**Dictyostelium discoideum** Developmentally Regulated Genes Whose Expression Is Dependent on MADS Box Transcription Factor SrfA

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Received 26 June 2003/Accepted 16 September 2003

The MADS box transcription factor SrfA is required for spore differentiation in *Dictyostelium discoideum*. srfA null strains form rounded spores that do not resist adverse environmental conditions. Five genes whose expression is dependent on SrfA have been isolated by differential hybridization. One of these genes, sigC, is identical to *phg1b*, previously characterized in mutants with altered adhesive properties and found to encode a nine-transmembrane-domain protein. This gene is transcribed into two mRNAs as the result of alternative splicing of two internal exons. The slower-migrating mRNA codes for a shorter protein that lacks the first transmembrane fragment and is not expressed in srfA null strains. The other four genes (sigA, sigB, sigD, and 45D) are expressed only during late developmental stages. In situ hybridization experiments showed that expression of sigA, sigB, and sigD is restricted to the sorus of developing structures. sigA codes for a protein with significant similarity to the GP63 metalloproteinase of *Leishmania*, leishmanolysin. The sequence of SigD is highly similar to that of several spore coat proteins of *D. discoideum*, and it may play a role in that structure. The gene 45D codes for an RNA-binding protein homologue whose expression is also dependent on the GATA transcription factor stalky (StkA). The expression of sigB is also dependent on both SrfA and StkA. The expression of 45D, but not of sigA, sigB, sigC, and sigD, can be induced in srfA null cells by constitutive protein kinase A activation. Strains in which either sigA, sigB, or sigD is disrupted were isolated and found to form spores that are not detectably different from those of wild-type strains.

The social amoeba *Dictyostelium discoideum* is one of the simplest eukaryotic models used to study morphogenesis and cell differentiation (14). *D. discoideum* cells grow as individual amoebae in rich environments. Under starving conditions, groups of up to 10^5 cells aggregate and enter into a multicellular developmental process. The end result is the formation of a fruiting body composed of a basal disk adhered to the substrate, a stalk that stands over the basal disk, and, on top of it, a sporocarp. Resistance forms, called spores, differentiate from individual amoebae inside the sporocarp. Spores are disseminated throughout the environment, where they germinate to give rise to individual amoebae, closing the biological cycle.

*D. discoideum* development occurs in about 24 h and is relatively simple. Two main differentiated cell types are present in the fruiting body, stalk cells, which are components of the basal disk and stalk, and spores. Despite the few cell types involved, *D. discoideum* development has many aspects in common with the developmental processes of more complex organisms. There are counting mechanisms that regulate the size of the structure (6), mechanisms that maintain a fixed proportion of stalk and spore cells (7, 9, 19), morphogenetic pathways that regulate the progression through the different developmental stages (18, 25), and a coordination between stalk and spore differentiation so that spore cells differentiate only when the sporocarp is being lifted on top of the stalk (14, 36).

The MADS box transcription factor SrfA is required for *D. discoideum* development and spore differentiation (13, 15). *D. discoideum* strains in which the *srfA* gene has been disrupted show developmental defects such as a delay of about 4 h in the process and deficient spore differentiation. srfA− spores are rounded, present a less stable coat, and do not resist heat and detergent treatments. *D. discoideum* SrfA shows a high similarity to animal serum response factors (SRFs) and yeast MCM1 and ARG80 proteins in the amino acid sequences of their DNA-binding and dimerization domains (MADS boxes) (13, 32, 37). Animal SRFs are involved in cellular responses to extracellular signals, such as growth factors, and in differentiation processes (3, 17, 34, 39). The phenotype of the srfA− strains and the similarity of the protein to other SRFs make SrfA a good candidate for involvement in the coordination of the last steps of *D. discoideum* development and in the regulation of spore differentiation. In agreement with this hypothesis, srfA expression has been shown to be regulated by several extracellular signals and regulatory pathways involved in the control of development. srfA expression is repressed during early development by the extracellular signals cyclic AMP (cAMP) and DIF-1 (11). During late development, signal transduction pathways, leading to the activation of protein kinase A (PKA) through an increase in the intracellular cAMP concentration, induce srfA expression in prespore cells (2, 12, 13, 30, 33, 38).

srfA mRNA is expressed in a complex pattern during development. mRNA accumulation is first observed after 8 h of

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development and continues to accumulate at constant levels during later development until midculmination (20 h), when there is a strong induction (13). In situ hybridization experiments have shown that the mRNAs is enriched in prespore cells (13). A more detailed study showed that the srfA regulatory region contains three functional elements that drive reporter gene expression in different cell types at different developmental stages (15). Among them, the distal promoter element drives expression to prespore cells at late developmental stages. We have previously proposed that srfA would regulate the expression of genes involved in late events of spore maturation (12). One of these genes is spfA, which is involved in spore stability and whose expression is greatly reduced in srfA null strains (13). However, the phenotype of spfA-deficient spores is much less severe than that of srfA null spores (28), suggesting the existence of additional genes whose expression is induced by SrfA. The identification of SrfA-dependent genes has been approached in this article through the generation of a cDNA library enriched in genes expressed in late developmental stages of wild-type, but not srfA null strains (13). Five genes whose expression is dependent on SrfA are described. All these genes are specifically expressed at the latest stages of D. discoideum development.

MATERIALS AND METHODS

Subtractive library construction. RNA was isolated from structures of wild-type (AX4) and srfA− strains developed for 21 and 24 h, respectively, using Trizol reagent (Gibco-BRL). Four micrograms of poly(A)+ RNA, isolated from both RNAs by use of an mRNA purification kit (Amersham Pharmacia Biotech), was utilized for the synthesis of a cDNA subtraction library by using a PCR-Select cDNA subtraction kit (Clontech). Wild-type cDNA was used as the tester cDNA, and srfA− cDNA was used as the driver cDNA to isolate clones from mRNAs expressed in wild-type, but not srfA−, strains. Subtracted cDNA clones were ligated into a pGEMT-Easy vector. Twenty of the clones that were obtained were analyzed for expression in wild-type and srfA− strains. Five genes whose expression is dependent on SrfA are described. All these genes are specifically expressed at the latest stages of D. discoideum development.

Isolation of SrfA-dependent genes. The isolation of genes whose expression is dependent on the transcription factor SrfA was approached by screening for genes that were differentially expressed in wild-type, but not in srfA−, structures. The analysis was focused on late development since the srfA− strain showed defects in spore differentiation. RNAs were isolated from wild-type structures at 21 h of development and from srfA− structures at 24 h of development; they presented similar midculmination structures. A PCR-Select cDNA subtraction kit (Clontech) was used to generate a cDNA library that was enriched in genes expressed in wild-type, but not srfA−, structures. Twenty clones from this library were analyzed by Northern blotting, and four were found to be SrfA dependent (data not shown) (see Fig. 2). These genes were named sigA (SrfA-induced gene A), sigB, sigC, and sigD. A comparison of the nucleotide sequences of these genes with the sequences of D. discoideum in the data banks of the Sanger Center, the Baylor College of Medicine, and the Department of Genome Analysis at Jena, Germany, allowed the identification of overlapping cDNA and genomic clones containing the complete open reading frames. The sequences of the proteins encoded by these open reading frames were compared to sequences at the National Center for Biotechnology Information protein data bank, and the most similar sequences found are indicated in Table 1.

The sigA gene (GenBank accession number AY387644) is located in chromosome 2 and codes for a 544-amino-acid-long protein that is very similar to the malic enzyme from many organisms and most probably represents the malic enzyme homologue in D. discoideum.

The sigB gene (GenBank accession number AY387645)
codes for a 635-amino-acid-long protein. The gene is located in chromosome 6 and is identical to a previously described cDNA clone whose expression is dependent on the transcription factor StkA (20). The encoded protein shows an extended similarity (26 to 27% identity) to GP63 cell surface proteases from Trypanosoma cruzi, Leishmania spp., and Arabidopsis thaliana.

The sigC gene (GenBank accession number AY387646) codes for a 587-amino-acid-long protein that is highly similar (46% identity) to members of a family of transmembrane proteins characterized by the presence of nine transmembrane fragments, such as human TMSF1 to -4. D. discoideum proteins of the TM9 family have been described recently, and a direct sequence comparison has shown that sigC codes for the Phg1b protein (5).

sigD (GenBank accession number AY387647) codes for a 445-amino-acid-long protein that is up to 28% identical to the previously isolated D. discoideum spore coat proteins SP87, DP87, SP96, SP70, and SP60. Figure 1 shows a diagram of the structure of the protein encoded by sigD. The presence of the conserved domains is indicated. The first domain (C7C) is very similar to those found in all the spore coat proteins (40) and is slightly different from the other domains. The rest of the protein contains six repeats of a cysteine-containing domain (C4C, CX4CPX4CVX) conserved in all spore coat proteins (40). SigD domains 1 and 2 show a divergence with the consensus domain since the conserved proline has been changed to a glutamine. The conservation of the amino acid sequence of the six SigD domains is higher than that observed in other spore coat proteins, as shown in Fig. 1B. These data indicate that sigD codes for a new member of the spore coat protein family.

One of the cDNA clones (sigB) was identical to a cDNA clone previously isolated in a screening for StkA-dependent genes (20). We looked for the SrfA dependence of the other three StkA-dependent genes isolated. Only the expression of one of them, coding for a protein with homology to RNA-binding proteins, was also found to be dependent on SrfA. This clone (45D) has been included in Table 1, although it was not isolated in the screening for SrfA-dependent genes.

Developmental expression of SrfA-dependent genes. The temporal pattern of expression of SrfA-dependent genes was analyzed by Northern blotting by using RNAs isolated from vegetative cells and from structures collected at different stages of development. The results are shown in Fig. 2. Genes sigB, sigD, and 45D are expressed at late developmental stages (20 to 24 h) in the wild-type strain. Their expression is barely detectable in the srfA− strain. The gene sigA, which codes for the malic enzyme homologue, is expressed at low levels in vegetative cells (time zero). Its expression decreases in the first hours of development and is strongly induced at later stages (20 to 24 h) in wild-type structures. The srfA− strain also showed low expression in vegetative cells and a moderate induction at later
stages of development, but this induction was significantly lower than that in the wild-type strain.

The sigC gene showed a more complex pattern of expression, since two RNAs of different migrations were observed in Northern blots (Fig. 2). The faster-migrating RNA is expressed in vegetative cells and at all stages of development, in both wild-type and srfA strains. Expression levels are fairly constant except for a small induction around 12 to 13 h of development that is observed in the wild-type strain. The induction was also observed in the srfA strain at 16 h of development, in agreement with the 3- to 4-h developmental delay observed for this strain. The slower-migrating RNA was observed exclusively in the wild-type strain and at late developmental stages (20 to 24 h). These data indicate that the expression of this RNA is SrfA dependent. The nature of these two mRNAs has been further studied, as described below.

The spatial pattern of expression of the sig genes was studied by using in situ hybridization. The results obtained for the sigA, sigB, and sigD genes are shown in Fig. 3. Hybridization was observed in the sori of culminant structures for the three genes, suggesting that they are expressed at high levels in differentiated spores. No hybridization was detected with the 45D probe. The expression of the developmentally induced sigC RNA could not be analyzed because of the lack of probes specific for the slower-migrating RNA (see below).

**Developmentally regulated alternative splicing of the sigC mRNA is dependent on SrfA.** Comparison of sigC cDNA and genomic sequences, obtained from the sequence data banks previously described, showed that this gene is divided into four exons (Fig. 4A). The two different RNAs observed in Northern blots may be the consequence of alternative splicing of some of these exons. Other possible origins may be the use of alternative promoters or polyadenylation sites. The possible presence of different exons in sigC mRNAs was analyzed by reverse transcription (RT)-PCR. The use of oligonucleotides that hybridize in exons 1 (sigC-1) and 4 (sigC-2 and sigC-3) (Fig. 4B) amplified two fragments of different sizes in RT-PCRs made from wild-type RNA isolated at 24 h of development. Nucleotide sequencing of the amplified fragments showed that the longest one contained exons 2 and 3, the end of exon 1, and the beginning of exon 4. The shorter fragment contained the end of exon 1 fused to the beginning of exon 4. These data indicated that the two mRNAs obtained in Northern blots could be the consequence of the alternative splicing of exons 2 and 3 (Fig. 4B). Northern blot analyses were performed to confirm this possibility. Two different probes were used: an exon 4 probe that should recognize both mRNAs (probe 1) (Fig. 4B) and a probe from exons 2 and 3 that should recognize only one of the mRNAs (probe 2) (Fig. 4B). The results obtained by using RNAs from different developmental stages of wild-type and srfA strains are presented in Fig. 4B. The exon 4-containing probe 1 hybridized to both mRNAs as expected. Probe 2, derived from exons 2 and 3, hybridized only with the faster-migrating mRNA. These data indicate that the faster-migrating mRNA contains exons 1 to 4 and is expressed through development in wild-type and srfA strains. The slower-migrating mRNA does not contain exons 2 and 3. The expression of this mRNA is developmentally regulated and SrfA dependent (Fig. 2 and 4). The analyses of the amino acid sequence encoded by exons 2 and 3 indicated that they code for over half of the predicted extracytoplasmic domain and for the first putative transmembrane region. The upper panel of Fig. 4A shows two diagrams indicating the location of the predicted

![FIG. 2. Developmental expression and SrfA-dependence of sig genes. Wild-type (AX4) and srfA null (srfA−) cells were placed on filters and incubated for the time indicated in hours at the top of each lane. RNA was extracted, and the expression of the sigA, sigB, sigC, sigD, and 45D genes was analyzed by Northern blotting. The bottom panel shows ethidium bromide staining of a representative RNA gel.](http://ec.asm.org/)
transmembrane domains 1 to 9 and the region encoded by exons 2 and 3.

Regulation of the expression of SrfA-dependent genes by PKA activation. Previous results from our laboratory had shown that the \( \text{splA} \) spore-specific gene is expressed at very low levels in \( \text{srfA}^- \) strains and that its expression can be induced by the activation of the PKA through treatment of dissociated cells with 8-Br-cAMP (13). We wanted to know if the isolated SrfA-dependent genes may also be induced by 8-Br-cAMP treatment of disaggregated cells. Wild-type and \( \text{srfA}^- \) strains were analyzed for gene expression by Northern blotting (Fig. 5). All the SrfA-dependent genes were induced by 8-Br-cAMP treatment of wild-type cells. This result was not unexpected, since 8-Br-cAMP treatment induces \( \text{srfA}^- \) expression (12).

The expression of the SrfA-dependent genes \( \text{sigA} \), \( \text{sigB} \), \( \text{sigC} \) (slower-migrating mRNA), and \( \text{sigD} \) was not induced by 8-Br-cAMP treatment of \( \text{srfA}^- \) cells. \( \text{sigA} \) showed a slight induction in \( \text{srfA}^- \) cells, but it was comparable to the induction obtained in this strain at 24 h of development (Fig. 2) and much lower than that observed in AX4 cells.

**DISCUSSION**

This report describes the isolation of five SrfA-dependent genes that are expressed in wild-type strains but not in \( \text{srfA}^- \) null strains. The expression of four of these genes (\( \text{sigA} \), \( \text{sigB} \), \( \text{sigD} \), and \( \text{45D} \)) is induced at late developmental stages (between 20 and 24 h). Three of these genes, \( \text{sigB} \), \( \text{sigD} \), and \( \text{45D} \), are expressed at detectable levels only at these late developmental stages. The gene coding for the malic enzyme homologue (\( \text{sigA} \)) is expressed in vegetative cells; its expression is repressed during aggregation and strongly induced again at 20 h of development. In situ hybridization experiments have shown that three of the genes that were studied (\( \text{sigA} \), \( \text{sigB} \), and \( \text{sigD} \)) are expressed at high levels in the spores of 24-h-old structures. The promoter regions of the genes \( \text{sigA} \), \( \text{sigB} \), \( \text{sigD} \), and \( \text{45D} \) have been cloned in LacZ reporter vectors. These constructs show specific LacZ expression in the spore (data not shown), confirming the results obtained by in situ hybridization for the genes \( \text{sigA} \), \( \text{sigB} \), and \( \text{sigD} \) and suggesting a spore-specific expression for \( \text{45D} \). These data are in agreement with the hypothesis that SrfA induces the expression of a number of spore-specific genes at the final steps of spore differentiation.

The regulation of the expression of \( \text{sig} \) genes by SrfA could be through direct binding to their promoters, which activates their expression. Alternatively, SrfA could activate the expression of other transcription factors that would, in turn, activate the expression of \( \text{sig} \) genes. However, the strong induction of \( \text{srfa} \) expression in prespore cells at culmination, a short time before the induction of \( \text{sig} \) genes, suggests a direct regulation by SrfA. Animal SRF transcription factors bind to a conserved DNA element, the CaR box, whose consensus sequence is CC(A/T)\( \mu \)GG (27). This sequence is not present in the available promoter regions of \( \text{sig} \) genes. However, several amino acids involved in DNA binding have diverged in vertebrate and *D. discoideum* SRFs (13, 26), which might have changed the DNA-binding specificity of SrfA with respect to animal SRF. A comparison of the nucleotide sequences of \( \text{sig} \) gene promoter regions did not allow the identification of conserved elements.
Therefore, establishing the mechanisms of activation will require a detailed functional analysis of the promoter regions of these genes.

Surprisingly, the alternative splicing of phg1b (sigC) is also dependent on SrfA. phg1b codes for a protein with nine predicted transmembrane regions, homologous to the TM9 family of endoplasmic proteins. Mutation of this gene causes temperature-dependent defects in cell adhesion and phagocytosis (5). The SrfA-dependent alternatively spliced mRNA codes for an internally deleted protein that lacks part of the predicted first extracytoplasmic domain and the first transmembrane fragment. The absence of the first transmembrane fragment is predicted to reverse the orientation of the other transmembrane fragments, which could impair the functionality of the protein. It is not clear how SrfA can regulate mRNA splicing. One possibility is that SrfA induces the expression of proteins involved in the regulation of splicing. The 45D-encoded protein, homologous to RNA-binding proteins, may be a good candidate for this function. However, strains in which 45D has been interrupted still show wild-type alternative splicing of phg1b (data not shown). Alternatively, SrfA could activate phg1b slower-migrating mRNA transcription from a distal promoter region during culmination to originate a larger mRNA. Transcription from this promoter could be associated with the splicing of exons 2 and 3.

Two of the SrfA-dependent genes isolated, sigB and 45D, also depend on the GATA transcription factor stalky (StkA) for their expression (20). The expression of the spore-specific gene spc4 is also dependent on both SrfA and StkA (13, 20). The transcription factor StkA is specifically expressed in cells of the prespore region, and its mutation produces the conversion of prespore cells into prestalk cells (8, 23). The existence of genes whose expression is dependent on both SrfA and StkA suggests the interaction of both transcription regulatory pathways in spore differentiation. Cooperation of the vertebrate SRF with GATA 4 and GATA 6 has been described previously (4, 21, 22). In D. discoideum, the two pathways are not completely overlapping, however, since there are genes whose expression is dependent on StkA but not on SrfA. The phenotypes derived from the alteration of both pathways are very different, and StkA seems to be required at an earlier step of spore differentiation than is SrfA, since stkA mutants do not form spores while srfA mutants are able to accomplish the first stages of spore differentiation (8, 13, 23; unpublished results). Therefore, it is also possible that the SrfA pathway could not be activated in StkA mutants because prespore cells dedifferentiate before srfA induction.

The interaction of SrfA with other pathways regulating spore differentiation is also indicated by the consequences of PKA activation in srfA null strains. It is intriguing that the activation of PKA by 8-Br-cAMP induces the expression of two SrfA-dependent genes but not the others. The more likely
interpretation for these results is that PKA is activating several transcription pathways. Previous results indicate that one of them involves SrfA, since PKA activation is required to induce srfA expression in prespore cells during midculmination (12). However, other pathways might also be activated, since 8-Br-cAMP induces the expression of some SrfA-dependent genes in srfA null strains. These results might be explained if the expression of these genes simultaneously depends on both SrfA and PKA. High-level artificial activation of PKA by 8-Br-cAMP treatment might override the requirement for SrfA. The proposed PKA-dependent alternative pathway is not the StkA pathway, since some StkA-dependent genes, such as spiA or 45D, are induced by 8-Br-cAMP while another, sigB, is not induced. The possible relation between PKA and SrfA pathways is summarized in Fig. 6.

The identification of SrfA-dependent genes is an important step towards the characterization of the biological processes regulated by this transcription factor. The similarities found between the SrfA-dependent genes and other genes of known function can provide some information in this respect. One of the more interesting genes is sigD, which demonstrates a very significant similarity to the family of D. discoideum spore coat proteins both in its sequence and in the predicted domain structure. However, this gene is expressed at a later developmental stage than the other described spore coat genes (16), and thus the secretory pathway used by the SigD protein may be different from the classical prespore vesicles (35). Knockout strains have been generated for this gene, but they did not present any obvious phenotype. The morphology of developing structures and spores, spore viability, and the stability at the sporocarp and after prolonged incubation in water and detergent treatment were similar to those of wild-type strains. These results may be the consequence of functional redundancy with other spore coat proteins, given their similarity.

Null strains were also generated for the malic enzyme homologue (SigA) and the homologue to the GP63 metalloprotease from Leishmania (SigB), but again, they did not present any obvious phenotype in either morphogenesis, spore differentiation, viability, or stability. Malic enzyme replenishes the tricarboxylic acid cycle when intermediates are used for biosynthesis and may be involved in the synthesis of the monosaccharides that are required for cellulose synthesis or in the

FIG. 5. The expression of some SrfA-dependent genes can be induced by PKA activation in srfA null strains. Structures obtained at the Mexican hat stage of development, either from wild-type (WT) or srfA null (srfA−) strains, were dissociated, and the cells were incubated in medium alone (−) or in the presence of 20 mM 8-Br-cAMP (+) for 2 h. Cells were collected after incubation, and RNA was extracted. The expression of each of the genes indicated to the right of the gel was analyzed by Northern blotting. The lower panel shows the ethidium bromide staining of a representative RNA gel.

FIG. 6. Schematic model of the proposed interaction between PKA activation and the SrfA pathway of gene expression regulation. Induction of srfA expression by PKA activation has been reported previously (12). The data presented in this article show that SrfA is necessary for the activation of the transcription of the sigA, sigB, sigD, and 45D genes. The spore-specific gene spiA is also not expressed in srfA− strains (13). Activation of PKA by 8-Br-cAMP treatment of disaggregated cells indirectly induces expression of spiA and 45D in srfA− strains through undefined pathways. In addition, the alternative splicing of sigC mRNA is dependent on SrfA. Genes whose expression is dependent on the transcription factor StkA (20) are indicated with asterisks. Dashed lines indicate indirect transcriptional regulation, while continuous lines indicate possible direct regulation.
generation of reduction potential (NADPH). The proteinase GP63 homologue might be required for several processes, including the degradation of the extracellular matrix, protein processing, or the degradation of the spore coat during germination. A null strain for the ribonucleoprotein homologue-encoding gene 4SD has been described previously and also has no detectable phenotype (20). Biochemical redundancy in these processes may explain the lack of phenotype of the null strains. Besides, the phenotype of srfA spores might be the consequence of the lack of expression of many target genes. So far, the absence of only one SrfA target gene, spiA, has shown defects in spore stability, and the severity of the phenotype is much lower than that of srfA− spores. There is the possibility that only the accumulative effect of the absence of several SrfA-dependent genes would give a distinct phenotype.

A knockout strain for phg1b (sigC) has also been studied (5). This strain presents a temperature-sensitive defect in adhesion and phagocytosis that probably corresponds to the absence of the protein isoform encoded by the faster-migrating mRNA that is expressed in vegetative cells and throughout development. The effect of the absence of the deleted protein, encoded by the slower-migrating mRNA, should be observed at the late developmental stages when this isoform is expressed. A defect in the morphology of the stalk has been observed in this strain (data not shown), although more experiments need to be done to unambiguously correlate the phenotype with the absence of the alternatively processed mRNA.

In summary, the results described in this article strongly suggest that SrFA participates in one of the transcriptional regulatory pathways that are required for spore differentiation. The determination of the mechanisms through which SrFA induces the expression of these genes and of the function of the encoded proteins will require further studies.

ACKNOWLEDGMENTS

R. Escalante and N. Moreno contributed equally to this work. We are indebted to Pierre Cosson and Julian Gross for providing the phg1b and 4SD null strains, Christopher West for help in the analyses of the SigD amino acid sequence, and Juan Jesús Vicente for critical reading of the manuscript.

This work was supported by grants PB98-0517 and BMC2002-01501 from the Dirección General de Investigación, Ministerio de Ciencia y Tecnología. Sequence data for D. discoideum were obtained from the Genome Sequencing Centers of the University of Cologne; the Institute of Molecular Biotechnology, Department of Genome Analyses, Jena (http://genome.imb-jena.de/dictyostelium/); the Baylor College of Medicine in Houston, Tex.; and the Sanger Center in Histon, Cambridge, United Kingdom (http://www.sanger.ac.uk/projects/D_discoideum).

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