Surface cAMP Receptors Mediate Multiple Responses during Development in *Dictyostelium*: Evidenced by Antisense Mutagenesis

Tze Li J. Sun, * Peter J. M. Van Haastert, † and Peter N. Devreotes*

*Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; and † Division of Biochemistry, University of Groningen, Groningen, The Netherlands

**Abstract.** Cell surface cAMP receptors (cARs) have been implicated in multiple aspects of development in *Dictyostelium*. Antisense mutagenesis has recently provided strong evidence that cARs are necessary for aggregation (Klein et al., 1988. Science (Wash. DC). 241:1467-1472). We show here that the expression of cAR1 antisense mRNA which prevents the appearance of cAR1 antigen also prevents the expression of cAMP-binding activity and blocks multiple cAMP-mediated responses. Chemotactic sensitivity to cAMP was lost as were stimulus-induced cAMP and cGMP production. Furthermore, the expression of developmentally regulated marker genes, dependent on repeated cAMP stimulation, was altered. As a result, the developmental program was severely impaired; most of the cells failed to aggregate and undergo further differentiation.

Materials and Methods

**Antisense Constructs and Transformation**

In addition to the previously described construct, (Klein et al., 1988) which uses the actin 6 (A6) promoter, two other constructs were also designed. cDNA of cAR1 was inserted into the Bgl II site of vector BS18 (a gift from Richard Firtel, Department of Biology, University of California at San Diego) which uses an actin 15 promoter to generate complementary RNA in the transformed cell. For the third construct, a 1.9-kb fragment derived from the 5' region of a cAR1 genomic clone was inserted in the Xba I site of p6B (cAR1 cDNA in Bluescript, see Klein et al., 1988) and cotransformed with BS18. Since cells transformed with this genomic clone did not over-express cAR1 with a developmental pattern similar to wild type (our unpublished observation), it is likely the upstream fragment contains the cAR1 promoter. Cells (strain AX-3) were transformed with these vectors according to Knecht and Loomis, 1987. During prolonged passage of some antisense cell lines, efficiency of antisense decreased as evidenced by a shortening in the duration of the aggregation block. This effect appears to be due to the loss of copies of the integrated vector. Although amebae can usually...
be stored in liquid nitrogen, this process somehow interferes with the retention of the phenotype of antisensed cells. Therefore, new transformations were performed every 3 mo.

In analyses of genomic DNA, using the full-length cAR1 cDNA as a probe, both the control and the antisense transformants had a 3.5-kb Xba I fragment and a 12.9-kb Eco RV fragment (data not shown). Because these fragments are identical to those of the parental cell line, AX-3, we concluded that the antisense vector did not integrate into the cAR1 gene by homologous recombination. In the antisense transformants a second darker band was observed in both restriction digests. Based on the size of this band (7.8 kb) and the restriction enzyme used (single site in the vector), we concluded that the majority of the vector integrated in tandem repeats of up to 50 copies.

**Development of Cells**

Cells were allowed to develop in shaken suspension (100 rpm) in DB (5 mM Na2HPO4, 5 mM KH2PO4, 2 mM MgSO4, 0.2 mM CaCl2) at 2 × 10⁷ cells/ml, or on the surface of starvation plates (DB, 1% agar) at 6.4 × 10⁵ cell/cm². Cells developed on starvation plates were photographed as described (Gross et al., 1976).

**RNA, DNA Preparation, and Analysis**

Total RNA of developed cells was prepared as described (Klein et al., 1988). Approximately 10 µg of RNA per lane were electrophoresed in 1.2% formaldehyde agarose gels and blotted to nitrocellulose (Maniatis et al., 1982). Total DNA was prepared by a modification of the DNA minipreps (Nellen et al., 1987), then digested with restriction enzyme overnight, electrophoresed in 0.7% agarose gel, and blotted to nitrocellulose. The nitrocellulose was baked in an 80°C vacuum oven for 2 h, and hybridized with specified probes. RNA probes were prepared as suggested by the manufacturer (Promega Biotec, Madison, WI). DNA probes were made by the random priming method (Feinberg and Vogelstein, 1983) from fragments isolated from agarose gels.

**Immunoblot**

Total protein of developing cells was prepared by lysis directly into sample buffer (10% glycerol, 5% DTT, 3% SDS, 6.25 mM Tris, pH 6.8, 0.25% Bromophenol blue) at a final density of 5 × 10⁶/ml. Extracts were subjected to SDS-PAGE and transferred to nitrocellulose. Polyclonal rabbit antisera against cAR1 (Klein et al., 1988) and 125I-protein A were used to detect the receptor protein.

**cAR1 Functional Assay**

cAMP binding assays were carried out as previously described (van Haastert and Kien, 1983). Both ammonium sulfate and phosphate buffer assays were used. cAMP-stimulated cAMP production was determined by an isotope dilution assay using 5 × 10⁻⁶ M 2'-deoxy-cAMP as stimulus in the presence of 5 mM DTT (Van Haastert, 1984b). The time course and amount of cGMP production was determined by a RIA (van Haastert and van der Heijden, 1983) after stimulation with 10⁻⁷ M cAMP.

**Chemotaxis**

Cells were developed for 3–7 h and then washed off the agar and used in the small population assay to determine chemotaxis responsiveness towards cAMP (Konijn, 1970).

**Results**

**Characterization of Antisense Cell Lines: Absence of [³H]cAMP Binding Activity**

As previously reported, control and antisense transformed cell lines displayed dramatically different phenotypes. The controls underwent normal development; characteristic waves

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**Figure 1.** Antisense cell lines fail to aggregate. Cell lines stably transformed with cAR1 antisense constructs or control vectors derived from vector A6, were grown in HL5 medium supplemented with 20 µg/ml of G418 and 30 µg/ml of dihydrostreptomycin. Amoebae were harvested and washed in DB and plated at 5 × 10⁷ cells per dish (100 mm) of DB-agar. Plates were incubated at 22°C. Control and antisense transformants are shown in row C and A, respectively. The hours of development are indicated. Each panel shows a 2 × 5 cm portion of the monolayer.
Absence of cAR1 antigen in antisense cell lines. Cells were transformed with either vector BS18 or the antisense construct derived from vector BS18. Individual clones were developed on the starvation plates and harvested at 0, 3, 6, 8, or 10 h. Extracts of $5 \times 10^6$ cells were immunoblotted with specific cAR1 antiserum as described under Materials and Methods.

and streams were apparent within a few hours after starvation. The multicellular stages of development such as mounds, fingers, slugs, Mexican hats, and fruiting bodies (at 24–26 h) appeared normal (Fig. 1). During the same time period the antisense transformed cell lines remained as uniform monolayer. By 23 h, a small fraction (<5%) of the cells entered into tiny aggregates which did not form streams, but eventually gave rise to fingers and slugs, and small fruiting bodies (Fig. 1). The remaining cells had not aggregated after two weeks.

As reported previously, normal expression of cAR1 antigen (mol wt, 40,000) was detected in immunoblots of the control transformed cells; it rose from undetectable levels in growing cells to a maximum during the aggregation phase of development. In contrast, there was no detectable cAR1 antigen in the antisense cell lines at any time (Fig. 2). We found that the $[^3H]$cAMP binding activity of the control transformants increased in parallel to the expression of cAR1 antigen reaching a maximum of $\sim 10^5$ sites/cell at 6–8 h of development; however, the $[^3H]$cAMP binding activity of antisense transformants remained close to the basal level throughout the developmental program (Fig. 3).

We also examined the mRNA in the antisense transformants. The control transformants showed a developmentally regulated expression of cAR1 mRNA which, similar to wild type, peaked at 3 h, declined until 9 h, and increased slightly at 12 h (Fig. 4 b, bottom). The mechanism by which the antisense vectors, A6 and BS18, inhibit production of cAR1 protein may differ. In antisense transformants made from the vector A6 construct, there was a negligible amount of endogenous cAR1 mRNA (Fig. 4 a, bottom), while in those made from the vector BS18 construct, there was a considerable amount of endogenous cAR1 mRNA present (Fig. 4 b, bottom), although we did not determine its integrity. In spite of these differences in RNA blots, both types of constructs led to a complete loss in cAR1 antigen and $[^3H]$cAMP binding activity as exemplified in Figs. 2 and 3.

**Antisense Mutagenesis Arrests the Developmental Program by Inhibiting Multiple Responses**

Binding of cAMP to surface receptors triggers activation of internal adenylate and guanylate cyclases leading to increased accumulation of cAMP and cGMP (Theibert and Devreotes, 1986; van Haastert and van der Heijden, 1983). When control transformants were stimulated, cAMP levels increased from undetectable levels to $\sim 100$ pmol/10^7 cells within 2 min (Fig. 5 a). cGMP levels rose from 5 pmol to a peak of 25 pmol/10^7 cells within 10 s, and returned to the basal levels by 50 s (Fig. 5 b). In contrast, similarly stimu-
labeled antisense transformants displayed only marginal increases in cAMP production to 2 pmol/10^7 cells (Fig. 5a, inset); cGMP levels did not increase (Fig. 5b).

The chemotactic responses of growing cells toward folic acid and of early developmental stage cells toward cAMP have been well documented (26, 27). The role of cAR1 in chemotaxis was assessed in the antisense transformants. Cells starved on agar for 7 h were assayed by the "small population" method. The control transformants began to show positive responses with 10^{-8}-10^{-4} M cAMP; 100% of the populations responded to 10^{-7} M (Fig. 6). In contrast, the antisense transformants did not respond to concentrations of cAMP below 10^{-6} M, and only 10% of the populations responded at the highest doses (Fig. 6). However, when folic acid was tested as a chemoattractant for undifferentiated cells, both control and antisense transformants responded identically (data not shown). Thus, the chemotactic response to cAMP is specifically impaired in the antisense transformants.

The expression of several marker genes was compared in the control and antisense cell lines. The absence of cAMP receptors greatly reduced the cAMP-induced expression of the gene D2 (Fig. 7a), a serine esterase, normally induced during early development, and completely prevented the expression of the spore coat proteins sp96, sp70, and sp60 (Fosnaugh and Loomis, 1990). Surprisingly, the major Go2 mRNA during development are nearly coincidental, suggesting that the components of the signal transduction pathway are coordinately expressed. We pursued these mechanistic studies further.

Recent observations indicate that cAMP signals are transduced through cAR1 via the Go2 protein (Kumagai et al., 1989; Pupillo et al., 1989). The time of peak production of cAR1 and the major Go2 mRNA during development are nearly coincidental, suggesting that the components of the signal transduction pathway are coordinately expressed. We were surprised to find that induction of Go2 does not appear to require expression of cAR1 at the cell surface, since its RNA levels increased normally in the antisense transformants. However, there was no decline in the antisense trans-

Discussion

Our characterization of the antisense transformed cell lines demonstrates the essential role of cAMP receptors in the developmental program. The presence of complementary RNA in these cell lines inhibited the expression of cAR1 and resulted in a characteristic phenotype. The cells displayed severely retarded aggregation, negligible cAMP binding, little ligand-induced cAMP or cGMP accumulation, very weak chemotactic responses, and failure to express or properly regulate a number of developmental marker genes. During starvation, the control cells developed cAMP binding activity in parallel with the appearance of cAR1 protein (mol wt, 40,000) as detected by the cAR1 antisera in immunoblots. Under the same conditions, antisense cells displayed very low cAMP binding activity and no observable cAR1 protein. Proper developmental expression of cAR1 mRNA (2 kb) was observed in control transformants. In the A6 vector construct transformants, only weak hybridization to fragments smaller than 2 kb was detected. Although a considerable amount of apparent 2-kb mRNA persisted in the cell lines transformed with the BS18 vector construct, the absence of cAR1 protein showed that it was not translated. These observations suggest that antisense mutagenesis may block production of the protein either by destabilizing the mRNA or by preventing its translation. The analysis of genomic DNA showed identically sized cAR1 bands in both the antisense and the wild-type cells in several digests, suggesting that gene disruption did not occur in the antisense transformants, and that the phenotype is most likely due to the presence of antisense RNA. Since our major goal was to elucidate the role of cAR1 in development, we have not pursued these mechanistic studies further.
Figure 7. Expression of developmentally regulated marker genes. RNA blots were prepared as described in Fig. 4 b and probed with full-length cDNA (a) D2 (gift from Richard Firtel) or (b) Go2. Formants suggesting that cAR1 may control the repression of Go2, which occurs after aggregation.

When individual clones from a transformation were examined, the phenotypes of some appeared weaker or stronger than the mean. That is, in some clones there was a long delay before aggregation rather than a permanent block and a large percentage of the cells eventually differentiated. The phenotype of each clone was reproducible and those that were delayed least contained significant levels of cAR1 antigen. As noted above, even in the transformants with strong phenotypes a small fraction of the cells eventually aggregated. However, it was not possible to determine whether these few cells were expressing cAR1, and thus we cannot conclude whether the small aggregates require this receptor. This phenotype, in which cells within small territories aggregate without streams, may be of interest because it is similar to another cAR may also be prevented because it may be dependent on cAR1. We are attempting to construct null mutants of the individual cARs by homologous recombination to address these important issues.

We would like to thank Richard Firtel for providing the BS18 vector and cDNA probe, D2; Ron Johnson for the construction of BS18 antisense vector, and contribution of the 5' fragment cAR1; Fanja Kesbeke for the technical help; and Peggy Ford for the typing of this manuscript.

This study was supported by National Institutes of Health Grant GM-34933 to P. N. Devreotes and a grant to P. J. M. Van Haastert from the C. and C. Huygens Fund, which is subsidized by the Netherlands Organization for Scientific Research.

Received for publication 31 October 1989 and in revised form 25 January 1990.

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