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Underexpression of a Novel Gene, *Dia2*, Impairs the Transition of *Dictyostelium* Cells from Growth to Differentiation

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In *Dictyostelium discoideum* Ax-2 cells, a specific point (PS-point) in the cell cycle from which they initiate differentiation in response to starvation has been specified. Using synchronized Ax-2 cells and the differential display method, a novel gene (differentiation-associated gene 2; *dia2*) was isolated as one of the genes expressed specifically during the shift of Ax-2 cells from growth to differentiation. The *dia2* gene codes a lysine- and leucine-rich protein with a predicted molecular mass of 16.9 kDa. Northern blot analysis has shown that the *dia2* mRNA, of 0.7 kb, accumulates in differentiating cells starved just before the PS-point, while there is no detectable expression in vegetatively growing cells. Antisense-mediated gene inactivation of *dia2* greatly inhibited the progress of differentiation, presumably through the reduced expression of cAMP receptor 1 (*car1*). Thus, the *DIA2* expression was suggested to have an essential role in the initiation of differentiation, closely relating to the cAMP signaling system.

In general, growth and differentiation are mutually exclusive, and understanding the transition mechanism of cells from a proliferative status to differentiation is a crucial issue to be solved in developmental biology and cancer research. *Dictyostelium discoideum* (strain Ax-2) cells grow and multiply by binary fission as long as nutrients are supplied. Upon exhaustion of nutrients, starving cells differentiate to acquire aggregation-competence and they form multicellular structures by means of chemotaxis to cAMP (1) and EDTA-resistant cohesiveness (2). The growth and differentiation phases are temporally separated from each other and easily controlled by nutritional conditions. Also, a temperature-shift method for inducing synchronous growth of cells has been established (3), and a specific point (referred to as a putative shift point; PS-point) has been specified in the cell cycle of Ax-2 cells (4). This is the point from which cells enter the differentiation phase when placed under conditions of nutritional deprivation. Thus, *Dictyostelium* offers a particularly useful system for elucidating the cellular and molecular mechanisms of growth/differentiation transition.

Gene expressions associated with the initial step of growth/differentiation phase transition were analyzed using Ax-2 cells synchronized by the temperature-shift method and differential screening. Four genes (*Quit1*, *Quit2*, *Quit3*, and *annexin VII*) were isolated, as being expressed specifically or differentially in cells that had been starved just before the PS-point and that were now in the initial step of cell differentiation (5–7). *Quit1* has been identified to be the cAMP receptor 1 (*car1*) gene (5), that is essential for differentiation (8, 9). CAR1-dependent events include receptor phosphorylation and influx of extracellular Ca^{2+} (10). *Quit2* encodes a novel Ca^{2+}-binding protein (calfumirin-1; CAF-1) with four EF-hand domains (6), and its overexpression enhances differentiation in a Ca^{2+}-dependent manner (11). Interestingly, *Quit3* has no coding region and encodes the complementary sequence of *annexin VII* (7) that is believed to be needed for cellular Ca^{2+}-homeostasis (12, 13), thus regulating annexin VII synthesis via a natural antisense transcript (7).

Recently, we isolated two genes (*DAPS-1* and *DdEF-1β*) expressed differently during the transition from growth to differentiation of *Dictyostelium* Ax-2 cells (14,15), using Ax-2 cells synchronized by the temperature-shift method (3) and the differential display method (16). *DAPS-1* encodes a proteasome subunit and was found to be expressed predominantly in response to differentiation (14), while the expression of *DdEF-1β*encoding for EF-1β, an essential factor for protein synthesis, decreases during the transition of

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cells from growth to differentiation (15). In this paper, we report the structure and function of a novel gene, *dia2*, (differentiation- associated gene 2), which is specifically expressed during the phase-shift of Ax-2 cells from growth to differentiation.

MATERIALS AND METHODS

Cell culture and developmental conditions. Vegetative cells of *Dictyostelium discoideum* Ax-2 were grown axenically in HL-5 medium (17) supplemented with 1% glucose. Transformants expressing either the antisense RNA of *dia*2 or the original vector construct (*pAct15-gal*) were grown axenically by shake culture in HL-5 medium containing 20 μg/ml G418. To allow cells to differentiate, cells were harvested at the exponential growth phase, washed twice in BSS (Bonner's salt solution; 18), and settled down either in a 24-well titer plate (Falcon, #3047) or on 1.5% nonnutrient agar. This was followed by incubation at 22°C, as previously described (14).

Synchronization of the cell-cycle phase. Cell synchronization was performed using the temperature shift method (3) with a slight modification. Exponentially growing cells ($1.0–1.5 \times 10^6$ cells/ml) at 22.0°C with a doubling time of about 7.6 h were shifted to 9.4°C, shaken for 14.5 h and then reshifted to 22.0°C. Under these conditions, cell doubling occurred over about a 2-hr period after a lag phase of about 1 h. T7 cells, 7 h after the shift-up from 9.4 to 22.0°C, were harvested just before the PS-point, starved by washing twice in 20 mM phosphate buffer (PB) (KH_2PO_4/Na_2HPO_4, pH 6.5), and then shaken at 1×10^7 cells/ml for 2 h at 150 rpm. This yielded T7 + 2 cells, i.e., newly differentiating cells. T4 + 2 cells were prepared by starving T4 cells for 2 h in PB, as starved but not differentiated cells. As another control, T9 cells obtained by incubating T7 cells for 2 h in the growth (HL-5) medium were used.

Isolation of total RNAs and Northern hybridization. Total RNAs were prepared according to the method of Nellen *et al.* (19) from T7 + 2 cells, T9 cells, and T4 + 2 cells. They were also prepared from nonsynchronized cells at various developmental stages. Northern blot analysis was carried out, as previously described (14).

Differential display and cDNA sequencing. Differential display was performed by the method of Liang *et al.* (20) with the RNAimage kit (GenHunter), using DNA-free total RNAs of T4 + 2 cells, T7 + 2 cells, and T9 cells. cDNA sequencing was carried out using the ABI PRISM Dye Terminator cycle sequencing kit (Perkin–Elmer) and the ABI Prism 310 Genetic Analyzer (Perkin–Elmer).

Plaque hybridization and in vivo excision. The cDNAs encoding the full length of mRNAs were screened by the plaque hybridization method from T7+2 cDNA library (5) that was constructed in λ-ZAPII (Stratagene). Plaque hybridization was performed, as previously described (14). A phagemid containing the cloned insert was recloned by *in vivo* excision of the pBluescript SK(−) phagemid from the λ-ZAPII vector (Stratagene).

Sequencing of the deleted 5′- site of dia2. cDNA clones containing *dia2* sequences were isolated by plaque hybridization from the T7 + 2 cDNA library (5). However, we failed to obtain a full length of the cDNA clone from 9 positive clones. The *dia2* cDNA isolated by plaque hybridization lacked the 5′-end. Thereupon, the longest positive clone with a 440-bp insert with a stop codon was firstly isolated and sequenced. The missing 5′-site of *DIA2* was directly determined from the T7 + 2 cDNA library by the 5′-RACE PCR method. The PCR was done using a reverse primer (5′-GGAAACAGCTATGACCATG-3′) of pBluescript II SK(−) and a DA4A1 primer (5′- AGCAGAA-TGTGTAATCAT-3′). As a result, two DNA fragments with a length of 400 and 340 bp were obtained. The DNA fragments were reamplified, and the reamplified DNAs were cloned into the pGEM-T Easy vector (Promega). Since an additional 110 bp of DNA fragment containing a start codon (ATG) was obtained, its sequence was determined.

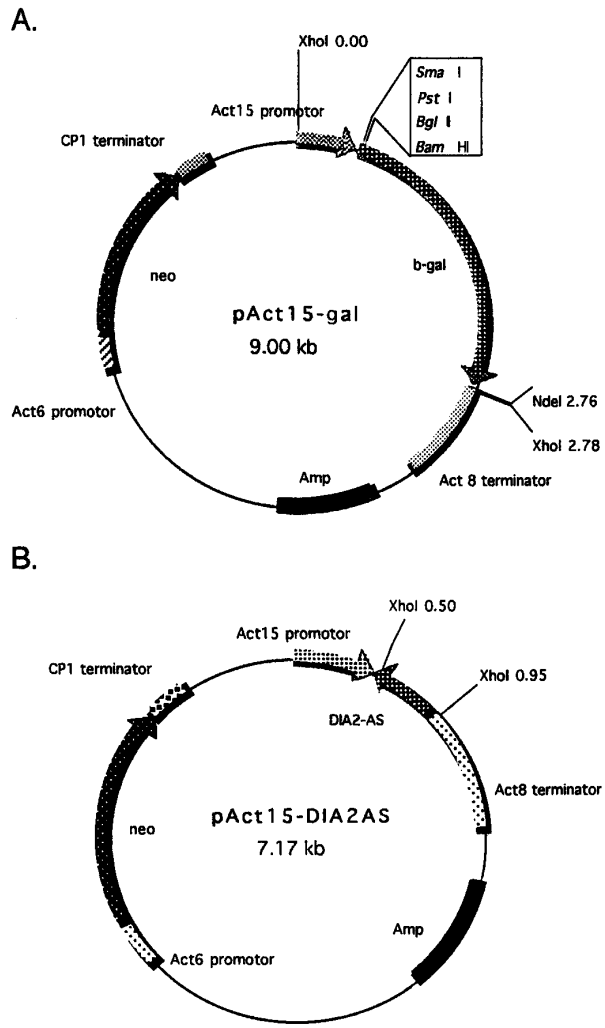

FIG. 1. An antisense *dia2* transformation vector (pAct15-DIA2AS) and its original vector (pAct15-gal). (A) The vector *pAct15-gal* consists of the *Dictyostelium* actin-15 promoter and confers resistance to the antibiotic G418 on *Dictyostelium* cells. (B) The *dia2* cDNA was inserted into the region of deleted *lacZ* of *pAct15-gal* in antisense direction, as described under Materials and Methods.

Extraction and purification of plasmid DNAs. Extraction and purification of plasmid DNAs were performed using alkaline lysis (21, 22).

Plasmid construction, transformation and selection of transformants. *pAct15-gal* (23) was used as the original vector for transformation (Fig. 1). The β-galactosidase gene was removed from this plasmid by digesting with *Bam*HI and *Nde*I, and the remaining vector was blunt-ended using a DNA blunting kit (Takara). This vector was dephosphorylated using bacterial alkaline phosphatase (BAP) and then ligated overnight with the blunt-ended *dia2* cDNA fragment that had been digested with *Apa*I and *Sma*I. These ligates were inserted into XL1-blue competent cells. To produce cells underexpressing the *dia2* mRNA, Ax-2 cells were transformed with the antisense construct, as described by Nellen *et al.* (19). Transformed cells were selected in 10 ml of HL-5 medium containing 10–20 μg/ml G418 in Petri dishes.

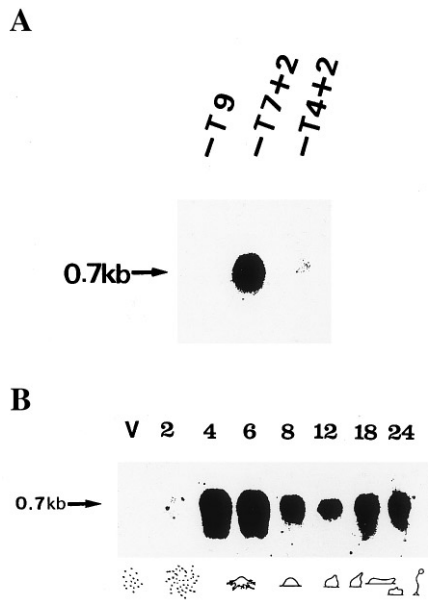

FIG. 2. (A) Specific expression of the *dia2* mRNA in just-differentiating cells from the PS-point. Synchronized Ax-2 cells were withdrawn at T4 + 2, T7 + 2, and T9, and total RNAs were isolated. The RNAs (20 mg for each) were size-separated on 1% formaldehyde agarose gel and blotted for hybridization to the double-stranded*dia2* cDNA probe. (B) Developmental change of the *dia2* mRNA expression. Non-synchronized Ax-2 cells growing in HL-5 medium were harvested, washed and allowed to develop on 1.5% nonnutrient agar plate. Northern analysis of *dia2* mRNA was performed, as described under Materials and Methods. Lane V; vegetative growth phase. The numbers indicate incubation times (h) after starvation. The gross morphology of each developmental stage is shown at the bottom of the figure.

RESULTS

Dia2 is a novel gene expressed specifically in response to cell differentiation. From Northern analysis of *dia2* cDNA, it is evident that the mRNA of 0.7 kb is markedly expressed in T7 + 2 cells (differentiating cells) starved just before the PS-point, while that expression is never detected in vegetatively growing T9 cells (Fig. 2A). The *dia2* mRNA was also expressed in T4 + 2 cells, but only very weakly compared with that in T7+2 cells (Fig. 2A). It is possible that in the RNA isolated from T4 + 2 cells there may be T7 + 2 RNA as a contaminant, because of imperfect cell synchrony. Thus, we may be under-estimating the enrichment of *dia*2 mRNA in T4 + 2 cells.

The developmental change in *dia2* expression was examined using nonsynchronized Ax-2 cells. The *DIA2* mRNA began to be expressed 2 h after starvation, and the maximal expression was attained at 4–6 h (around the aggregation-stream stage) after starvation (Fig. 2B). This was followed by a temporal decrease and re-increase at the early slug stage (18–24 h after starvation) (Fig. 2B).

The *dia2* cDNA sequence was determined as described under Materials and Methods). It encodes for a protein (DIA2) of 151 amino acids with a predicted molecular mass of 16.9 kDa. The deduced amino acid sequence of DIA2 shows that it is lysine-rich (14.6% of the total amino acid residues) and also leucine-rich (10.6% of the total amino acid residues) (Fig. 3). From homology searches, *dia2* was concluded to be a novel gene without any significant similarity to other genes thus far reported. The hydropathy profile of DIA2, determined by the method of Kyte and Doolittle (24), indicated that the DIA2 protein has three hydrophobic portions at amino acid positions (1–25, 110–117, 127–134). Since the first 1–25 portion has a relatively high hydrophobicity, this protein is most likely to be anchored to the membrane at the N-terminus.

Developmental role of the dia2 expression in the growth/differentiation transition. An antisense-mediated gene inactivation strategy was adopted for analysis of the function of *dia2*. Exponentially growing Ax-2 cells were transformed with the antisense construct (*pAct15-DIA2AS*) or the original vector construct (*pAct15-gal*) (see Fig. 1), and transformants were selected in HL-5 medium containing 20 μg/ml of G418. Among 96 clones examined, one clone (AS-2) was found to exhibit aberrant development after starvation. Production of the *dia2*-antisense transcript in AS-2 cells was confirmed by the fact that AS-2 cells are able to

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TTTTAATTATTTTTATTTTTCAAATATAAATTAAAAAATGAAACAAATTATTAGATTAAT   60
                                    M  K  Q  I  I  R  L  I     8

AACTACATTATTATTATTATCACTCATTGGTATTACTTGTGCAGCAGTAGCCAAACTTCA  120
 T  T  L  L  L  L  S  L  I  G  I  T  C  A  A  V  A  K  L  H    28

TGAAACAGATAAAAAATTTAAAGGTGTTGAATTGCCTGAAAAATTTTTAGATGATTTTAA  180
 E  T  D  K  K  F  K  G  V  E  L  P  E  K  F  L  D  D  F  N    28

TTTAGAGGTTGGTAAACTTCAATATGCAAATCTTTTCCGTTCTAAAAAAGATGATATTAC  240
 L  E  V  G  K  L  Q  Y  A  N  L  F  R  S  K  K  D  D  I  T    48

ACATTCTGCTGGTGAAATAAGAATTGATCAACAAGGTTCATTTTCAAGAGGTGAAAAAAA  300
 H  S  A  G  E  I  R  I  D  Q  Q  G  S  F  S  R  G  E  K  K    68

GAAAAAGTTTAAGGTATACAATCTTCAAGCTCAAACAAATGGTAAATCAAGTAAAACAGT  360
 K  K  F  K  V  Y  N  L  Q  A  Q  T  N  G  K  S  S  K  T  V    88

TGCTCATGTTGAAGTATTTGATACAATTGATGCAAAAACAAAAGCTGAACAAAATGAAGT  420
 A  H  V  E  V  F  D  T  I  D  A  K  T  K  A  E  Q  N  E  V   108

ATTGGCATTAGCTAAAGAAGCATTCACAAAAAGTAAAGATTCTGGAAAATTATATCAAGT  480
 L  A  L  A  K  E  A  F  T  K  S  K  D  S  G  K  L  Y  Q  V   128

TATTCCAAACTAATAAAGAACATACATTTGTTTTAAAAAATTAAATATTTAAATAAATAA  540
 I  P  N  *                                                    131

TAATTTTGAA                                                     550
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FIG. 3. cDNA sequence of *dia2* and deduced amino acid sequence. *DIA2* was isolated and sequenced, as described under Materials and Methods. The putative polypeptide deduced from the 550-bp nucleotide sequence is consisting of 151 amino acids with a molecular mass of 16.9 kDa. This nucleotide sequence is deposited in the DDBJ, EMBL, and GenBank databases with Accession No. AB007027.

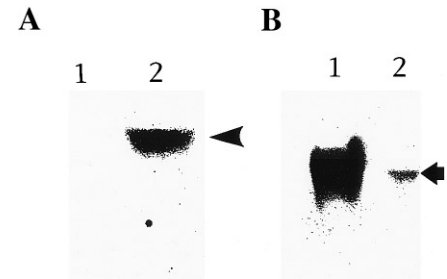

FIG. 4. (A) Enforced expression of *dia2*-antisense RNA in the transformant (clone AS-2) at the vegetative growth phase. Total RNAs were extracted from exponentially growing AS-2 cells and parental Ax-2 cells. This was followed by electrophoresis and transfer on to a nylon membrane. The antisense transcript of *dia2* was detected using the ^{32}P-labeled *dia2* cDNA probe. The *dia2*-antisense RNA (size, 2.9 kb; lane 2, arrowhead) was expressed in vegetatively growing AS-2 cells (lane 2), but not in Ax-2 cells (lane 1). (B) Underexpression of the *car1* mRNA in AS-2 cells. Nonsynchronized AS-2 cells and parental Ax-2 cells at the exponential growth phase were separately harvested, washed and allowed to develop on 1.5% nonnutrient agar. Total RNAs were extracted from cells 4 h after starvation. The *car1* mRNA (size, 1.9 kb; arrow) expression was greatly reduced in AS-2 cells (lane 2) than in Ax-2 cells (lane 1), as probed by ^{32}P-labeled *Quit1*(*car1*) cDNA.

synthesize a mRNA that hybridizes to the double-stranded *DIA2* cDNA probe even during the vegetative growth phase, where parental Ax-2 cells never produce the mRNA (Fig. 4A). Here it is of interest to note that in AS-2 cells the *car1* expression is greatly suppressed (Fig. 4B).

When the transformant AS-2 and its parental strain Ax-2 were separately harvested at the exponential growth phase, washed in BSS and incubated under submerged conditions at various cell densities they exhibited quite different behaviors in a density-dependent manner (Fig. 5). At 1.6×10^5 cells/cm^2, AS-2 cells showed no sign of cell aggregation after 10 h of incubation, while Ax-2 cells were able to form aggregation-streams (Figs. 5E and 5F). Below lower densities around 8×10^4 cells/cm^2, neither AS-2 nor Ax-2 cells aggregated by 10 h after starvation. After a prolonged time (24 h) of incubation, Ax-2 cells formed tight aggregates, while AS-2 cells still remained as single cells (data not shown). AS-2 cells were found to form aggregates at a higher density (6.4×10^5 cells/cm^2), but their development was somewhat delayed compared with that of Ax-2 cells (Figs. 5A and 5B). Incidentally, cells transformed with the vector, *pAct15-gal*, exhibited essentially the same developmental kinetics as Ax-2 cells, when they were starved and incubated under submerged conditions in BSS.

On agar, starving AS-2 cells displayed a peculiar morphogenesis. At a density of $2–8 \times 10^5$ cells/cm^2 relatively large aggregates were formed after 12 h of starvation, being delayed 2–3 h than in the case of parental Ax-2 cells. Subsequently, the aggregate of AS-2 cells subdivided, to form a mass of smaller aggregates, and no tips were formed on the aggregates. In due course the small aggregates seemed to fuse with each other to form large amorphous aggregates.

When starved AS-2 and Ax-2 cells were mixed at various number-ratios and incubated on 1.5% nonnutrient agar at a density of about 5×10^5 cells/cm^2, no synergism was observed between the two. In a mixed culture of AS-2: Ax-2 (10:1), they completely sorted out after aggregation: A few number of small tips, consisting of Ax-2 cells were formed on the amorphous cell masses composed of AS-2 cells. Below $1/10^3$ Ax-2 cells tips were never formed, and therefore the inability of AS-2 cells to form tips seemed not to be compensated for by the presence of Ax-2 cells.

DISCUSSION

The novel gene, *dia2*, was found to be specifically expressed in the initial step of cell differentiation as cells exit from the PS-point of the *Dictyostelium* cell cycle. The *dia2* mRNA is predicted to encode a unique protein (deduced molecular mass; 16.9 kDa) with a high content of lysine (14.6%) and leucine (10.6%) residues. The hydropathy profile of DIA2 protein suggested that it might be anchored to the membrane at the N-terminus.

In nonsynchronized Ax-2 cells, the expression of *dia2* mRNA fluctuated in a bimodal fashion during the course of development (Fig. 3), being quite similar to the expression pattern of the cAMP receptor 1 (*car1; Quit1*) gene (5). Louis *et al.* (25) have demonstrated that two *car1* mRNAs (referred to as early mRNA and late mRNA) are regulated by separate promoters activated at different developmental stages and are alternatively spliced, generating different transcripts in *D. discoideum* NC-4 and Ax-3 cells. CAR1, a G-protein-linked surface cAMP receptor, is known to be essential for *Dictyostelium* development, including cell aggregation; its disruption by homologous recombination or antisense RNA results in a failure of transformed Ax-3 cells to develop (8, 9). Expression in T7 + 2 cells (starved just before the PS point) as previously exemplified by *car1* (*Quit1*) is believed to be one of the earliest events realized in differentiation (5). The present work has shown that forced underexpression of the *dia2* mRNA, by an antisense transcript greatly impairs the *car1* expression as well as the formation of cell aggregates by cAMP-mediated chemotaxis: particularly at low cell densities (Fig. 5). In addition, tip formation was never observed in AS-2 cells expressing the anti-*dia2* mRNA. Taken together these data strongly suggest that the DIA2 protein is closely coupled with CAR1-associated events.

As to gene expressions associated with the growth/differentiation transition, the *V* gene and *I* gene have both been reported as being specifically expressed in vegetative cells and starved cells, respectively, and,

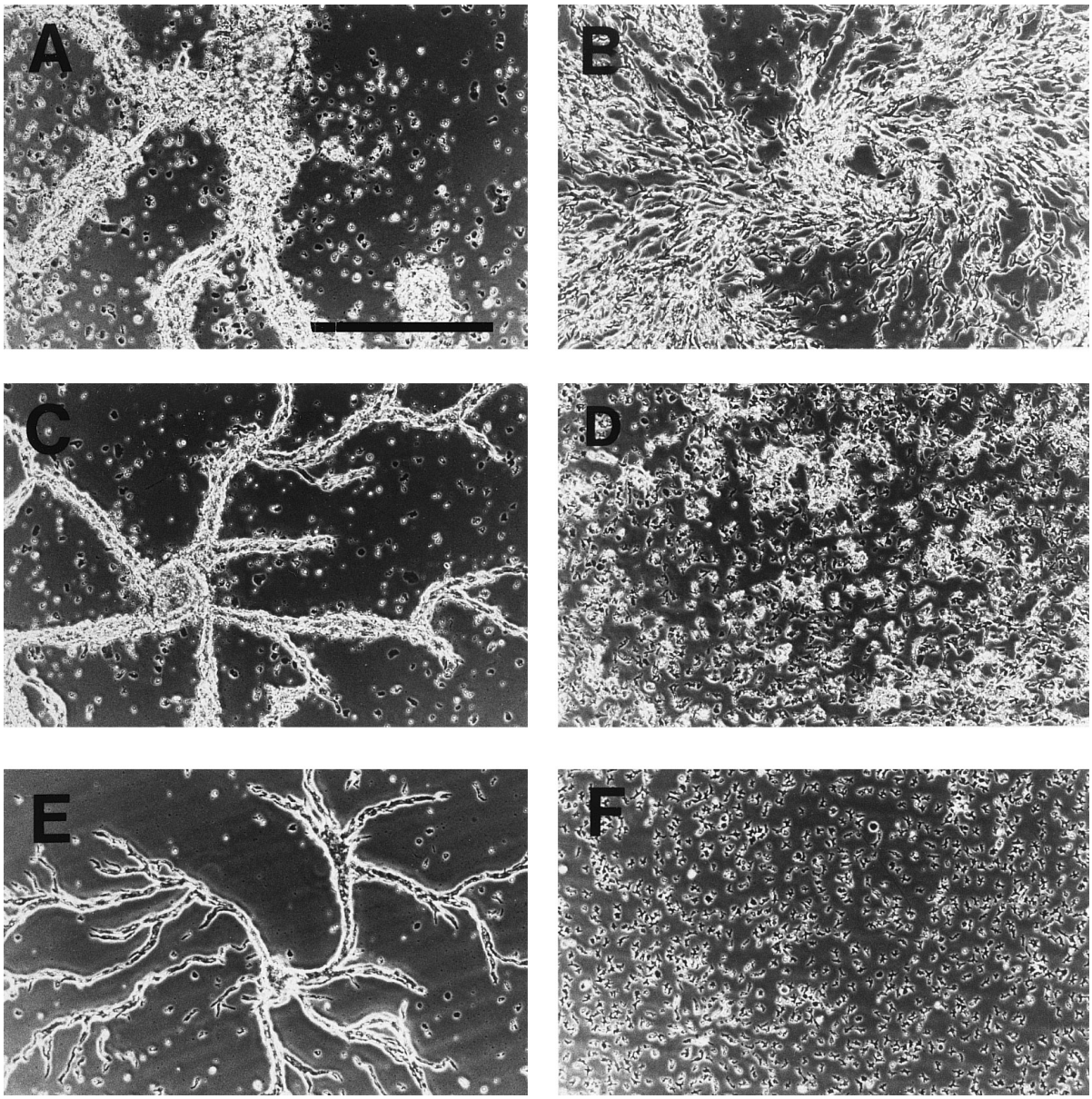

FIG. 5. Developments of starved AS-2 cells and parental Ax-2 cells under submerged conditions at different cell densities. Ax-2 and AS-2 cells were harvested at the exponential growth phase, washed twice in BSS, and plated in a 24-well plate at various cell densities. This was followed by incubation for 10 h at 22°C. (A, C, E) Ax-2 cells. (B, D, F) AS-2 cells. Cell density: (A, B) 6.4×10^5 cells/cm^2; (C, D) 3.2×10^5 cells/cm^2; (E, F) 1.6×10^5 cells/cm^2. Bar, 150 μm.

somewhat surprisingly, cells transformed with an antisense expression vector against the *V4* gene fail to develop after starvation (26, 27). Recently, Souza *et al.* (28) have demonstrated that a protein kinase, *YakA* (a homolog of *Tak 1p,* a growth-regulating protein kinase in *Saccharomyces cerevisiae*) is essential for the proper regulation of both growth and differentiation, and that the *YakA* expression from a conditional promoter causes cell-cycle arrest in nutrient-rich medium and promotes differentiation-associated events, such as the expression of genes required for cAMP signaling. These studies were done using nonsynchronized cells. As noted, however, it is generally difficult to precisely analyze the transition mechanism using nonsynchronized cells, because Ax-2 cells in any phase of the cell cycle at the onset of starvation progress through the cell cycle to the PS-point, and enter the differentiation phase from that specific point. Therefore, we have attempted to isolate genes expressed specifically in the initial step of differentiation, from the PS-point, using synchronized cells and the differential display method. The structural and functional characterization of genes thus far found offers us strong indications as to the importance of cAMP, Ca^{2+} and their related events for

the transition of *Dictyostelium* cells from growth to differentiation. To analyze more precisely the function of *dia2*, we are now planning to prepare *dia2*-null mutants by homologous recombination and also to obtain the DIA2 protein and its specific antibody.

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