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Precise expression of the cAMP receptor gene, *CAR1*, during transition from growth to differentiation in *Dictyostelium discoideum*

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Abstract

The gene expressions associated with the switch-over from cell proliferation of *Dictyostelium discoideum* to differentiation have been analyzed using temperature shift and differential plaque hybridization methods. *Quit1* was cloned as a specifically expressed gene when cells were starved just before the PS point (putative shift point from growth to differentiation). The coding region of the *Quit1* gene is identical to that of the cAMP receptor 1 (*CAR1*) gene, which is essential for development. *Quit1* mRNA was specifically expressed just after starvation of cells at around the PS point, thus indicating the importance of *CAR1* for cellular differentiation as well as the actual existence of the PS point.

Key words: Cell cycle; Putative shift (PS) point; Differentiation; cAMP receptor 1; *Dictyostelium*

1. Introduction

Understanding how cells change from a proliferative status to differentiation is a principal issue in developmental biology, and also in cancer research. The life cycle of the cellular slime mold, *Dictyostelium discoideum* Ax-2, consists of a growth phase and a differentiation phase, which are temporally separated from each other. When growing cells are harvested by removal of extracellular nutrients, the starving cells initiate differentiating to form a multicellular aggregate. The cell aggregate passes through multiple stages on its way to terminal differentiation into either spores or stalk cells. Cell aggregation is mediated by a cAMP signaling system. The stimulation of receptors by cAMP promotes the synthesis and secretion of cAMP, and regulates the expressions of various early genes [1].

Evidence has been obtained indicating that the commitment of Ax-2 cells to differentiation depends on the cell's position in the cell cycle at the initiation of development [2–8]. Using Ax-2 cells synchronized by the temperature shift method [9], we have previously shown that cells progress through the cell cycle to a particular point (referred to as the putative shift point or PS point), irrespective of the presence or absence of nutrients, and enter the differentiation phase from this point under starvation conditions [5]. 101 kDa and 90 kDa proteins have been shown to fail to phosphorylate at the PS point under starvation conditions, and dephosphorylation of a 32 kDa protein, as well as low phosphorylation levels of the 101 kDa and 90 kDa proteins might be required for the phase-shift of Ax-2 cells from cell proliferation to differentiation [10]. In this study, we have focused on gene expressions associated with the exit of cells from proliferation into differentiation. Screening of the cDNA library by the method of differential plaque hybridization has demonstrated that the cAMP receptor 1 gene (*CAR1*) is specifically expressed in cells starved just before the PS point, providing evidence of the involvement of this gene in growth/differentiation transition and also tot he specific existence of the PS point in the cell cycle.

2. Materials and methods

2.1. Cells and culture conditions

Vegetative cells of *D. discoideum* Ax-2 (clone 8A) were grown axenically in HL-5 medium (10 ml) supplemented with 1.5% gulucose in a 200-ml Erlenmeyer flask coated with Sigmacote (Sigma). Cell synchronization was performed using the temperature shift method [9] with a slight modification. Exponentially growing cells ($1–2 \times 10^6$ cells/ml) at 22.0°C were shifted to 9.0°C, shaken for 14 h and then re-shifted to 22.0°C. Under these conditions, cell doubling occurred over about a 2-h period after a lag phase of about 1 h. Synchronized cell populations from various phases of the cell cycle (referred to as Tt cells, after shift-up from 9.0 to 22.0°C), were either (i) allowed to grow for another 2 h in HL-5 medium or (ii) washed twice in 20 mM Na/K-phosphate buffer (PB), pH 6.2, as starvation medium. For example, T7 cells, 7 h after the shift-up from 9.0 to 22.0°C, were withdrawn, washed twice in PB, and shaken for 2 h at 1×10^7 cells/ml at 150 rpm to obtain T7 + 2 cells. Incidentally, the PS point from which a cell enters the differentiation phase in response to nutritional deprivation is supposed to be located just after T7 [5]. As a reference, T1 + 2 cells were prepared by starving T1 cells for 2 h in PB.

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2.2. RNA extraction, differential screening, and Northern hybridization

Total RNA was extracted as described by Nellen et al. [11], and a cDNA library was prepared from the poly(A)$^+$ mRNA of T7 + 2 cells. The library was constructed in ZAPII according to the manual of the cloning kit (Stratagene). The library was plated with *E. coli* XL-1 blue on agar and transferred on to two nylon membranes (Amersham). For differential screening, these membranes were hybridized to a ^{32}P-labeled single-stranded cDNA probe prepared from poly(A)$^+$ RNA of T7 + 2 cells or T9 cells (obtained by incubating T7 cells in HL-5 medium for 2 h). After washing twice in 0.1 × SSPE containing 0.1% SDS at 65°C, the membranes were exposed to X-ray films (New A; Konica) for 3–10 days at −80°C. For Northern hybridization, 5 μg of total RNA samples were separated on a 1% formaldehyde agarose gel and transferred on to the nylon membranes. Probes were radiolabeled by the random primer method using [α-^{32}P]dCTP, and hybridized to RNA blots at 65°C in 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, and 20 μg/ml denatured salmon sperm DNA. This was followed by washing and autoradiography as described above.

2.3. DNA sequencing

The cDNA sequence of *Quit1* was determined using a DNA sequencer (ABI, 373A).

3. Results and discussion

Single-stranded cDNA probes were prepared from poly(A)$^+$ RNA of T9 cells and T7 + 2 cells, and then the T7 + 2 cDNA library was screened by the differential plaque hybridization method. Of 260,000 cDNA clones examined, ten clones hybridized more strongly to the T7 + 2 probe than to the T9 probe. No clones hybridized more strongly to the T9 probe than to the T7 + 2 probe. Three cDNA clones were found to be expressed specifically or predominantly in response to starvation of cells at around the PS point, and the most notable one was referred to as *Quit1*. Northern analysis of *Quit1* expression showed that the *Quit1* mRNA, 1.9 kb, was strikingly accumulated in T7 + 2 cells that had been starved just before the PS point, while *Quit1* mRNA expression was not noticable in growing cells such as T3 and T9 cells, (Fig. 1A). *Quit1* mRNA was also expressed in T1 + 2 cells, but only slightly. The slight expression in T1 + 2 cells might be due to imcompleteness of cell synchrony; or mixing up of T7 + 2-like cells in the cell population. Alternatively, it is also possible that the *Quit1* mRNA might be expressed slightly in concert with starvation even in synchronized cells that had been located at cell cycle positions other than the PS point (near T7).

To examine the developmental change of *Quit1* expression, non-synchronized Ax-2 cells were harvested in the exponential growth phase (1–2 × 10^6/ml), washed, and allowed to develop on 1.5% non-nutrient agar. This was followed by extraction of total RNA from developing cells at 2-h intervals. As shown in Fig. 1B, *Quit1* mRNA (coding region) levels increased in a bimodal fashion during development; the first peak occurred 4 h (just before cell aggregation) after starvation and the second one at the tight-aggregate stage (12 h after starvation).

The sequence data of *Quit1* revealed that it encodes a

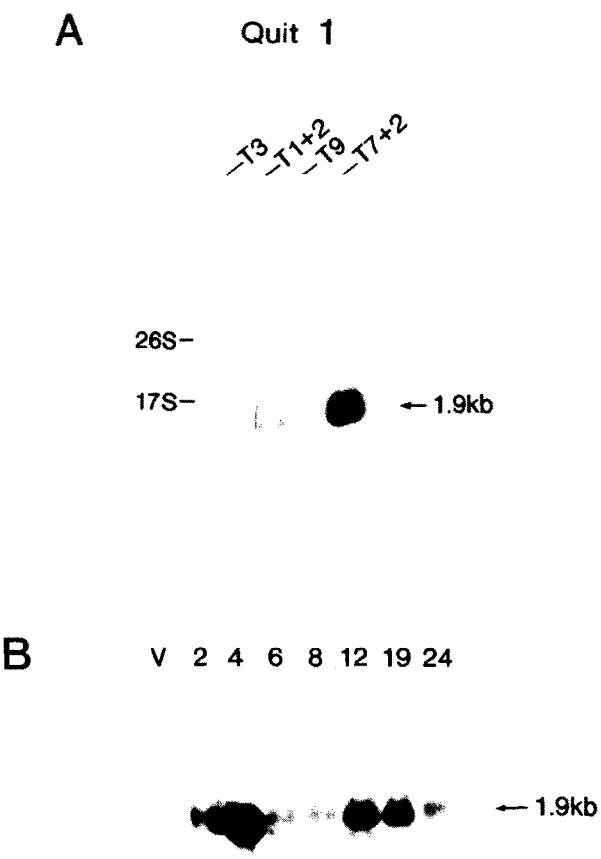
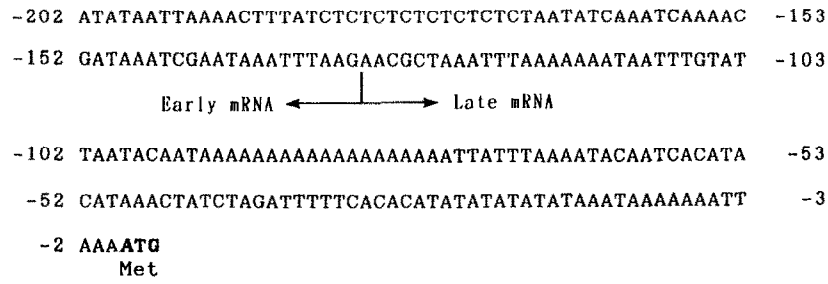


Fig. 1. (A) Differential expressions of *Quit1* mRNA after starvation in a cell cycle-dependent manner. Synchronized Ax-2 cells were collected at T3, T1 + 2, T9, and T7 + 2 as described in section 2, and total RNA was isolated. RNAs were size-separated on denaturing gels and blotted for hybridization to the *Quit1* cDNA probe. It is clear that *Quit1* mRNA is predominantly expressed in T7 + 2 cells starved just before the PS point. (B) Developmental expressions of *Quit1* mRNA. Non-synchronized Ax-2 cells growing in HL-5 medium were harvested, washed, and allowed to develop on 1.5% non-nutrient agar. Lane V, vegetative growth. At the indicated time (h) of incubation, total RNA was isolated from cells and blotted for Northern hybridization, as described above. The gross morphology of each developmental stage is shown at the bottom of the figure.

protein equivalent to the cAMP receptor 1 (CAR1), but that the 5′ untranslated region is somewhat different from previous results [12] (Fig. 2). Recently, it has been 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 [12]. As shown in Fig. 2B, *Quit1* mRNA has a unique 5′ untranslated sequence which was presumably formed by conjunction of the 5′ side of the splicing site of the early *CAR1* mRNA and the 3′ side of the late *CAR1* mRNA. Since the *Quit1* mRNA probed with the total length of *Quit1* cDNA, it remains to be elucidated whether or not the mRNA and/or the late *CAR1* mRNA are expressed later in development arount the tight aggregate stage.

A



B

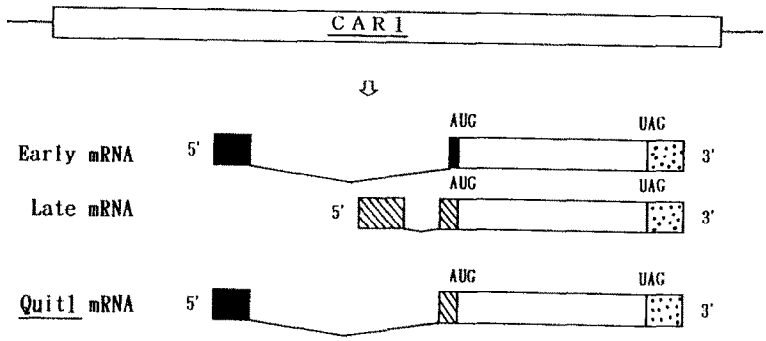


Fig. 2. (A) DNA sequence organization in the 5′ untranslated region of *Quit1*. Arrows indicate the 5′ and 3′ splicing junctions for the early and late *CAR1* mRNAs reported by Louis et al. [12]. The translation initiation site of *Quit1* is shown as ATG in bold letters. (B) Difference in the 5′-untranslated regions between the *Quit1* and *CAR1* genes. The *Quit1* mRNA has a unique 5′ untranslated sequence that is presumably framed by conjunction of the 5′ side of the splicing site of the early *CAR1* mRNA and the 3′ side of the late *CAR1* mRNA.

Although the reason why the 5′ untranslated region differs in organization between *Quit1* mRNA and early *CAR1* mRNA is presently unknown, it might be, at least partly, due to differences in the strains used.

CAR1, a G-protein-linked surface cAMP receptor, exerts a central role in *Dictyostelium* development, including cell aggregation; its disruption by homologous recombination and antisence RNA results in the failure of transformed Ax-3 cells to develop [13,14]. As exemplified by *CAR1* (*Quit1*) expression in T7 + 2 cells (starved just before the PS point), the expression is believed to be one of the earliest events induced by starvation. Therefore, it is possible that CAR1 might be one of the candidates involved in the growth/differentiation transition. The data presented here also suggest the real existence of the PS point in the *Dictyostelium* cell cycle.

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