Correction. In the article "Cloning and cDNA sequence of the regulatory subunit of cAMP-dependent protein kinase from *Dictyostelium discoideum*" by Rupert Mutzel, Marie-Lise Lacombe, Marie-Noëlle Simon, Jean de Gunzburg, and Michel Veron, which appeared in number 1, January 1987, of *Proc. Natl. Acad. Sci. USA* (84, 6–10), the authors wish that the following correction be noted. In Table 1, the units for the amount of cAMP bound should be pmol/mg of protein, not nmol/mg of protein.
ABSTRACT  cDNA clones encoding the regulatory subunit of the cAMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) from Dictyostelium discoideum were isolated by immunoscreening of a cDNA library constructed in the expression vector Agt11. High-affinity cAMP-binding activity was detected in extracts from bacteria lysogenized with these clones. Nucleotide sequence analysis of three overlapping clones allowed the determination of a 1195-base-pair cDNA sequence coding for the entire regulatory subunit and containing nontranslated 5' and 3' sequences. The open reading frame codes for a protein of 327 amino acids, with molecular weight 36,794. The regulatory subunit from Dictyostelium shares a high degree of homology with its mammalian counterparts, but is lacking the NH2-terminal domain required for the association of regulatory subunits into dimers in other eukaryotes. On the basis of the comparison of the regulatory subunits from Dictyostelium, yeast, and bovine tissues, a model for the evolution of these proteins is proposed.

Considerable information has accumulated on the biochemistry of cyclic nucleotide-dependent protein kinases, which are found in all eukaryotic cells (reviewed in refs. 1 and 2). Best known are the mammalian cAMP-dependent protein kinases, which are composed of regulatory (R) and catalytic (C) subunits. Two major types of R subunits have been found (R1 and R2). The holoenzyme is a tetramer (R2-C2) which, in the presence of cAMP, dissociates into active monomeric C subunits and R2 dimers. Each R subunit carries two high-affinity cAMP binding sites. The determination of the amino acid sequence of bovine R1 and R2 subunits (3, 4) has provided strong structural arguments for the organization of each subunit into distinct domains responsible for dimerization of R, interaction with C, and cAMP binding (5).

cAMP-dependent protein kinases have also been found in lower eukaryotes, including Neurospora and yeast (6–8). Of particular interest is the enzyme from Dictyostelium discoideum, since cAMP is known to play a crucial role in the expression of developmentally regulated genes in this primitive eukaryote (9, 10). In contrast to their mammalian counterparts, R subunits from Dictyostelium are isolated as monomers carrying only one high-affinity binding site for cAMP (11–13). Reconstitution experiments using purified R and C subunits have led to the proposal that the Dictyostelium holoenzyme is a dimer (RC) composed of only one R and one C subunit (13). This structure is (so far) unique, since the cAMP-dependent protein kinase from yeast, although carrying only one cAMP binding site per R subunit, was shown to be a tetramer (6).

In order to analyze its structure in more detail and to study its role in the regulation of differentiation, we have isolated cDNA clones for the R subunit from Dictyostelium.

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MATERIALS AND METHODS

Materials, Phages, and Bacterial Strains. Nitrocellulose filters were from Schleicher & Schuell. 5-Bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-Gal), isopropyl β-d-thiogalactopyranoside (IPTG), and restriction enzymes were from Boehringer Mannheim. The replicative form (RF) of phage M13 mp19 was purchased from Pharmacia. Sequencing reactions were performed using the M13 sequencing kit from Amersham. The "Cyclone" kit used for the M13 deletions was from International Biotechnologies (New Haven, CT). [32P]GTP (>400 Ci/mmol), and [α-32P]GTP (>3000 Ci/mmol) were from Amersham.

λ phage and M13 DNA were purified by mini methods as described (14–16). Agt11 phages were lysogenized into Escherichia coli Y1089 (17) as described by Huynh et al. (18).

Screening of the Agt11 Library. The Agt11 library used was constructed by M.-L. L., G. J. Podgorski, J. Franke, and R. H. Kessin (39). In brief, cDNA was synthesized by the method of Gubler and Hoffman (19) from poly(A)+ RNA isolated from D. discoideum Ax3 starved for 3 hr in the presence of 1 mM cAMP. The cDNA was ligated into the EcoRI site of Agt11, packaged in vitro, and used to infect E. coli Y1088 (17).

Rabbit antibodies against the purified R subunit from Dictyostelium (13) were preabsorbed with extracts from E. coli BTA282(λAp3) (20) in order to eliminate crossreaction with phage- and bacteria-encoded proteins. For this, the total immune serum was diluted 1:10 in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.3/0.15 M NaCl) and incubated 1 hr at 4°C with an equal volume of bacterial extract (50 mg of protein per ml). Cell debris was removed by centrifugation, and the procedure was repeated twice. The absorbed immune serum (diluted 1:250) detected as little as 0.5 ng of purified R subunit spotted together with 20 μg of E. coli protein onto a nitrocellulose filter, whereas no reaction occurred with E. coli protein alone.

The cDNA library was screened according to ref. 17, using strain Y1090 and 3 × 1010 plaque-forming units per 100-mm plate. A positive control consisting of 1 ng of R subunit was also spotted on each filter. After blotting as described (17), the filters were successively rinsed twice, incubated with 2% bovine serum albumin (1 hr at 37°C) and then with preabsorbed anti-R immune serum (1 hr at 37°C) followed by 16 hr at 4°C. Each filter was treated separately in a Petri dish containing 20 ml of the appropriate medium: after five 5-min washes with TBS containing 5% powdered skimmed milk

Abbreviations: R subunit, regulatory subunit; C subunit, catalytic subunit; IPTG, isopropyl β-d-thiogalactopyranoside; kb, kilobase(s).

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(Regilait, Lyon, France) (21), the filters were incubated with 1 μCi of 125I-labeled protein A at room temperature for 1 hr. They were then washed five times with 5% milk/TBS and three times with TBS alone, dried, and exposed for 16 hr to Kodak X-Omat S films for autoradiography.

**Determination of cAMP-Binding Activity in Crude Bacterial Extracts.** Crude extracts from Y1089 cells lysogenized with various λgt11 recombinants were prepared before and after lac induction by IPTG as described (18). cAMP-binding activity was measured in 20 mM potassium phosphate (pH 7.5) as in ref. 13, by incubating 10−25 μg of protein with 0.2 μM [3H]cAMP for 1 hr at 4°C. Binding data were corrected for background radioactivity (without extract) and for non-specific binding (0.1 mM nonradioactive cAMP present).

**Subcloning and Sequencing of cDNAs.** cDNA inserts from the λgt11 clones of interest were excised with EcoRI, ligated into M13 mp19 RF DNA previously cut with EcoRI, and transformed into E. coli strain JM103 according to Messing (16). For selection of complementary cDNA strands, single-stranded DNAs purified from individual recombinant M13 phages were hybridized two by two and analyzed by agarose gel electrophoresis (16). DNA sequence analysis was performed by the method of Sanger et al. (22) as modified by Biggin et al. (23), using either the full-length inserts or progressive deletions of these DNAs obtained according to Dale et al. (24). The nucleotide sequence of a short 3′ sequence was determined according to Maxam and Gilbert (25).

**RESULTS AND DISCUSSION**

**Isolation of cDNA Clones for the R Subunit.** Screening of 3.6 × 10^5 plaque-forming units from the λgt11 cDNA library with specific immune serum against the R subunit from *Dictyostelium* allowed the detection of 38 clones. In order to check that these clones indeed coded for the R subunit, high-affinity cAMP-binding activity was assayed in lysates of bacteria lysogenized with the clones containing the longest inserts. Binding assays were performed with 0.2 μM cAMP. Under these conditions cAMP binding to bacterial catabolite gene activator protein (if any) would not have been detected (26).

Table 1 shows extracts from bacteria lysogenized with clones 11.2, 1.1, 2.2, and 2.1, harboring inserts 1.08−1.15 kilobases (kb) long, contained high cAMP-binding activity after induction by IPTG. In the absence of induction, cAMP binding was very low, similar to the control values. These results are exactly those expected for the expression of a LacZ/R-subunit cDNA-encoded fusion protein. Surprisingly, clones 7.4, 2.3, and 10.1, with inserts >1.2 kb, exhibited significant cAMP-binding activity (at least 10 times background) even in the absence of the lac inducer. Instead of increasing binding activity, addition of IPTG slightly reduced it. Notwithstanding this unusual regulation, which will be discussed below, the cAMP-binding activity in these clones clearly indicated that the corresponding inserts also coded for the R subunit. Therefore, no further characterization of the clones shown in Table 1 (e.g., by hybrid-selected translation) was considered necessary.

**Nucleotide Sequence of the Complete cDNA of the R Subunit.** Nucleotide sequence analysis was performed using three clones (Fig. 1). Clone 2.1 was selected for its high inducible cAMP-binding activity, whereas clones 10.1 and 2.3 were chosen for the large size of their cDNA inserts (see Table 1). Fig. 2 shows the complete nucleotide sequence of the cDNA of the R subunit, which contains an open reading frame of 981 nucleotides. Both 5′ and 3′ non-translated sequences are extremely A+T-rich, as previously observed in *Dictyostelium* (27). The 5′ end contains two stop codons (TAA) in-frame with the first ATG at position 139. The nucleotide sequence surrounding this ATG agrees with the consensus sequence (ANATG) for initiation of translation often found in eukaryotes (28). Moreover, the ATG is preceded by an A, as has been found for most *Dictyostelium* genes (27). We thus conclude that translation of the R-subunit mRNA starts at this ATG codon.

The isolation by immunoscreening of a λgt11 cDNA library of clones containing a 5′ nontranslated sequence is unexpected. Indeed, this sequence contains nonsense codons, which will stop translation, in all three reading frames. To explain the synthesis of R subunit and the lack of IPTG induction of the cAMP-binding activity in clones 2.3 and 10.1, we hypothesize that the very A+T-rich 5′ sequence contains signals that can serve for transcription termination as well as reinitiation of both transcription and translation.

The coding region predicts a protein of 327 amino acids, with molecular weight 36,794. The codon usage is analogous to that observed with other *Dictyostelium* genes (29), with the exception of two codons (CTG and GCG) that had not been found previously.

**Comparison of the Primary Structure of Dictyostelium and Bovine R Subunits.** Since *Dictyostelium* diverged from the mainstream of eukaryotic descent at the earliest branch point yet characterized by molecular phylogeny (30), the compar...
ison of the amino acid sequence of the R subunit from *Dictyostelium* (R \textsubscript{D}) with that of its mammalian counterparts (R\textsubscript{I} and R\textsubscript{II}) gives information on the evolution of these proteins. Fig. 3 shows that an optimal alignment of these three sequences can be obtained by introducing a few gaps in only two regions of the sequence.

Most striking is the absence in R\textsubscript{D} of the 70 residues corresponding to the NH\textsubscript{2} terminus of R\textsubscript{I} or R\textsubscript{II}. It has been proposed (31) that the domain responsible for the association of bovine R subunit into dimers is located at the NH\textsubscript{2} extremity of R\textsubscript{I} and R\textsubscript{II}; this domain has been localized in R\textsubscript{II} to the 45 NH\textsubscript{2}-terminal residues (32, 33). The absence of the corresponding sequence in R\textsubscript{D} provides structural evidence for its monomeric nature and consequently for the organization of *Dictyostelium* cAMP-dependent protein kinase as an RC dimer.

Starting from the NH\textsubscript{2} terminus in R\textsubscript{D}, the first highly conserved sequence (residues 27–34) corresponds to the so-called "hinge region" previously shown (34) to be involved in the interaction of the C subunit both with R\textsubscript{I} and R\textsubscript{II} and with the heat-stable inhibitor protein. Homology in this region is in agreement with the observation that hybrid holoenzymes can be reconstituted from R\textsubscript{D} and bovine C subunit (13). Considering that the residues phosphorylated in R\textsubscript{I} and R\textsubscript{II} are, respectively, Ser-99 and Ser-95 (4, 5), it is likely that *in vitro* phosphorylation of R\textsubscript{D} (12) occurs at Ser-32. The maximal homology extends throughout the sequence from Phe-67 to the COOH terminus of R\textsubscript{D}. In R\textsubscript{I} and R\textsubscript{II}, the analogous sequence (starting at Phe-136) corresponds to the two cAMP-binding domains (4, 5, 35). When defined by their residue number in the R\textsubscript{D} sequence, these domains extend approximately from Phe-67 to Leu-190 (domain A).

![Fig. 2. Nucleotide sequence of the cDNA for the R subunit. Stop codons are underlined. Amino acid sequence corresponding to the open reading frame is shown above the nucleotide sequence.](image-url)
### Type of Regulatory Subunit

<table>
<thead>
<tr>
<th>Type</th>
<th>Amino Acid Number</th>
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<tbody>
<tr>
<td>R.D</td>
<td>0-0</td>
</tr>
<tr>
<td>R.I</td>
<td>1-38</td>
</tr>
<tr>
<td>R.II</td>
<td>1-37</td>
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### Fig. 3

Comparison of the primary structures of R subunits from Dictostelium and bovine heart. Amino acid sequences of R_I and R_D are from refs. 3 and 4. Identities of R_D with one or both of the mammalian proteins are boxed. Standard one-letter abbreviations are used.

and from Arg-191 to the COOH terminus (domain B). The presence of two cAMP-binding domains in R_D is unexpected, since binding experiments have shown only one high-affinity cAMP-binding site (11–13). This site is likely to correspond to the NH_2-proximal domain (domain A), since fast dissociation kinetics are observed both for R_P (11, 13) and for cAMP binding to domain A in bovine R subunit (36). On the basis of the sequence similarities between the two domains, we hypothesize that domain B in R_P could also bind cAMP, but with a much lower affinity than domain A. In fact, binding of the nucleotide with a K_d in the micromolar range could have failed to be detected under the conditions used for measurement of cAMP binding to purified R_D (12, 13).

On the basis of a comparison of the R subunits from Dictostelium, yeast, and mammals, it is now possible to propose a more detailed scheme for the evolution of these proteins (Fig. 4). The presence of two cAMP-binding domains in R_D indicates that duplication of a gene for a putative ancestral cAMP-binding protein occurred earlier than the acquisition of the dimerization domain D. Therefore, the presence of two cAMP-binding domains in the yeast R subunit is predicted by its similarity to bovine R_I (6), although for the yeast protein only one high-affinity cAMP binding site has been demonstrated and no amino acid sequence data are yet available. Since the affinity of cAMP for catabolite gene activator protein is in the micromolar

### Fig. 4

Hypothetical pathway for the evolution of the regulatory subunits of cAMP-dependent protein kinases. Proteins are designed as sequences of functionally and structurally homologous domains. Arrows show the acquisition of new functions. Evolution of R subunits is assumed to start from an ancestral low-affinity cyclic nucleotide-binding protein (X) and to proceed by a series of genetic events involving gene duplication, gene fusion, and modification by point mutation. Note that the presence of two cAMP-binding domains in R subunit of yeast is predicted by the model (see text) but not yet established on the basis of amino acid sequence data. A and B are cAMP-binding domains carrying low-affinity (circles) or high-affinity (squares) cAMP-binding sites; I is the domain of interaction between R and C subunits; D (boxed) is the domain responsible for dimerization of R subunits.
range (26), we hypothesize that the original duplicated cAMP-binding protein also had a "low" affinity for cAMP. Evolution leading to the primitive eukaryotic cAMP-dependent protein kinase would have involved both acquisition of an I domain required for interaction with the catalytic subunit and modification of domain A to a high-affinity cAMP-binding site with $K_D$ in the nanomolar range. Finally, a second high-affinity binding site in domain B would have appeared later to give mammalian cAMP-dependent protein kinases additional regulatory flexibility through cooperativity between the two cAMP-binding sites.

This model takes into account most of the biochemical properties of the cAMP-dependent protein kinases. However, it leaves open two important questions. What is the advantage conferred by the dimerization domain, and what is the role of cAMP-binding domain B in primitive eukaryotes? We have no answer at present, since the Dictyostelium kinase seems as sensitive to in vitro activation by cAMP as its more sophisticated homologues (37). The possibility that R subunits could serve another function in addition to the regulation of the catalytic activity has been considered (38). It is tempting to speculate that this (these) putative other function(s) for R subunits is contributed by cAMP-binding domain B and that its importance in the regulation of eukaryotic cell functions is responsible for the conservation of the corresponding sequence from slime molds to mammals.

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