The TPR (tetratricopeptide repeat) family became widespread during evolution, having been found from bacteria to mammals. By means of restriction enzyme-mediated integration, we have identified a Dictyostelium gene (trfa) highly homologous to a Saccharomyces cerevisiae gene encoding a TPR protein, Ssn6 (Cycc8), which functions as a global transcriptional repressor for diverse genes. The deduced amino acid sequence of the Dictyostelium gene product, TRFA, contains 10 consecutive TPR units as well as Gln repeats, Asn repeats, and a region rich in Glu, Lys, Ser, and Thr. The sequences of some of the 10 TPR units in TRFA are more than 70% identical to the corresponding units in Ssn6.

The trfa− cells produced smooth plaques on a bacterial lawn and failed to aggregate normally when starved on a plain agar plate. Individual trfa− cells also failed to correctly respond to cAMP, although the adenyl cyclase of trfa− cells was expressed upon starvation and activated by stimulation with cAMP as in the wild-type cells. When cultured in a rich medium in suspension, they grew more slowly and stopped growing at a lower density than the wild-type cells. Furthermore, they divided into cells of various sizes and tended to be much smaller than the wild-type cells. These pleiotropic defects of the trfa− cells suggest the possibility that Dictyostelium TRFA may regulate the transcription of diverse genes required for normal growth and early development.

The cellular slime mold Dictyostelium discoideum is an ideal organism for studying molecular mechanisms regulating developmental decisions. Dictyostelium cells start the developmental process and finally form fruiting bodies when they are starved. At an early stage of development, the cells undergo chemotactic aggregation and start developing into multicellular organisms without cell division, responding to relayed pulses of cAMP. This process is controlled through a series of integrated signal transduction pathways (1). A number of novel Dictyostelium genes involved in this process have already been identified by means of an efficient random tagging method called restriction enzyme-mediated integration (REMI) (2).

The TPR (tetratricopeptide repeat) motif composed of 34 amino acid residues with the consensus sequence WX_{L}GX_{Y}AX_{P}FX_{A}AX_{P} (X, any amino acid) is found in many proteins of a variety of organisms from bacteria to mammals (3–5). The TPR family members generally contain 3–19 TPR units, often arranged in tandem. Comparison of the TPR units in a TPR protein shows minimal homology limited to the consensus sequence. However, when the corresponding TPR units are compared among TPR proteins, there is striking homology beyond the consensus sequence. It has been proposed that the consensus sequence in the TPR unit functions as a “knob and hole” for the formation of a tightly packed helix-turn-helix domain (3, 6) and that the residues outside the consensus sequence play roles in protein-protein interactions (3, 7). Individual TPR units may interact with particular target proteins (8) and play a wide range of roles in cell cycle regulation, transcription, splicing, or protein import (8–14).

One of the well studied TPR proteins is Ssn6 (Cycc8) of Saccharomyces cerevisiae (9, 15). Genetic studies have shown that it forms a transcription complex with Tup1 (16), which then recognizes specific DNA-binding proteins and represses a set of unrelated promoters (17). A distinct set of TPR units is responsible for the repression of a distinct set of unrelated promoters (8). In this way, the Ssn6-Tup1 complex functions as a global repressor. In yeast cells, Ssn6 is required for normal growth, sporulation, mating, and other glucose-repressible phenotypes (9, 15, 18).

By means of the REMI method with the bsr marker (19–21), we identified a Dictyostelium gene, trfa, that is highly homologous to Ssn6. It is likely that the gene product (TRFA), the first Dictyostelium TPR protein ever found, may be a global repressor for promoters of genes required for normal growth and early development of Dictyostelium cells.

EXPERIMENTAL PROCEDURES

Cell Growth, Development, and Chemotaxis—All the strains used in this study were derived from D. discoideum AX2 cells (22). The wild-type AX2 cells and mutant cells were grown axenically in HL5 medium containing penicillin and streptomycin at 6 units/ml and 6 μg/ml, respectively. Transformants were selected with blasticidin S at 10 μg/ml in axenic medium.

The developmental phenotype was observed on a DM-agar plate (23) covered with an Escherichia coli B/r lawn or on a phosphate-buffered agar plate (2% Bactoagar, pH 6.7). The bacterial lawn was seeded with a drop of the Dictyostelium cells (2 × 10^6 cells). On a phosphate-buffered agar plate (diameter = 60 mm), 9 × 10^5 cells were dispersed.

Small population assays were carried out as described (24, 25). The wild-type and mutant Dictyostelium cells (5 × 10^4 cells/0.2 μl) were placed as drops on an agar plate (1% Bactoagar containing 1 mM CaCl_2 and 1 mM MgCl_2), 4 mm from the edge of a well filled with cAMP (10 μM).

The abbreviations used are: REMI, restriction enzyme-mediated integration; TPR, tetratricopeptide repeat; kb, kilobase pair(s); ACA, adenyl cyclase A; bsr, blasticidin S-resistance.
After 6 h, distribution of cells in each drop was examined and recorded. Assays for Adenylyl Cyclase—Activation of adenylyl cyclase by cAMP was examined as described (26). Briefly, the wild-type and mutant cells were shaken in 15 ml of 12 mM sodium potassium phosphate, pH 6.2, for 6 h. The density of the wild-type cells was 10^7 cells/ml. Many of the mutant cells were smaller than the wild-type cells, their density was adjusted to give the same turbidity as that of the wild-type cells. They were then washed and suspended in 1.5 ml of 12 mM sodium potassium phosphate, pH 6.2. The cells were aerated for 10 min and then stimulated with 2-deoxy-cAMP (100 nM) as described previously (29). Ten to 40 μg of total RNA was separated by 1% agarose, 6% formaldehyde gels and transferred to a nitrocellulose membrane. A 0.4-kb fragment of the cAR1 gene (31) was also amplified by PCR from genomic DNA and used as a probe for the Northern blot analysis.

**Molecular Cloning and Sequence Analysis of the trfA Gene**—Figures 1 and 2. GenBank/EMBL/DDJB accession number AB009080. The deduced amino acid sequence of the TRFA protein.

**Dictyostelium Homologue of Yeast SSN6**

**FIG. 1.** Restriction map of Dictyostelium genomic DNA around the trfA gene. The insertion site of the tagging plasmid is shown. The trfA coding sequence consists of four exons (thick lines), i.e., the genomic DNA fragment rescued in pBcClone; ii, the genomic DNA fragment rescued in pEcclone; iii, the genomic DNA fragment used in the targeting strategy, the XbaI/PstI fragment used as a probe for Southern blotting. Ba, BamHI; Bg, BgI II; E, EcoRI; H, HindIII; P, PstI; S, SpeI; V, EcoRV; X, XbaI.

**FIG. 2.** The deduced amino acid sequence of the TRFA protein. GenBank/EMLDBDBJ accession number AB009080. The boxed region indicates the 10 consecutive TPR motifs (171–521).
coli B/r cells. Colonies that formed smooth plaques were picked and selected further with HL5 medium containing blasticidin S (10 μg/ml). They were recloned on DM agar plates with an E. coli B/r lawn.

RESULTS

Molecular Cloning of a Dictyostelium Gene Involved in Aggregation upon Starvation—Dictyostelium cells were mutagenized by randomly inserting a BamHI-cut tagging plasmid, pUCBsrΔBam (Fig. 1), into the genome. The insertion of the tag was stimulated by adding a restriction enzyme, DpnII. Transformants were selected on agar plates covered with an E. coli lawn and containing blasticidin S. From these transformants, one strain, CC01, was identified as a clone that formed smooth plaques.

Two genomic DNA fragments, pEcoclone and pBgclone, were then rescued from the CC01 genome by means of the plasmid rescue. They contained the 4.0- and 1.5-kb genomic DNA segments flanking the inserted tag (Fig. 1). Thus, a 5.5-kb fragment of the Dictyostelium genomic DNA disrupted by the insertion event was finally rescued. To confirm the sequence around the insertion site, the corresponding fragment was amplified by polymerase chain reaction using the genomic DNA of AX2 cells, cloned, and sequenced. The sequence showed that two bases were deleted at the insertion site in the CC01 genome.

Homologous Recombination and Phenotype of the Resulting Mutant—Two genomic DNA fragments flanking the tagging plasmid in the CC01 strain were ligated to pUCBsrΔBam to generate a targeting plasmid, SHR6 (Fig. 1). The plasmid was linearized and then introduced into AX2 cells without addition of a restriction enzyme. Among 150 transformants selected with blasticidin, 12 colonies formed smooth plaques on an E. coli lawn. Of the 12 transformants, 1 clone designated as K07 was used for the detailed analysis as below.

Southern blot analysis was carried out to confirm the homol-

![Fig. 4. Phenotypes of the wild-type and mutant cells at the developmental stage.](image-url)
ogous recombination of the targeting plasmid into AX2 genome. Digestion of AX2 and CC01 genomes with BamHI and EcoRV generated 3.0- and 7.6-kb fragments containing the trfA gene, respectively. The difference of the size (4.6 kb) corresponded to the length of the inserted plasmid. The same digestion of K07 genome generated the 7.6-kb fragment, indicating that the targeting plasmid was correctly inserted into AX2 genome by homologous recombination. Since insertion of the targeting plasmid into the Dictyostelium genome by homologous recombination resulted in the same phenotype as that of the original REMI mutant (CC01 cells), it is concluded that the cloned DNA fragment contained a gene responsible for the mutant phenotype.

The Disrupted Gene Encodes a TPR Family Gene, trfA—The sequences of the rescued genomic DNA fragments revealed an open reading frame interrupted by three introns (Fig. 1). The intron-exon boundaries were confirmed by sequencing cDNA clones prepared from mRNA at the vegetative stage (data not shown). In the original REMI mutant, the tagging plasmid was inserted into the third exon of the gene (Fig. 1). The open reading frame encoded a 160-kDa protein comprising 1390 amino acid residues (Fig. 2). We designated this gene as trfA and the protein as TRFA. Homology searches of SWISS PLOT and Protein Information Resource using BLASTP (34) revealed that TRFA showed the highest similarity scores with S. cerevisiae SSN6 (9), a member of the TPR family. Like Ssn6, TRFA contained 10 copies of the TPR unit in tandem (Fig. 3A). The amino acid sequences of the 10 TPR units within TRFA were 58% identical to those in Ssn6. Like other TPR motifs analyzed by Sikorski et al. (4), the TPR units in TRFA contained small, uncharged amino acid residues at positions 8 (Gly or Ala), 20 (Ala), and 27 (Ala), aromatic residues at positions 4 (Trp), 11 (Tyr or Phe), and 24 (Tyr or Phe), a large aliphatic residue at position 7 (Leu or Ile), and a Pro residue at the border of the TPR units, particularly at position 32 (Fig. 3, B and C).

Outside the consensus sequence, no significant homology was detected among these 10 TPR units of TRFA (Fig. 3B). The divergence of TPR units outside the consensus sequence is common for other TPR family members. However, when each TPR unit, from TPR1 to TPR10, in TRFA was compared with that in Ssn6, each pair showed striking identity even outside the eight consensus residues (Fig. 3C). In particular, the third, fourth, and ninth TPR units of TRFA were more than 70% identical to the corresponding units of Ssn6.

TRFA contained Gln-rich (residues 1–170) and Asn-rich (522–937) regions (Fig. 3A). Gln-rich and Asn-rich sequence fragments are frequently observed in other Dictyostelium proteins (35, 36). In Ssn6, two Gln-rich regions (1–45 and 399–681) flank the TPR region. Furthermore, like SSN6, TRFA had multiple PXXXQ repeats, where X was uncharged (Fig. 2). Gln- and Pro-rich sequences are common in transcription factors (37–40) and thought to mediate transcriptional activation (41, 42).

The C-terminal region of TRFA (938–1390) had high contents of Glu (19%), Lys (14%), Ser (16%), and Thr (13%) residues (Fig. 3A). It is noteworthy that the residues in this EKST-rich region were highly charged (45% charged residues) and included three repeats of (R/K)(R/K)(S/Y)(X, any amino acid), which are thought to be the sites phosphorylated by cAMP- or cGMP-dependent protein kinases (underlined in Fig. 2) (43). This region also included putative phosphorylation sites for casein kinase II (43). The C-terminal region of Ssn6 (682–966) also has high contents of Glu (15%), Ser (11%), and Thr (12%) residues (Fig. 3A). This region of Ssn6 is also charged (26%) and includes putative phosphorylation sites for casein kinase II (10).

Northern Blot Analysis of the trfA Gene—The expression pattern of the trfA gene in the wild-type cells before and after initiating the development was examined by the Northern blot analysis. The gene was expressed at the vegetative stage and remained to be active at an early developmental stage, although its expression level was lower than that at the vegetative stage (data not shown).
**Phenotype of trfA\(^{-}\) Cells**—The original REMI mutant (CC01 cells) as well as the homologous recombinant (K07 cells) formed smooth plaques on bacterial lawns (Fig. 4B). Consistent with this observation, these cells rarely formed large aggregates when they were washed free of medium and plated on phosphate-buffered agar plates (Fig. 4, C-F). Only a small fraction of trfA\(^{-}\) cells aggregated into small mound-like structures, which did not develop into fruiting bodies even on prolonged incubation (data not shown). These results indicate that the trfA\(^{-}\) cells have defects in an early developmental process, especially at the aggregating stage.

Small population assays were carried out to determine whether the observed defect in cell aggregation arose from a defect in autonomous chemotactic response of individual trfA\(^{-}\) cells to cAMP. The wild-type cells on an agar plate exhibited a clear chemotactic response toward a well filled with cAMP (10 \(\mu\)M) (Fig. 5A). However, under the same conditions, the trfA\(^{-}\) cells exhibited little chemotactic response to cAMP (Fig. 5A), although some of them started to show weak response after prolonged incubation. Thus, individual trfA\(^{-}\) cells seem to be defective in correctly responding to the chemotactic signal.

The growth of the wild-type and trfA\(^{-}\) cells in a rich medium was compared by following turbidity changes in suspension cultures, which is a measure of the cell mass (Fig. 6B). When the cells were starved free of medium and plated on phosphate-buffered agar plates (Fig. 4, C-F). Only a small fraction of trfA\(^{-}\) cells aggregated into small mound-like structures, which did not develop into fruiting bodies even on prolonged incubation (data not shown). These results indicate that the trfA\(^{-}\) cells have defects in an early developmental process, especially at the aggregating stage.

**Expression of cAR1 and ACA Genes in trfA\(^{-}\) Cells upon Starvation**—The fact that trfA\(^{-}\) cells had defects in cell aggregation at the early developmental stage suggests two possibilities. One is that the cells did not enter the developmental stage because they failed to express genes such as cAR1 (the cAMP receptor gene) (30) and ACA (the adenyl cyclase A gene) (31), which were crucial for generating the cAMP pulses. The other is that the cells entered in the developmental stage but failed to aggregate because they had defects in their signal transduction pathway to induce the chemotactic response to the cAMP signal.

To determine which is the case, expression of the two critical genes, cAR1 and ACA, in trfA\(^{-}\) cells upon starvation was examined. As shown in Fig. 7, expression of these genes was rarely detected in the wild-type and trfA\(^{-}\) cells at the vegetative stage. When the cells were starved by washing free of the medium, the cAR1 and ACA genes were expressed in the wild-type cells as observed before (30, 31). When the starved wild-type cells were stimulated by repeated addition of cAMP (100 \(\mu\)M every 6 min) (29), their expression pattern did not change much because the starved cells generated their own cAMP pulses. Expression of the cAR1 and ACA gene in trfA\(^{-}\) cells followed the similar pattern upon starvation, and repeated addition of cAMP did not change this pattern, indicating that the developmental stage was actually initiated in trfA\(^{-}\) cells upon starvation.

**Transient Increase of the cAMP Level in trfA\(^{-}\) Cells upon Stimulation with cAMP**—When the wild-type cells were starved, the newly induced ACA became ready to respond to the cAMP signal. As shown in Fig. 5B, the cAMP concentration in the wild-type cells increased transiently upon stimulation with 2-deoxy-cAMP and then quickly returned to its original level, a typical response to the cAMP signal. The cAMP level in the starved trfA\(^{-}\) cells also exhibited a similar response to the stimulation with 2-deoxy-cAMP, although the maximal level was higher.

**DISCUSSION**

Yeast strains bearing mutations in the SSN6 gene have multiple mutant phenotypes, including constitutive expression of glucose-repressible genes, calcium-dependent flocculation, mating defects of MAT \(\alpha\) cells, inability of homozygous diploids to sporulate, and poor growth (9, 15, 18, 44). These diverse phenotypes result from a failure to repress different kinds of genes (17). Genetic analysis has revealed multiple functional interactions between distinct subsets of TPR units in Ssn6 with target proteins in this process (45). When the TPR units in TRFA were compared with those in Ssn6, striking homology was detected not only in the TPR consensus sequence but also in the region outside this sequence. In particular, the third, fourth and ninth TPR units of TRFA and Ssn6 shared high proportions of identical residues. The TPR consensus sequence may be required to maintain the basic structural architecture of the TPR domain, whereas residues outside the consensus sequence would be required as a surface for the interaction between TPR units and target proteins (8). Thus, the fact that the TPR units of TRFA and Ssn6 are highly homologous beyond the consensus sequence implies that these proteins interact with similar target proteins.

It must be also noted that the sequence similarity between TRFA and Ssn6 extends even outside the homologous TPR units. Both TRFA and Ssn6 have poly(Gln) stretches, which also contain interspersed Pro residues. Consequently, this region contains many PX(QX) repeats, where X is an uncharged residue (9). Such a Gln- and Pro-rich segment is present in many transcription factors (38–40). Moreover, in the C-termi-
nental regions of TRFA and Sn6, there are EKST-rich or EST-rich sequences containing putative phosphorylation sites for cAMP- and cGMP-dependent kinases as well as for casein kinase II.

As in yeast cells bearing mutations in SSN6, trfA- cells exhibited multiple defects. First, the mutant cells grew poorly, and cells with abnormal sizes were generated in suspension culture. Second, the mutant cells showed a defect in an early developmental process, especially at the aggregation stage. The cells rarely associated into large aggregates to form mounds and slugs of a normal size. Consistent with this defect in the early development, individual trfA- cells exhibited a defect in the autonomous chemotactic response toward cAMP, independent of the cAMP relay between cells.

Northern blot analysis showed that upon starvation, trfA- cells started expressing two critical genes for the developmental stage, i.e. cARI and ACA, an indication that the cells entered in the early developmental stage. Adenyl cyclase (ACA) of trfA- cells thus induced was transiently activated by cAMP or its analog with a similar time course to that of the wild-type cells, suggesting that the cAMP relay in trfA- cells functioned normally. Taken together, it is likely that the major defect in trfA- cells at the early developmental stage was not in the system for generating the cAMP signal but in the system required for correctly responding to it.

The striking structural similarity between TRFA and Sn6 as well as the pleiotropic phenotypes of trfA- cells indicate that TRFA may function as a multiple regulator for multiple cellular functions like Sn6. Several transcription factors regulating the developmental process of Dictyostelium cells such as the G-box-binding protein and STAT protein have been already cloned (46, 47). The coordination among these factors would be essential for normal growth and development of Dictyostelium cells.

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**Dictyostelium** TRFA Homologous to Yeast Ssn6 Is Required for Normal Growth and Early Development

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