Regulation of the Synthesis of Two Carbohydrate-binding Proteins in Dictyostelium discoideum*

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(Received for publication, December 12, 1977)

The relative rate of de novo synthesis of two membrane-associated carbohydrate-binding proteins (CBP) has been examined during Dictyostelium development. The results show that the relative rate of CBP synthesis is minimal during the vegetative stage and increases to represent approximately 3.5 to 5% of newly synthesized protein during the aggregation stage after which the relative rate decreases. Analysis of the relative rates of synthesis of CBP-26 and CBP-24 indicate that at the peak period of synthesis (approximately 5 to 9 h of development) CBP-26 is synthesized at a rate which is approximately eight times greater than CBP-24.

In addition, we have examined the relative amount of CBP-26 and CBP-24 mRNA during development as assayed by its ability to direct CBP synthesis in in vitro protein-synthesizing systems. We show that there is no detectable CBP mRNA in vegetative cells and that during the pre-aggregating stages, assayable CBP mRNA appears and accumulates with a maximal level at the period of peak in vivo CBP synthesis. These results suggest that the rate at CBP synthesis in vivo is controlled by the relative amount of functional mRNA.

The cellular slime mold, Dictyostelium discoideum, grows and multiplies as unicellular amoebae. Upon starvation, amoebae differentiate over a period of approximately 9 h into cells which are capable of forming stable intercellular contact, after which they form multicellular organisms. Rosen et al. (1973) showed that concomitant with the development of cohesiveness, cells synthesize a lectin which will agglutinate sheep erythrocytes. The lectin binds free and modified galactose residues and can be purified from crude extracts of Dictyostelium cells by affinity chromatography on Sepharose 4B (Rosen et al., 1973). Its ability to agglutinate sheep erythrocytes is inhibited by several derivatives of D-galactose, but not glucose or mannose. This lectin or carbohydrate-binding protein has been shown to be on the cell surface by lactoperoxidase-catalyzed iodination and immunoprecipitation, Siu et al. (1976) demonstrated that CBP appears on the cell surface during development. Moreover, using radioimmunoassays to quantitatively determine the amount of intracellular CBP, it was demonstrated that CBP accumulates from less than 0.01% of the soluble protein in vegetative cells to about 1% of soluble protein in aggregated cells (10 to 16 h), representing 1.2 × 10⁸ molecules/cell at maximum concentration (Siu et al., 1976). Since estimations of CBP in vegetative cells represent the limit of resolution by radioimmunoassays (1 ng of CBP/500 µg of cell protein or 5 × 10⁶ molecules/cell, Siu et al., 1976), we feel that CBP may be absent in growing cells. This implies that there may be qualitative as well as quantitative regulation of CBP synthesis during development.

The first indication that these lectins are regulated in development came from the correlation of agglutination activity with the development of cohesiveness in Dictyostelium cells (Rosen et al., 1973). Using surface labeling of intact cells with lactoperoxidase-catalyzed iodination and immunoprecipitation, Siu et al. (1976) demonstrated that CBP appears on the cell surface during development. Moreover, using radioimmunoassays to quantitatively determine the amount of intracellular CBP, it was demonstrated that CBP accumulates from less than 0.01% of the soluble protein in vegetative cells to about 1% of soluble protein in aggregated cells (10 to 16 h), representing 1.2 × 10⁸ molecules/cell at maximum concentration (Siu et al., 1976). Since estimations of CBP in vegetative cells represent the limit of resolution by radioimmunoassays (1 ng of CBP/500 µg of cell protein or 5 × 10⁶ molecules/cell, Siu et al., 1976), we feel that CBP may be absent in growing cells. This implies that there may be qualitative as well as quantitative regulation of CBP synthesis during development. Preliminary studies with inhibitors of protein and RNA synthesis suggest that CBP accumulation is achieved by de novo synthesis, and its expression is regulated, at least in part, at the transcriptional level (Siu et al., 1976).

To further understand the level at which CBP is regulated, we have examined the relative de novo rate of synthesis of CBP-26 and CBP-24 during Dictyostelium development and the developmental kinetics of accumulation of CBP mRNA. For simplicity, we have defined the developmental cycle of Dictyostelium in terms of cell cohesiveness; vegetative cells (0 h), noncohesive cells (1 to 4 h), cohesive cells (4 to 10 h), aggregated cells (after 10 h). The relative rate of CBP synthesis is surface of cohesive cells, the remainder is present in an intracellular "pool" (Siu et al., 1977). CBP is a tetramer in its native state with an apparent molecular weight of about 100,000. Upon electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate, the partially purified preparation gives rise to two Coomassie blue staining bands: a major band of molecular weight 26,000 (CBP-26) which accounts for 90% of the staining material, and a minor band of molecular weight about 24,000 (CBP-24) which accounts for 10% of the staining material (Simpson et al., 1974). Frazier et al. (1975) showed that these two components are distinct proteins as indicated by differences in their isoelectric points, electrophoretic mobilities, amino acid composition, tryptic peptide maps, subunit molecular weight, agglutination specificities for different types of erythrocytes, sensitivity of their agglutination activity to inhibition by different sugars, and the developmental time course of their appearance. Each CBP appears to be a homotetramer in its native state (Frazier et al., 1975). These carbohydrate binding proteins are also known as discoidin I and II (Simpson et al., 1974; Frazier et al., 1975).

To further understand the level at which CBP is regulated, we have examined the relative de novo rate of synthesis of CBP-26 and CBP-24 during Dictyostelium development and the developmental kinetics of accumulation of CBP mRNA. For simplicity, we have defined the developmental cycle of Dictyostelium in terms of cell cohesiveness; vegetative cells (0 h), noncohesive cells (1 to 4 h), cohesive cells (4 to 10 h), aggregated cells (after 10 h). The relative rate of CBP synthesis is
low in noncohesive cells; increases to a maximum in cohesive, but preaggregated cells, and is low again in aggregated cells. Quantitation of CBP mRNA in Dictyostelium cells at various stages of development suggests that the rate of CBP synthesis appears to be controlled by the intracellular concentration of translatable CBP mRNA. No functional CBP mRNA can be detected in vegetative cells as assayed by the cell-free protein-synthesizing system of the wheat embryo. In this analysis, we resolved the developmental kinetics of the two carbohydrate-binding proteins.

MATERIALS AND METHODS

Cell Strain and Growth Conditions—Wild type D. discoideum (NC4) was used in all experiments except where noted. Amoebae were grown on SM agar in association with Klebsiella aerogenes (Sussman, 1966). Cells were grown for approximately 36 h at 21°C until the bacterial lawn was partially cleared (a yield of about 1 × 10^9 cells/9-cm Petri dish).

Developing Cells—Development was initiated by harvesting cells with cold 0.2% NaCl. Bacteria were removed from the cells by repeated differential centrifugation. Cells were resuspended at about 2 × 10^7 per ml in ND 4/6 buffer (Sussman et al., 1973). Various times of experiments, about 5 to 8 × 10^6 cells were deposited and spread evenly on 1 liter of 1.9% agar on metal trays (40 × 28 cm). For in vivo labeling experiments, 1 × 10^6 cells were deposited on 47-mm Whatman No. 50 filters supported in absorbent pads saturated with PDF containing the following antibiotics: rifampicin (100 μg/ml), chloramphenicol (100 μg/ml), kanamycin (100 μg/ml), and tetracycline (10 μg/ml).

The plated cells were incubated at 22°C.

Isolation of Carbohydrate-Binding Protein—CBP was isolated by a modified procedure of Simpson et al. (1974) from axenically grown A3 strains of D. discoideum as previously described (Su et al., 1976).

Preparation of Antiserum and IgG—Antiserum to purified CBP was prepared in rabbits by immunization with complete Freund's adjuvant by injections directly into lymph nodes (Gondie et al., 1966). IgG fraction of the antiserum was prepared by precipitation with 35% (NH_4)_2SO_4. The precipitate was dissolved in 50 mM phosphate buffer, pH 4, and dialyzed against this buffer extensively at 4°C prior to chromatography on DEAE cellulose equilibrated with the same buffer. Elution of IgG was monitored by assessing peak fractions that were pooled and stored frozen at −20°C.

Characterization of Anti-CBP Antibody—The antiserum has been previously shown to be specific toward CBP by double diffusion analysis using pure CBP and crude extracts of Dictyostelium (Su et al., 1976). This is confirmed by the fact that CBP-24 and CBP-26 are only proteins detected in immunoprecipitates of crude extracts of Dictyostelium in presence of absence until the bacterial lawn was partially cleared (a yield of about 1 × 10^9 cells/9-cm Petri dish).

Quantitation of CBP mRNA in low in noncohesive cells; increases to a maximum in cohesive, synthesizing system of the wheat embryo. In this analysis, we resolved the developmental kinetics of the two carbohydrate-binding proteins.
cbp-24 bands were located by fluorography and then quantitated. In the third, crude cell extracts were analyzed on 10% polyacrylamide slab gels followed by fluorography. The CBP band was localized and relative incorporation into CBP was determined using densitometer tracings of the fluorographs.

Fig. 1 shows the gel profiles of anti-CBP immunoprecipitates analyzed on 10% sodium dodecyl sulfate-polyacrylamide tube gels. As can be seen, only a single labeled peak which comigrated with marker CBP can be detected. This system cannot resolve the two CBPs. In order to resolve CBP-26 and CBP-24, the immunoprecipitates were analyzed on 7 to 20% gradient slab gels, followed by fluorography (see Fig. 2). No other proteins, except CBP-26 and CBP-24 were detected. Since immunoprecipitated CBP is free of contaminating labeled proteins, we also quantitated radioactivity in CBP by solubilizing and counting immunoprecipitates directly in order to obtain sufficient counts to give accurate estimations of the relative rate of CBP synthesis. This analysis (data not shown) corroborated those obtained after polyacrylamide gel analyses, as do densitometer tracings of fluorographs of total cell extracts (see Fig. 3). Table I and Fig. 4 summarize these results and show the rate of CBP labeling relative to total protein synthesis estimated by the three different methods. As shown, CBP synthesis is low in noncohesive cells (1 to 3 h of development), less than 0.5% of newly synthesized proteins. Incorporation into CBP increases to 3.5 to 6% of total incorporation as the developing cells acquire cohesiveness. Maximum CBP labeling occurs between 7 and 10 h of development just prior to the formation of tight aggregates, after which CBP synthesis falls back to a low level of less than 1% of newly labeled proteins. After correcting for the fact that CBP is relatively deficient in leucine compared to total Dictyostelium proteins (7.5 mol%, Loomis, 1975), the rate of CBP labeling relative to total protein labeling at peak value (from now on this will be referred to as peak rate of synthesis) is estimated to be approximately 5 to 6% of total protein synthesis.

Fig. 2 shows the fluorography of immunoprecipitated CBP analyzed on 7 to 20% gradient sodium dodecyl sulfate-polyacrylamide slab gels. As can be seen, the two proteins CBP-24 and CBP-26, are well resolved. Further analysis of the fluorograph shown in Fig. 2 indicates that a differential incorporation of label into CBP-24 and CBP-26 occurs during development. These results, shown in Table I, are summarized in Fig. 5. As can be seen, the ratio of incorporation of label into CBP-24 and CBP-26 changes during development. Analysis of the ratio of CBP-24 to CBP-26 indicates that CBP-26 is the more abundant form of the lectin synthesized throughout the synthetic period in cohesive cells.

Quantitative Assay for CBP mRNA—Total RNA was extracted from developing cells and polyadenylated [poly(A)] mRNA was enriched and separated from ribosomal RNA by affinity chromatography on poly(U)-Sepharose. The poly(A)^- mRNA and the non-polyadenylated [poly(A)^-] fractions were used to direct protein synthesis in the wheat embryo extract system (see "Materials and Methods"). In vitro synthesized products were assayed for CBP by direct immunoprecipitation with cold carrier CBP. The immunoprecipitates were analyzed by fluorography after separation on gradient slab gels. No CBP synthesis could be detected using the poly(A)^- RNA fraction from 6-h developing cells to program the wheat germ system as analyzed by immunoprecipitation and fluorograph (data not shown), while poly(A)^+ RNA and total RNA from 6-h cells directed the synthesis of immunoprecipitable CBP. These results show that CBP mRNA is polyadenylated and can be assayed by its ability to direct CBP synthesis in an in vitro protein-synthesizing system.

In order to establish that biologically active CBP mRNA may be assayed quantitatively, the following experiments were done. Poly(A)^+ RNA was extracted from cells at a time when in vivo CBP synthesis is high (6 h) and low (12 h) and assayed for CBP mRNA as a function of input RNA concentration. Fig. 6A shows that increasing amounts of RNA added to the
protein-synthesizing system resulted in greater amounts of total in vitro synthesized proteins for both RNAs. Further, the amount of CBP immunoprecipitated from the in vitro products is linearly proportional to input RNA, up to optimal concentrations (Fig. 6B). Although the ability to direct total incorporation is essentially the same for the RNA from 6- and 12-h cells (Fig. 6A), the ability to direct CBP synthesis is lower in the 12-h RNA (Fig. 6B), which is consistent with the fact that in vivo CBP synthesis is comparatively lower at 12 h of development. In addition, pairwise mixtures of poly(A)+ RNA from 6-h cells (high rate of CBP synthesis in vivo), and from vegetative or 12-h cells (low rate of CBP synthesis) were assayed for CBP mRNA as described. The results of this experiment show that for all combinations of RNA, the amount of CBP synthesized was that expected from the summation of the amount synthesized by the RNAs assayed independently. From these results, we conclude that the RNA preparations from these particular stages did not contain a specific species of RNA or other types of factors which preferentially compete with CBP mRNA in the system. Since the synthetic capacity of CBP mRNA is linearly proportional to input RNA concentration, and there does not appear to be
In vivo rate of CBP synthesis during Dictyostelium development.

Rate of CBP synthesis was determined as follows. For Series A, cells were pulse-labeled with \(^{3}H\)leucine at times indicated and CBP was immunoprecipitated with anti-CBP IgG by direct immunoprecipitation in presence of carrier CBP (see "Materials and Methods" for details). CBP-24 and CBP-26 were resolved by analysis in a 7 to 20% gradient sodium dodecyl sulfate-polyacrylamide slab gel by \(N,N'\)-diallyltartardiamide and located by fluorography. The specific bands were excised from the gel, solubilized by 2% periodic acid, and counted in Aquasol with \(^{3}H\)toluene as external standard. Whenever possible, cross-contamination of counts between the two CBP's was checked by determining the distribution of radioactivity in the two bands by densitometer tracing of the fluorograph (values not shown). The densitometer tracings agreed with the relative values obtained by exciting the bands. Radioactivity used for immunoprecipitations refers to total trichloroacetic acid-insoluble counts in the sample. Values in parentheses indicate CBP incorporation relative to total incorporation. For Series B, the immunoprecipitates were applied directly onto 10% polyacrylamide gels cross-linked with \(N,N'\)-diallyltartardiamide. Following electrophoresis, the gels were stained to localize CBP and IgG markers, sliced, and counted as described above. Shown are the total counts prior to immunoprecipitation and the amount isolated from the CBP peak on the gel. Background obtained by precipitation with nonimmune serum was subtracted. External standard was not used to determine counts per min.

<table>
<thead>
<tr>
<th>Time of pulse label (in development)</th>
<th>Radioactivity used for immunoprecipitation</th>
<th>Radioactivity in CBP-26</th>
<th>Radioactivity in CBP-24</th>
<th>Ratio of CBP-26:CBP-24</th>
<th>Newly synthesized protein</th>
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<td>1-3</td>
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<td>805 ((0.06%))</td>
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<tr>
<td>2-4</td>
<td>(1.25 \times 10^9)</td>
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<td>1221 ((0.10%))</td>
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<td>5.7 ((0.72)%)</td>
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<td>10.1</td>
<td>6.3 ((0.63)%)</td>
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<td>(8.52 \times 10^9)</td>
<td>1.24 ((1.46%))</td>
<td>2953 ((0.35%))</td>
<td>4.1</td>
<td>2.0 ((0.48)%)</td>
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<td>12-14</td>
<td>(7.06 \times 10^9)</td>
<td>2921 ((0.41%))</td>
<td>1290 ((0.18%))</td>
<td>2.1</td>
<td>0.56 ((0.24)%)</td>
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</table>

<table>
<thead>
<tr>
<th>Time of pulse label (in development)</th>
<th>Radioactivity used for immunoprecipitation</th>
<th>Radioactivity in CBP</th>
<th>Label in CBP</th>
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<tr>
<td></td>
<td>dpm</td>
<td></td>
<td>%</td>
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<tr>
<td>1-3</td>
<td>(54 \times 10^4)</td>
<td>270</td>
<td>0.5</td>
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<tr>
<td>4-6</td>
<td>(67 \times 10^4)</td>
<td>1210</td>
<td>1.8</td>
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<tr>
<td>4-9</td>
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<td>2400</td>
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<tr>
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<td>1.4</td>
</tr>
<tr>
<td>13-15</td>
<td>(140 \times 10^4)</td>
<td>300</td>
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<tr>
<td>5.5-0.5</td>
<td>(54.5 \times 10^4)</td>
<td>24,000</td>
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</table>

**Fig. 4.** Relative rate of CBP synthesis during development. The relative rate of CBP synthesis during development was determined as described in Figs. 1, 2, and 3 and as calculated in Table I. The relative rate of CBP is plotted versus the labeling time during development. Values have been corrected for leucine content in CBP versus total cellular protein as described in text. ■ values obtained from densitometer tracings of fluorographs of slab gel electrophoresis of total cell proteins (see Fig. 3); □ values obtained from immunoprecipitated CBP analyzed on 10% sodium dodecyl sulfate-polyacrylamide tube gels (Fig. 1 and Table I, Series B); Δ, values obtained from immunoprecipitated CBP analyzed on 7 to 20% gradient sodium dodecyl sulfate-polyacrylamide slab gels (Fig. 2 and Table I, Series A).
CBP Synthesis in Dictyostelium

CBP mRNA corresponded in size to the in vivo isolated CBP carrier. Table II and Fig. 8 show the quantitation of mRNA coding for CBP-24 and CBP-26. Results show that CBP mRNA is negligible or not detectable in vegetative cells (also see "Results") and is present at low levels at 3 h of development, which is consistent with earlier results on the de novo synthesis of CBP. CBP mRNA accumulates rapidly between 3 and 9 h of development and reaches a maximum value at 9 h of development. At this time, it represents a 5-fold increase over the amount of CBP mRNA present in the 3-h developing cells. This maximum level of CBP mRNA (at 9 h) also occurs at a time when relative rate of CBP labeling in vivo is greatest (8 to 10 h, see Table I). The amount of CBP mRNA then falls rapidly to a low level again at 12 to 15 h of development, a time at which in vivo CBP synthesis decreases. As is evident from comparing the results in Fig. 5 and Fig. 8, the pattern of mRNA accumulation for CBP-26 and CBP-24 correlates well with the patterns of their in vivo relative rate of synthesis.

**Figure 6.** Assay of CBP mRNA in the wheat germ in vitro protein-synthesizing system. A, incorporation of [3H]leucine into total proteins synthesized by the wheat embryo system as a function of input RNA concentration. Poly(A') RNA was extracted from developing 6-h cells (○), 12-h cells (●), and used to direct protein synthesis in the wheat embryo system (see "Materials and Methods"). Incorporation of [3H]leucine into total proteins was determined by quantitating radioactivity in total trichloroacetic acid (TCA)-insoluble material. B, incorporation of [3H]leucine into CBP synthesized by the wheat embryo system as a function of input RNA concentration. Poly(A') RNA was extracted from developing 6-h cells (○), 12-h cells (●), and used to direct protein synthesis in the wheat embryo system. CBP was precipitated by direct immunoprecipitation with cold carrier and analyzed on a 7 to 20% gradient slab gels as described under "Materials and Methods." Incorporation of [3H]leucine into CBP was determined by quantitating radioactivity under the CBP peaks.

**Figure 7.** Fluorograph of immunoprecipitated in vitro synthesized CBP. Poly(A') RNA was isolated from Dictyostelium cells at various stages of development (as indicated) and used to direct protein synthesis in the wheat embryo system. Three concentrations of poly(A') RNA from the linear portion of the concentration curve below the saturation level (8, 16, and 32 µg/ml) were used. The 100- and 200-liter assays with all three RNA concentrations were pooled and used to immunoprecipitate CBP as described under "Materials and Methods." The immunoprecipitates were analyzed on 7 to 20% gradient slab gels followed by fluorography. S, Coomassie blue-stained CBP extracted from axenically grown Dictyostelium cells by affinity chromatography on Sepharose 4B.

**Table II**

<table>
<thead>
<tr>
<th>Developmental time of mRNA used</th>
<th>Total cpm used in immunoprecipitation</th>
<th>Total cpm in immunoprecipitation (trichloroacetic acid)</th>
<th>CBP mRNA</th>
<th>cpm in 20,000</th>
<th>cpm in 24,000</th>
<th>Ratio of CBP-26/CBP-24</th>
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<tbody>
<tr>
<td>0</td>
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<td></td>
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<tr>
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</tr>
<tr>
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<td>4.55 × 10^5</td>
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<td>6,802</td>
<td>1,473</td>
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<td>5,821</td>
<td>1,182</td>
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**NB:** In order to determine whether the regulation of in vivo CBP synthesis occurs at the transcriptional or translational level, CBP mRNA was quantitatively assayed at various stages of development. Poly(A') RNA was isolated from vegetative (0 h) and 3-, 6-, 9-, 12-, and 15-h developing cells as described under "Materials and Methods." These RNAs were used to direct in vitro protein synthesis in the wheat embryo extract system at three RNA concentrations below the optimum. The linear portions of the total in vitro protein synthesis curve were pooled for the assay of synthesized CBP by direct immunoprecipitation. Fig. 7 shows a fluorograph of the immunoprecipitated in vitro synthesized CBP. As can be seen in Fig. 7, the in vitro synthesized CBP

**Figure 7.** Fluorograph of immunoprecipitated in vitro synthesized CBP. Poly(A') RNA was isolated from Dictyostelium cells at various stages of development (as indicated) and used to direct protein synthesis in the wheat embryo system. Three concentrations of poly(A') RNA from the linear portion of the concentration curve below the saturation level (8, 16, and 32 µg/ml) were used. The 100- and 200-liter assays with all three RNA concentrations were pooled and used to immunoprecipitate CBP as described under "Materials and Methods." The immunoprecipitates were analyzed on 7 to 20% gradient slab gels followed by fluorography. S, Coomassie blue-stained CBP extracted from axenically grown Dictyostelium cells by affinity chromatography on Sepharose 4B.

**Table II**

**Assay of CBP mRNA level**

CBP mRNA was assayed as described under "Materials and Methods" using a wheat germ in vitro protein-synthesizing label. CBP was immunoprecipitated using added carrier CBP and anti-CBP y-globulin. The immunoprecipitates were then analyzed and quantitated on polyacrylamide gradient slab gels as described in the legend to Table I. Densitometry tracing of the fluorograph (see Fig. 7) was used to confirm the ratios shown in the last column of the table. Total cpm used refers to total trichloroacetic acid-insoluble alkali-resistant counts.

<table>
<thead>
<tr>
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<th>cpm in 24,000</th>
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have determined the relative rate of de novo synthesis and measured the level of functional mRNA for two lectins, CBP-26 and CBP-24, during Dictyostelium development.

Attempts to measure the specific activity of trichloroacetic acid-soluble amino acid pool by amino acid analysis gave preliminary evidence that the [3H]leucine label is converted into other amino acid and unidentified products which is consistent with the suggestion that the major source of energy during development is derived from amino acid catabolism (Loomis, 1975). For these reasons, it was not technically possible to measure the absolute rate of synthesis of CBP during development. Therefore, the rate of synthesis of CBP was estimated by quantitating specific incorporation of [3H]leucine into immunoprecipitated CBP as a function of incorporation into total soluble protein at various times of development. Analysis of our methods of immunoprecipitation indicates that the methodology was quantitative as well as very specific. In control experiments, we showed that the CBP antibody is specific for CBP-26 and CBP-24 and that the washed immunoprecipitates are free of contaminating proteins. To ensure that the quantitation was accurate, immunoprecipitates were analyzed on either 10% cylindrical or 20% gradient sodium dodecyl sulfate-polyacrylamide gels, and relative incorporation into CBP was determined by measuring radioactivity under the CBP peaks. In addition, the supernatant from the primary immunoprecipitation was analyzed for residual, unprecipitated CBP as described under "Materials and Methods."

Frazier et al. (1975), on the basis of physical and physiological evidence (see introduction), concluded that CBP-26 and CBP-24 are distinct lectins and provides preliminary evidence that the developmental time course of the two lectins is different. We now provide additional evidence supporting this notion by quantitating the in vivo kinetics of incorporation of label into each of the lectins and the accumulation of functional mRNA for the two CBPs. We have demonstrated that the pattern of developmental regulation of CBP-26 and CBP-24 can be resolved from one another and that they are probably derived from unique genes. Moreover, that in vitro synthesis of CBP mRNA yields both the 26,000- and 24,000-dalton proteins supports that the 24,000 protein is not derived from the 26,000 protein and is translated from a separate mRNA.

Analysis of the relative rate of in vivo synthesis of CBP gave the following pattern. CBP synthesis is at a very low level in noncohesive cells, and increases rapidly between 4 and 10 h as cells acquire cohesiveness. Maximum CBP synthesis occurs between 7 and 10 h of development just prior to the formation of tight aggregates. At maximum synthetic period (5% to 9% h), synthesis of CBP is quite high, 3.5 to 6% of total newly synthesized protein. After the cells have aggregated, CBP synthesis falls back to a low level again. The pattern of CBP synthesis is consistent with the accumulation pattern of CBP established by radioimmunoassays. It should be pointed out that some variability is found from experiment to experiment with respect to the maximum rate of CBP synthesis although the qualitative aspects of the developmental kinetics is consistent. Most likely this variability is due either to asynchrony in the development of cells and/or to the presence of starved cells in the vegetative (0 h) cells due to early clearing of some areas of the bacterial agar plates.

We have determined the quantitative changes of CBP mRNA during development using an in vitro protein-synthesizing system. Control experiments established that stimulation of the synthetic capacity in the wheat embryo system is dependent upon exogenous RNA and there appears to be no preferential translation of competing RNA species at different

**Figure 8.** Radioactivity in CBP-26 and CBP-24 was quantitated by excising the appropriate gel regions, eluting the proteins, and counting in Aquasol (see "Materials and Methods"). Percentage of CBP mRNA was determined by the incorporation of [3H]leucine into CBP as a function of incorporation into total in vitro synthesized proteins. Concentration of CBP mRNA at different times of development was expressed as percentage of maximum concentration which occurs at 9 h of development. Data is shown in Table II. ▲ mRNA for CBP-26; □ mRNA for CBP-24.

These data suggest that the in vivo rate of synthesis for the two CBPs is controlled by the intracellular concentration of functional mRNA.

As is shown in Fig. 7, no CBP was detected by fluorography after prolonged exposure when total poly(A)+ RNA from vegetative cells were assayed for CBP mRNA. Similar experiments have been performed with polysomal poly(A)+ mRNA and total cell RNA from vegetative cells. In addition to the wheat embryo extract system, the messenger-dependent rabbit reticulocyte lysate system (Pelham and Jackson, 1976; a gift from T. Hunter) was also used for in vitro protein synthesis and CBP synthesis could not be detected by fluorography after long exposure or by direct counting of the gel area excising the appropriate gel regions, eluting the proteins, and counting radioactivity under the CBP peaks. In addition, the supernatant from the primary immunoprecipitation was analyzed for residual, unprecipitated CBP as described under "Materials and Methods."

**Disscussion**

A large number of proteins are differentially synthesized during the developmental cycle of Dictyostelium (see Loomis, 1975; Sussman and Brackenbury, 1976; Jacobson and Lodish, 1975; Firtel and Jacobson, 1977; for reviews). Of these, three (UDP-galactose pyrophosphorylase, glycogen phosphorylase, and actin) have been studied at the level of protein or enzyme activity accumulation and relative rate of de novo protein synthesis (Francke and Sussman, 1971 and 1973; Firtel and Bonner, 1972; Killick and Wright, 1974; Thomas and Wright, 1976). For one (actin), the relative level of mRNA has also been examined (Alton and Lodish, 1977). In this paper, we

3 K. L. Kindle and R. A. Firtel, manuscript in preparation.
times of development by this system. The quantitation of CBP mRNA were carried out under conditions where the protein synthetic capacity of mRNA is linearly proportional to total input RNA. Care was also taken that CBP was quantitatively immunoprecipitated from the in vitro synthesized proteins as determined by checking for CBP in "depleted" extracts and was free of contaminating proteins by analysis on 7 to 20% gradient slab gels. These experiments established a pattern of accumulation for CBP mRNA as seen in Fig. 8. We note that the relative level of CBP mRNA (the ratio of CBP synthesized in vitro to total [3H]leucine incorporation) as assayed in our system is much lower than that expected from the relative rate of CBP synthesis in vivo. The relatively low level of detectable CBP synthesized could be due to a combination of three factors: 1) the inability to precipitate a fraction of the CBP synthesized in vitro with our antibody; 2) that a large fraction of the total incorporation could represent incomplete products resulting from early termination; 3) that CBP mRNA may be poorly translated relative to other mRNAs in the wheat germ system. From our data, however, we conclude that the relative levels of CBP mRNA during development is reproducible from experiment to experiment and from different mRNA preparations. We thus conclude that we can accurately assay the relative changes, but not absolute levels, of CBP mRNA from different developmental stages.

Our results show that functional CBP mRNA is not detectable in either total or polysomal poly(A)+ RNA extracted from vegetative (0 h) cells. It is present at low levels in noncohesive cells. CBP mRNA accumulates rapidly between 3 and 9 h of development and reaches a maximum value at 9 h of development. At this time, biologically active CBP mRNA appears as a 5-fold increase over the amount present in the noncohesive cells.

The developmental pattern of CBP mRNA, the relative in vitro rate of CBP synthesis, and the accumulation of CBP protein established by radioimmunoassays (see Fig. 9, taken from Siu et al., 1976) are consistent with one another. When intracellular CBP is low (in noncohesive cells), both synthesis of CBP and concentration of functional CBP mRNA are low. Maximum rate of CBP synthesis and maximum CBP mRNA occurs at a time when CBP is accumulating rapidly and linearly in cells acquiring cohesiveness. The amount of CBP mRNA and the relative rate of CBP synthesis rapidly falls to a low level again after the cells have aggregated. At this time in development, the cellular CBP level is at a maximum and it remains high from 10 to 16 h (1% of total soluble protein, Siu et al., 1976), although both the relative rate of CBP synthesis and functional CBP mRNA are low. This suggests that CBP may be a relatively stable protein with a half-life of longer than 6 h.

The pattern of change in CBP mRNA accumulation and relative rate of CBP synthesis corresponds closely with one another during development. Further, as CBP is expressed during development, there is a 5- to 10-fold increase in both the relative rate of CBP synthesis and the intracellular concentration of CBP mRNA. The most simple and direct interpretation of these results is that in vivo CBP synthesis is regulated mainly by the intracellular concentration of functional CBP mRNA. Therefore regulation of CBP expression appears to operate at the level of mRNA. Additional preliminary experiments using actinomycin D and daunomycin (as in Firtel et al., 1975) to inhibit mRNA synthesis at various times of development showed that incorporation of [3H]leucine into CBP is inhibited if the cells are placed in the drugs between 0 and 1 h of development.2 These results together with the fact that no functional CBP mRNA from vegetative cells can be assayed by the wheat embryo system are all consistent with the idea that the synthesis of CBP mRNA initiates and increases rapidly between 1 and 2 h of development and that gene activation probably occurs between 1 and 2 h of development. Since in vitro protein synthesis systems recognize "biologically active" mRNA, one cannot exclude possibilities that some inactive form or unprocessed form of CBP mRNA is present in vegetative cells and is activated during Dictyostelium differentiation. Although, there is no precedence for such a mechanism of regulation in eukaryotic development, these kinds of questions cannot be resolved until a "probe" for the CBP gene is obtained. We are presently trying to isolate recombinant plasmids that contain the CBP genes.

Acknowledgments—We would like to thank Drs. W. Rowekamp, C. H. Siu, R. A. Lerner, S. Rosen, and S. Barondes for helpful suggestions. We are indebted to Dr. A. Hunter for assistance in setting up the in vitro protein-synthesizing systems and for supplying some of the extracts. We also would like to thank Drs. G. Howell, E. Deuer, J. Siegel, and Ms. C. Macleod for discussions on the manuscript.

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* Unpublished data.

Fig. 9. Quantitation of total CBP with radioimmunoassay taken from Siu et al. (1976). NC4 cells were harvested from bacterial growth plates for development. Cells were collected at 2-h intervals and the soluble protein fractions were used for radioimmunoassays as described in Siu et al. (1976). The noncohesive mutant WL3, which was shown to lack CBP both on the surface and in the cytoplasm, was used as the control (△). The results of two experiments using NC4 were plotted together (●) and each experimental point represents the average concentration of CBP in the soluble protein fraction calculated from the values obtained with different dilutions of the protein sample. Cells at 0 and 2 h had no inhibitory effect on the antibody even when 250 µg of soluble protein/sample were used.

Regulation of the synthesis of two carbohydrate-binding proteins in Dictyostelium discoideum.
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