegel and Schleicher, unpublished) resulted in the isolation of contact site A deficient (Harloff, unpublished) or 120 kd gelation factor deficient transformants (Witke, unpublished).

α-Actinin-negative transformants were also obtained when a vector was used that contained the α-actinin fragment in sense orientation, showing that the event of recombination is independent of the orientation of the insert in the vector (Witke, unpublished). The frequency of negative transformants was comparable to the one observed in transformations with the antisense construct (Table I). The sense transformants showed an RNA and DNA pattern similar to the one described for the α-actinin-deficient antisense transformants, indicating a common mechanism of inactivation. This mechanism is clearly distinguishable from the inactivation of the myosin heavy chain gene where integration of a truncated gene occurred upstream of the resident gene (DeLozanne and Spudich, 1987).

The transformation-induced defect in the α-actinin gene can be explained by either one of two initial events that have been observed in other organisms. First, it could have arisen from gene transplacement. Gene transplacement has been found in yeast where an incoming gene (carrying a leu^+ phenotype) was

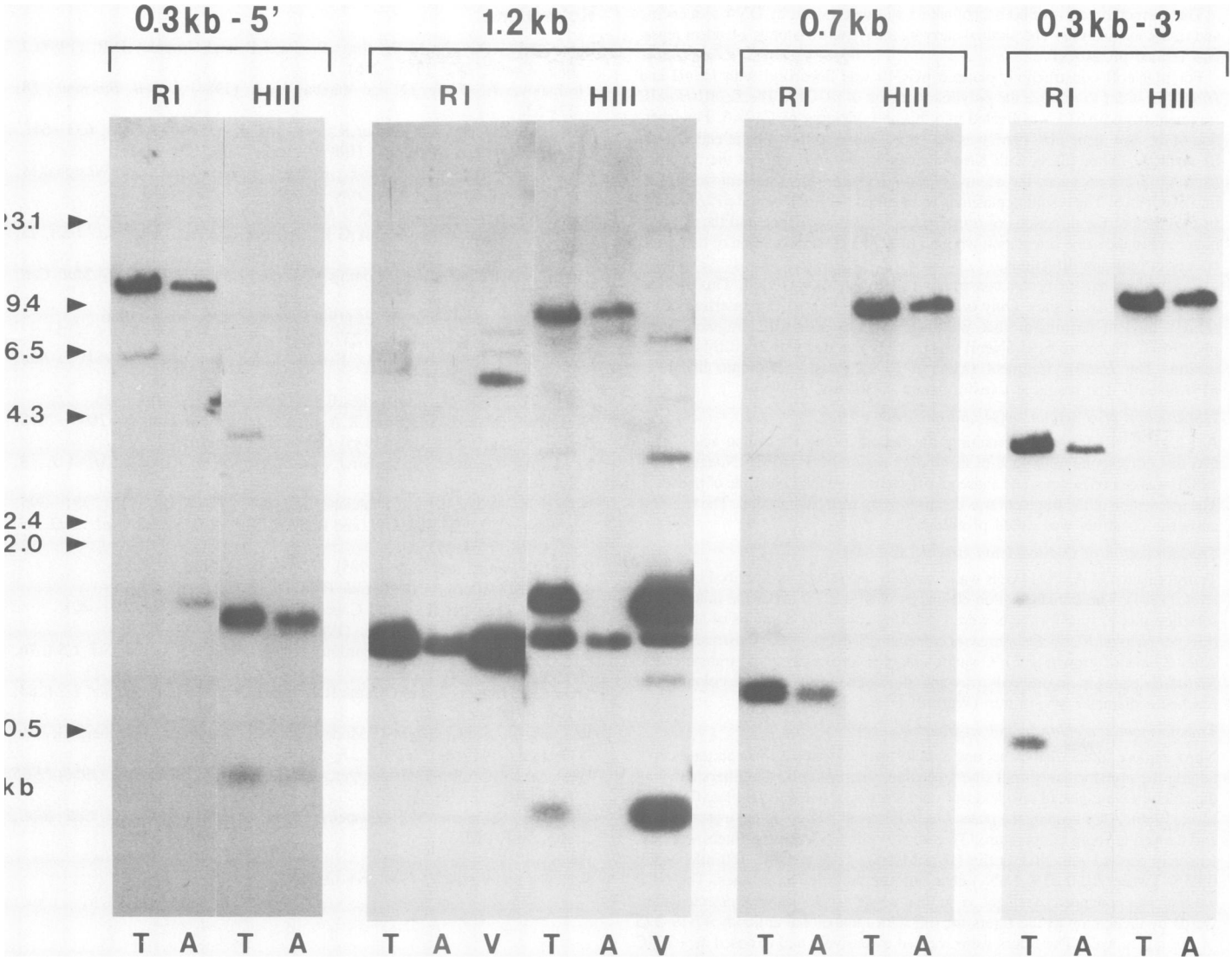

Fig. 5. Southern blot analysis of nuclear DNA of the α-actinin-negative transformant T-abα 3 (**lanes T**) and of non-transformed strain AX2 (**lanes A**). 10 μg of DNA, each were digested with *Eco*RI (RI) and *Hin*dIII (HIII), separated on a 0.7% agarose gel in Tris−phosphate buffer, pH 7.8, transferred to nitrocellulose and used for Southern hybridization. **Lanes V** contain the transformation vector pDabα 1.2 digested with the indicated restriction enzymes. The fragment used as a probe is indicated on top of each panel. The location of *Eco*RI and *Hin*dIII sites in the α-actinin gene is indicated in Figure 1. The weakly hybridizing bands in T-abα 3 correspond to vector sequences. The hybridization is presumably due to contaminating pUC19 sequences that were present in the nick translated probe.

exchanged with the resident gene (carrying a leu$^-$ phenotype) thereby leading to restoration of gene function (Hinnen *et al.*, 1978). Errors leading to mutation may be produced at the site of recombination. Second, a mechanism comparable to the 'heteroduplex-induced mutation' described for mammalian cells (Thomas and Capecchi, 1986) could have led to the observed changes. In this case contact between resident and introduced sequences takes place at one site only and afterwards the strands are not properly resolved. Common to both mechanisms is the generation of changes like base exchanges, insertions, deletions or duplications too small to be detected by Southern blot analysis, but sufficient to create a premature stop of transcription, thus leading to a shortened message. Sequencing of the α-actinin gene of the transformants will resolve the changes that have occurred. To distinguish between both mechanisms we will insert in frame an altered 1.2 kb α-actinin fragment that carries a marker, i.e. an antibody binding site, and screen for the expression of this marker. The successful isolation of transformants that express a marked α-actinin would be proof for the transplacement mechanism and would open a wide range of applications in the engineering of endogenous proteins in *Dictyostelium*.

Materials and methods

Construction of transformation vector pDNeoII

The *D.disoideum* transformation vector pDNeoll was constructed in the *E.coli* vector pUC19. Three fragments from different plasmids were introduced into this vector. The *Neo*^{r} gene was obtained from plasmid A15T by *Xba*I digestion. A15T contains the phosphotransferase gene of transposon Tn903 which can be transcribed in *D.discoideum* under the control of a 270 bp 5′ non-coding DNA fragment of the actin 15 gene (Knecht *et al.*, 1986). Termination of that gene is ensured by a 600 bp 3′ non-coding region of the actin 15 gene. The actin 6 promoter is used for transcription of inserted DNA into both sense or antisense RNA. It was isolated as an *Eco*RI/*Hin*dIII fragment from plasmid pA6T5 (W.Nellen, unpublished).

For transcription termination of sequences cloned into pDNeoII we chose the 3′ non-coding region of the actin 8 gene (Romans and Firtel, 1985; Nellen and Firtel, 1985). A 300 bp subcloned *Sau*96 fragment was obtained by *Pst*I/*Xba*I digestion of plasmid A83′ (W.Nellen, P.Howard, unpublished). This fragment was blunt ended by S1-nuclease and ligated into the *Nru*I site of pIC20H (Marsh *et al.*, 1984). Among several positive clones, one contained two copies of the terminator in a tandem array.

The correct direction of both terminators was determined by DNA sequencing and restriction analysis. The plasmid carrying the A8 terminator as a tandem repeat was termed pIC20A8T.

For pDNeoII construction, plasmid pUC19 was linearized with *Eco*RI and *Hin*dIII in order to remove the polylinker region of pUC19. The *Eco*RI/*Hin*dIII fragment from pA6T5 was ligated into this site, yielding pUC19A6. For insertion of the *Neo*^{r} gene pUC19A6 was linearized with *Eco*RI and blunt ended using S1-nuclease. After filling with Klenow enzyme the *Xba*I sites of the fragment from A15T that contained the neomycin resistance gene, this fragment was ligated into pUC19A6. The resulting plasmids were called p19A6NeoI and p19A6NeoII. In p19A6NeoI the directions of transcription of the *Neo*^{r} gene and the A6 promoter are in the same orientation while in p19A6NeoII transcription of both units occurs in opposite directions.

For further steps of vector construction p19A6NeoII was used. This plasmid contains two *Hin*dIII sites. Only one of them could be used for insertion of the actin 8 tandem terminator that was recovered as a *Hin*dIII fragment from pIC20A8T. Therefore p19A6NeoII was partially digested with *Hin*dIII for linearization. The *Hin*dIII fragment from pIC20A8T was ligated into linearized p19A6NeoII. The resulting transformation vector was pDNeoII.

Construction of α-actinin sense and antisense vectors

A 1.2 kb *Eco*RI fragment containing the region coding for amino acids 79 to 505 that correspond to ~45% of the coding region of α-actinin (Noegel *et al.*, 1987), was cloned in sense and antisense orientation into the *Eco*RI site of pDNeoII. The orientation of the fragment was determined by *Hin*dIII digestion. The resulting antisense construct was called pDabα 1.2.

Transformation of D.discoideum strain AX2-214

Transformation of AX2-214 cells was essentially carried out as described by Nellen *et al.* (1984). The transformation efficiency was $\sim 10^{-5}$. After the initial G418 selection phase, transformants were cloned by spreading dilutions of about 10^3 cells per plate on *Klebsiella aerogenes* containing SM-agar plates (Sussman, 1966). After 2 days of growth at 21°C single colonies were picked and transferred to *Klebsiella* plates in an ordered array and allowed to grow until the colonies had reached a diameter of ~5 mm (Wallraff *et al.*, 1986).

Transformant screening by colony-blotting

Nitrocellulose filters (Schleicher and Schüll) were laid onto *Klebsiella* plates containing the transformants until filters became wet. For cell lysis filters carrying the colonies were placed upside down onto a dry-ice cooled metal plate (Wallraff *et al.*, 1986). After keeping the plates frozen for about 30 min, filters were removed and thawed at room temperature. To remove cellular debris the filters were extensively washed with NCP buffer (10 mM Tris−HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 0.02% NaN_3) changing the buffer several times. The NCP washing was continued overnight. α-Actinin-negative colonies were detected afterwards by incubation of the filters for 6 h with monoclonal antibody 47-19-2 (5×10^5 c.p.m./ml). The filters were washed extensively and exposed at −70°C for 16 h on X-Omat AR5 film (Kodak). Colonies on the filters were localized by staining with 0.2% Ponceau S in 3% TCA (Gerisch *et al.*, 1985).

DNA and RNA analysis

Preparation of DNA and RNA samples were performed as described previously (Witke *et al.*, 1986). Single-stranded RNA probes were prepared using the pGem system (Promega Biotech). Hybridizations with single-stranded RNA probes were done for 16 h at 55°C in 50% formamide and 2 × SSC hybridization buffer. After washing the filters at room temperature with 2 × SSC containing 0.02% SDS, a stringent wash was performed at 50°C in 50% formamide wash buffer for 1 hour, changing the buffer once after 30 min.

Other methods

Total cell protein was prepared by boiling cells for 3 min in 1% SDS. Protein was determined by the method of Lowry *et al.* (1951). Total cell homogenates containing 10 μg of protein per lane were separated on 10% acrylamide/SDS gels (Laemmli, 1970) and blotted onto nitrocellulose (Towbin *et al.*, 1979). After saturation in NCP-buffer (1 h) blots were incubated with iodinated mAb 47-19-2 or mAb 47-62-17 specific for α-actinin, mAb 101-460-2 specific for severin or with unlabeled polyclonal anti α-actinin antibody from rabbits followed by iodinated goat-anti-rabbit IgG antibodies.

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References

Baron,M.D., Davison,M.B., Jones,P., Patel,B. and Critchley,D.R. (1987) *J. Biol. Chem.*, **262**, 2558−2561.
Ciechanover,A., Finley,D. and Varshavsky,A. (1984) *J. Cell. Biochem.*, **24**, 27−53.
Crowley,T., Nellen,W., Gomer,R. and Firtel,R. (1985) *Cell*, **43**, 633−641.
DeLozanne,A. and Spudich,J. (1987) *Science*, **236**, 1086−1091.
Gerisch,G., Hagmann,J., Hirth,P., Rossier,C., Weinhart,U. and Westphal,M. (1985) *Cold Spring Harbor Symp. Quant. Biol.*, **50**, 813−822.
Giorda,R. and Ennis,H.L. (1987) *Mol. Cell. Biol.*, **7**, 2097−2103.
Hinnen,A., Hicks,J.B. and Fink,G.R. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1929−1933.
Knecht,D.A., Cohen,S.M., Loomis,W.F. and Lodish,H.F. (1986) *Mol. Cell. Biol.*, **6**, 3973−3983.
Knecht,D.A. and Loomis,W.F. (1987) *Science*, **236**, 1081−1086.
Laemmli,U.K. (1970) *Nature*, **227**, 680−685.
Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) *J. Biol. Chem.*, **193**, 265−275.
Marsh,J.L., Erfle,M. and Wykes,E.J. (1984) *Gene*, **32**, 481−485.
Nellen,W., Silan,C. and Firtel,R.A. (1984) *Mol. Cell. Biol.*, **4**, 2890−2898.
Nellen,W. and Firtel,R.A. (1985) *Gene*, **39**, 155−163.
Noegel,A., Gerisch,G., Stadler,J. and Westphal,M. (1986) *EMBO J.*, **5**, 1473−1476.
Noegel,A., Witke,W. and Schleicher,M. (1987) *FEBS Lett.*, **221**, 391−396.
Orr-Weaver,T.L., Szostak,J.W. and Rothstein,R.J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6354−6358.
Romans,P. and Firtel,R.A. (1985) *J. Mol. Biol.*, **183**, 311−326.
Sussman,M. (1966) *Methods Cell Physiol.*, **2**, 397−410.
Thomas,K.., Folger,K.R. and Capecchi,M. (1986) *Cell*, **44**, 419−428.
Thomas,K.R. and Capecchi,M. (1986) *Nature*, **324**, 34−38.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350−4354.
Trevor,K., Linney,E. and Oshima,R.J. (1986) *Proc. Natl. Acad. Sci. USA*, **84**, 1040−1044.
Wallraff,E., Schleicher,M., Modersitzki,M., Rieger,D., Isenberg,G. and Gerisch,G. (1986) *EMBO J.*, **5**, 61−67.
Westphal,M., Müller-Taubenberger,A., Noegel,A. and Gerisch,G. (1986) *FEBS Lett.*, **209**, 92−96.
Witke,W., Schleicher,M., Lottspeich,F. and Noegel,A. (1986) *J. Cell. Biol.*, **103**, 969−975.

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