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Repetitive DNA sequences cotranscribed with developmentally regulated *Dictyostelium discoideum* mRNAs

(coordinated gene expression/cloned gene/DNA·RNA hybridization)

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ABSTRACT **We have isolated a clone of *Dictyostelium discoideum* genomic DNA, pB41-6, that contains sequences that are reiterated and interspersed in the genome. The expression of these reiterated sequences is developmentally regulated, and they appear to be linked only to developmentally regulated mRNAs. We have used clone pB41-6 to isolate a set of complementary cDNA clones. Analysis of two selected clones indicates that they encode developmentally regulated mRNA species and, by means of the repetitive elements they contain, hybridize to many developmentally regulated mRNAs. In addition, we have defined two families of mRNA species carrying these repetitive sequences: one is induced at 5.5 hr of development and the other is induced between 5.5 and 15 hr of development. Since most of the mRNA species that have sequences complementary to clone pB41-6 accumulate in a coordinated fashion during development, these sequences may play an important regulatory role in the developmental program of *Dictyostelium*.**

On removal of the food source, *Dictyostelium discoideum* cells initiate a complex developmental program. Chemotactic movement results in the formation of mounds of $\approx 10^5$ cells. These subsequently differentiate into the spore and stalk cells found in the mature fruiting bodies (1). Despite the complex morphogenetic and biochemical changes that occur during this developmental cycle, there is one time point—at the late aggregation stage—at which major changes in gene activity take place. Of the ≈80 predominant proteins resolved by two-dimensional gel electrophoresis whose synthesis is developmentally controlled, induction of synthesis of 40 occurs at the stage of late aggregation (2, 3). Analysis of the complexity and diversity of the mRNA populations throughout development has shown that polyribosomes of late aggregating cells contain 2500 mRNA species that are absent from growing cells (4, 5). Appearance of these mRNAs is regulated at the transcriptional level (6, 7). The coordinated control (and expression) of genes that are expressed at the same time during development seems logically to require the existence of common elements for their regulation.

The DNA sequence organization of many eukaryotic organisms displays an interspersed arrangement of repetitive and single-copy sequences. It has been proposed that gene expression may be regulated by networks of repetitive sequences (8). Approximately 50% of the *Dictyostelium* genome is organized as short (250–450 base pairs) repetitive sequences interspersed with single-copy DNA in a way similar to that of most higher eukaryotes (9). Recently, poly(A)$^+$RNA transcripts consisting of single-copy sequences covalently linked to short repetitive sequences have been observed in several different organisms including *Dictyostelium* (10–12). However, the biological role of these sequences remains to be elucidated.

We report here the characterization of a *Dictyostelium* genomic clone, pB41-6, that carries sequences that are repeated and interspersed in the *Dictyostelium* genome. Furthermore, expression of these sequences is developmentally regulated and they appear to be linked only to developmentally regulated mRNAs. The fact that most mRNA species complementary to pB41-6 are expressed only during differentiation suggests an important regulatory role for these sequences.

MATERIALS AND METHODS

Growth and Differentiation of *D. discoideum*. Cells of *Dictyostelium* strain AX3 (13) were grown axenically as described by Alton and Lodish (14), and development was initiated as described by Blumberg and Lodish (4).

Isolation of *Dictyostelium* Nuclear DNA and Cytoplasmic Polyadenylylated RNA. *Dictyostelium* nuclear DNA was isolated from vegetative growing cells as described by Blumberg and Lodish (4). Cytoplasmic RNA was extracted from vegetative growing cells and from developing cells as described (4) except that, after the first phenol/chlorophorm extraction, the aqueous phase was extracted once with an equal volume of chloroform. Poly(A)$^+$RNA was isolated by affinity chromatography on columns of oligo(dT)-cellulose (4).

Isolation of pB41-6. A random collection of genomic *Dictyostelium* λ recombinants (*Eco*RI-digested *Dictyostelium* nuclear DNA) was hybridized according to the procedure of Benton and Davis (15) with *in vitro* end-labeled cytoplasmic polyadenylylated RNAs (16) from 15-hr developing cells. Those clones that gave a positive signal were purified, and their DNAs were isolated and hybridized with ^{32}P-labeled poly(A)$^+$RNA isolated from cells labeled with ^{32}Pi for 1 hr beginning at 5, 15, and 22 hr of development (17). In addition, a replica set of filters was hybridized with *in vitro* end-labeled cytoplasmic polyadenylylated RNA from vegetative growing cells and from 5-, 15-, and 22-hr developing cells. One clone, SB41, hybridized very strongly to all labeled mRNAs from developing cells but not at all to labeled mRNA from growing cells. Clone pB41-6 is a 2.7-kilobase subclone in pBR322 of clone SB41 that contains all of the regulated sequences.

Restriction Enzyme Digestions and DNA Blotting. Digestions were carried out in the buffers suggested by New England BioLabs. Blotting of DNA fragments into nitrocellulose paper was carried out according to the method of Southern (16). DNA fragments were transferred to diazobenzyloxymethyl (DBM)-paper as described by Alwine *et al.* (18) with the exception that we used 0.2 M NaOAc, pH 4.3, as the transfer buffer and the

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Abbreviations: DBM, diazobenzyloxymethyl; NaCl/Cit, standard saline citrate.

nitrobenzyloxymethyl-paper was activated to the DBM form and stored at −70°C many days before its use.

Hybridization Reactions. The hybridization buffer was 50% formamide containing 0.75 M NaCl/0.075 M Na citrate (5× NaCl/Cit)/0.02% serum albumin/0.02% polyvinylpyrrolidone/0.02% Ficoll/0.1% $NaDodSO_4$/25 mM EDTA/20 mM sodium phosphate, pH 6.5. For hybridizations using DBM-paper, we added denatured salmon sperm DNA (1 mg/ml) to the hybridization buffer. The hybridization reactions were carried out for 3 or 4 days at 37°C in a sealed bag (Seal 'n' Save, Sears). Nitrocellulose papers were washed twice for 30 min with 50% formamide/3× NaCl/Cit at 37°C, then twice for 30 min with 2× NaCl/Cit/0.1% $NaDodSO_4$ at 50°C, and finally twice for 30 min with 0.1× NaCl/Cit/0.1% $NaDodSO_4$ at 50°C.

The DBM-papers were washed three times for 15 min each with 50% formamide/2× NaCl/Cit at room temperature and then three times with 0.2× NaCl/Cit/0.1% $NaDodSO_4$ at 50°C. Exposure was at −70°C with Kodak XR-5 film and DuPont Lightning Plus intensifying screens.

Preparation of ^{32}P-Labeled DNA. DNA was isolated from the *Dictyostelium* recombinants as described (19). Cloned DNAs were labeled by nick translation (20) except for the following modifications. Samples (0.1–1.0 μg) of DNA were incubated at 15°C for 3 hr in 0.1 ml of 0.05 M Tris·HCl, pH 7.8/5 mM MgCl/0.01 M 2-mercaptoethanol containing 50–100 μCi of [^{32}P]dTTP (600 Ci/mmol, 1 Ci = 3.7×10^{10} becquerels; Amersham); 30 nmol each of dCTP, dGTP, and dATP; and 0.1 mg of gelatin. The reaction was initiated by the addition of 2 units of DNA polymerase I (P-L Biochemicals) and 0.1–1.0 ng of DNAse I. After 3 hr at 15°C, 300 μl of 0.2 M NaCl/10 mM Tris·HCl, pH 7.5/0.5% $NaDodSO_4$ was added and the reaction mixture was extracted once with phenol and once with chloroform. Carrier RNA was added and the labeled DNA was precipitated with ethanol.

The DNA was resuspended in 100 μl of 0.1 M NaOH, heated at 100°C for 3 min, and desalted on a 1-ml Sephadex G-25 (fine) column preequilibrated with 10 mM Tris·HCl, pH 7.4/1 mM EDTA. The final specific activity was $0.2–1 \times 10^8$ cpm/μg of DNA.

Endonuclease S1 Digestions. Hybridization reactions and nuclease S1 digestions were carried out as described (21) except that the hybridization mixture was incubated for 3 or 4 hr at 39°C and endonuclease S1 (Bethesda Research Laboratories, Rockville, MD) was added to a final concentration of 2000 units/ml.

R_0t Hybridizations. R_0t hybridizations [where R_0t is the initial concentration of RNA (moles of nucleotide/liter) multiplied by the time (seconds)] were carried out in 70 μl of 80% formamide/0.4 M NaCl/0.04 M 1, 4-piperazinediethane sulfonic acid, pH 6.4/1 mM EDTA. The reaction mixture containing nick-translated DNA and excess RNA was denatured at 65°C for 10 min and then transferred to 37°C. At the appropriate times of hybridization, aliquots were diluted into 10 vol of S1 buffer [0.25 M NaCl/0.03 M NaOAc, pH 4.6/1 mM $ZnSO_4$/5% glycerol/denatured salmon sperm DNA (20 μg/ml)]. Nuclease S1 (Sigma) was added and the reaction was incubated for 60 min at 37°C. Nuclease S1-resistant material was precipitated with trichloracetic acid in the presence of carrier RNA and assayed in a scintillation spectrophotometer.

RNA Blotting onto Nitrocellulose Paper. RNA species were separated by electrophoresis in a formaldehyde gel (22). We used 1.5% agarose in a horizontal gel box with circulating buffer. After electrophoresis, the gel was soaked in 10× NaCl/Cit for 20 min. It was then blotted onto a nitrocellulose filter that had been soaked in distilled water and equilibrated with 10× NaCl/Cit. The mechanism of blotting was as described by Southern (16) except that the transfer buffer was 10× NaCl/Cit. Presoaking and hybridization of the nitrocellulose strips were carried out as described above for DBM-papers. The papers were washed with 0.1× NaCl/Cit/0.1% $NaDodSO_4$ for several hours at 50°C with several changes of buffer.

Screening of the cDNA Library. Transformed colonies were grown, transferred to nitrocellulose paper, hybridized, and washed as described by Hanahan and Meselson (23). Positive colonies were isolated, and single colonies were purified several times. The DNA was isolated from single colonies by the rapid isolation procedure (24). Spotting of the DNA into nitrocellulose filters was performed according to Mangiarotti *et al.* (17).

Gel Electrophoresis. Agarose gel electrophoresis was performed according to Sharp *et al.* (25); glyoxal treatment of nucleic acids and subsequent electrophoresis was performed according to McMaster and Carmichael (26).

RESULTS

mRNA Species Complementary to Clone pB41-6. Clone pB41-6 carries a single *Dictyostelium* nuclear DNA *Eco*RI fragment of ≈2.7 kilobases. To identify the cytoplasmic polyadenylylated RNA species complementary to this clone and to determine their relative abundances at the different developmental stages, we isolated cytoplasmic polyadenylylated RNAs from vegetative amoebae and from cells at different stages of the developmental program. The mRNAs were size fractionated on agarose/formaldehyde gels and blotted onto nitrocellulose paper. The nitrocellulose strips were then hybridized with pB41-6 labeled *in vitro* by nick translation. The results indicate that clone pB41-6 hybridizes to a very large number of different mRNA species of sizes 800–4500 bases (Fig. 1*A*). Fig. 1*B* shows the accumulation pattern of mRNAs complementary to clone pCZ22, a clone encoding sequences that are conserved throughout development. The discrete bands observed hybridizing to pCZ22 demonstrate the integrity of the mRNA preparations used in this analysis. As the number of mRNA species hybridizing to pB41-6 far exceeds its coding capacity, pB41-6 must carry sequences that are common to many mRNA species. From the hybridization pattern, we also conclude that cytoplasmic

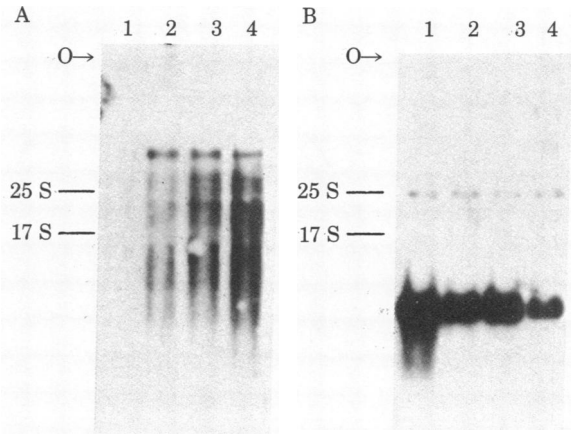

FIG. 1. Accumulation during development of mRNAs complementary to pB41-6. Cytoplasmic polyadenylylated RNA was extracted from vegetative growing amoebae and from cells developed for 5.5 hr, 15 hr, and 22 hr. (*A*) Nick-translated DNA from clone pB41-6 was hybridized to the filter. (*B*) Control experiment—RNA accumulation pattern for clone pCZ22 demonstrates the integrity of the mRNA population. Four micrograms of RNA was loaded on each lane of the gel, size fractionated, and blotted onto nitrocellulose paper. Lanes: 1, vegetative; 2, 5.5-hr; 3, 15-hr; and 4, 22-hr cytoplasmic polyadenylylated RNA, respectively. 25 S and 17 S refer to the migration of rRNAs; O, origin.

polyadenylylated RNAs that have sequences complementary to pB41-6 are rare during vegetative growth. Accumulation of these mRNAs starts ≈5 hr into development (streaming stage) and continues until at least the 22-hr stage (preculmination).

These data clearly indicate that a large number of developmentally regulated mRNAs contain sequences complementary to clone pB41-6. The relative abundances of these mRNA sequences can be determined by measuring the kinetics of hybridization of tracer amounts of labeled pB41-6 DNA with a vast excess of mRNA from either vegetative amoebae or developing cells. As can be seen in Fig. 2, there is a virtual absence of mRNAs having sequences complementary to pB41-6 in the vegetative mRNA population but a dramatic increase—at least 100-fold—of such sequences among 5.5-, 15-, and 22-hr mRNAs. It should be noted that this experiment, performed under nonstandard hybridization conditions using a double-stranded DNA probe, determines only the relative abundances of the mRNA sequences complementary to clone pB41-6 rather than the absolute abundances of these mRNA sequences at any given stage.

Approximately 38% of clone pB41-6 is insert, while 62% is vector DNA. Assuming asymmetric transcription, the maximum level of hybridization expected for clone pB41-6 in the experiment described above was 19%, a value very close to that obtained with 22-hr mRNAs (16%). Based on this result, we suggest that most of the sequences contained in pB41-6 are transcribed and are found as part of a large number of developmentally regulated mRNAs.

pB41-6 Contains Sequences that Are Found Linked only to Developmentally Regulated mRNAs. Because of the large number of different mRNA species hybridizing to pB41-6, we were interested in determining the size and number of the DNA sequences in pB41-6 complementary to such mRNAs. We hybridized unlabeled pB41-6 DNA to excess unlabeled cytoplasmic polyadenylylated RNAs. The hybrids were then treated with endonuclease S1 to digest all single-stranded molecules, and the nuclease S1-resistant material was denatured with glyoxal and fractionated on an agarose/acrylamide gel. The gel was then transferred to DBM-paper and hybridized to nick-translated pB41-6 DNA. The results demonstrate the existence of short DNA sequences, 300–500 bases long, contained in clone pB41-6, that are protected against nuclease S1 digestion by developmentally regulated mRNAs but not by mRNAs obtained from growing cells (Fig. 3A). Because excess RNA was used in the hybridization reaction, the experiment can detect the presence or absence of protected sequences but provides little quantitation of their level of expression. However, the use of RNA excess significantly increases the detection limits for RNA species in growing cells that have sequences complementary to pB41-6. As a control for the endonuclease S1 digestion experiment, the mapping results for clone pSC253, a clone encoding a single 2800-base mRNA species that is induced at 15 hr of development (7), are shown in Fig. 3B. Clone pSC253 does not contain short DNA sequences common to many mRNA species and, as shown in the nuclease S1 map, all of the nuclease S1-resistant material remains near the origin of the gel (>1500 bases). Moreover, vegetative mRNA does not protect pSC253 from nuclease S1 digestion while hybridization with 15-hr mRNA clearly results in the protection of large DNA fragments.

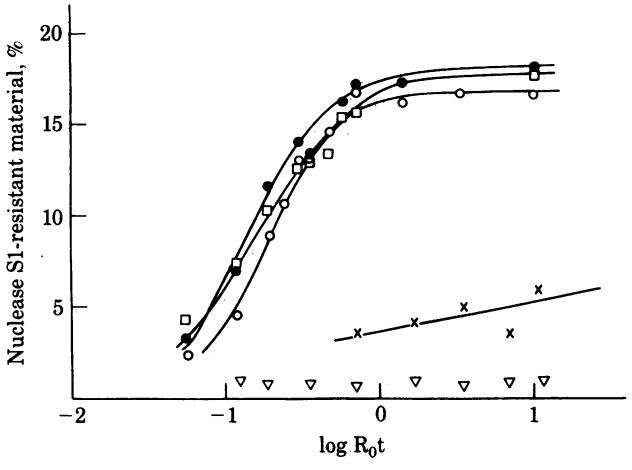

FIG. 2. Hybridization of labeled pB41-6 DNA to excess of cytoplasmic polyadenylylated RNA. Cytoplasmic polyadenylylated RNA from vegetative (×), 5.5-hr (○), 15-hr (□), and 22-hr (●) developing cells were hybridized with nick-translated pB41-6 DNA in 80% formamide. Percent hybridization was determined from the nuclease S1-resistant material after subtracting background levels. Controls [labeled pB41-6 DNA incubated with no RNA (▽)] yielded <1% nuclease S1-resistant material.

Clone pB41-6 Hybridizes to many Nuclear DNA Sequences. Because pB41-6 hybridizes to a large family of developmentally regulated mRNAs, we expect this clone to hybridize to many genes—i.e., those coding for the related developmentally regulated mRNA species. To determine whether clone pB41-6 carries sequences that are reiterated in the *D. discoideum* genome, nick-translated DNA from pB41-6 was hybridized to Southern DNA blots carrying restriction endonuclease-digested *D. discoideum* nuclear DNA. The results show that clone pB41-6 hybridizes to a large number of discrete fragments and therefore contains sequences that are reiterated in the *Dictyostelium* genome (Fig. 4). The restriction endonucleases *Bgl* II and *Hin*dIII cut only once within the pB41-6 sequence, and there is no internal recognition site for *Eco*RI restriction endonuclease.

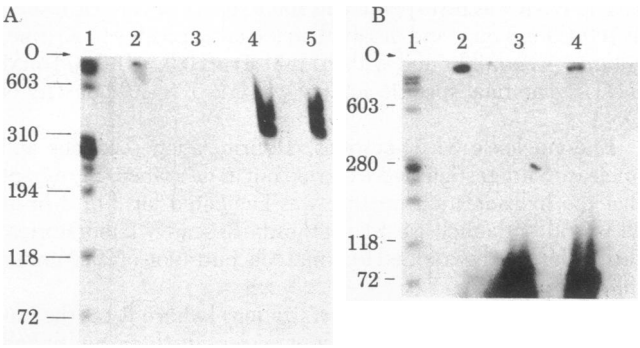

FIG. 3. Endonuclease S1 digestion of pB41-6–mRNA hybrids. (*A*) DNA from pB41-6 was hybridized to vegetative, 5.5-hr, and 15-hr cytoplasmic polyadenylylated RNAs in 80% formamide. Twenty micrograms of RNA was incubated with 0.3 μg of purified pB41-6 DNA (final volume, 60 μl) for 4 hr at 39°C. The samples were then treated with endonuclease S1, ethanol precipitated, resuspended, and denatured with glyoxal. The denatured samples were size fractionated on a 5% acrylamide/0.7% agarose gel, transferred to DBM-paper, and hybridized to nick-translated pB41-6 DNA. Lanes: 1, φX174 DNA digested with *Hae* III and end labeled was used as a size marker; 2, control—no RNA added; 3, 4, and 5, results of nuclease S1 digestion with vegetative, 5.5-hr, and 15-hr cytoplasmic polyadenylylated RNAs, respectively. The smearing of the digestion products is mainly due to the transfer of small DNA fragments onto DBM-paper. (*B*) As a control for the nuclease S1 digestion, DNA from clone pSC253 was hybridized as described above to vegetative and 15-hr cytoplasmic polyadenylylated RNAs. This clone encodes a single developmentally regulated mRNA induced at 15 hr of development. Lanes: 2, intact pSC253 DNA; 3 and 4, nuclease S1-resistant material obtained after hybridization to vegetative and 15-hr cytoplasmic polyadenylylated RNA, respectively. O, origin.

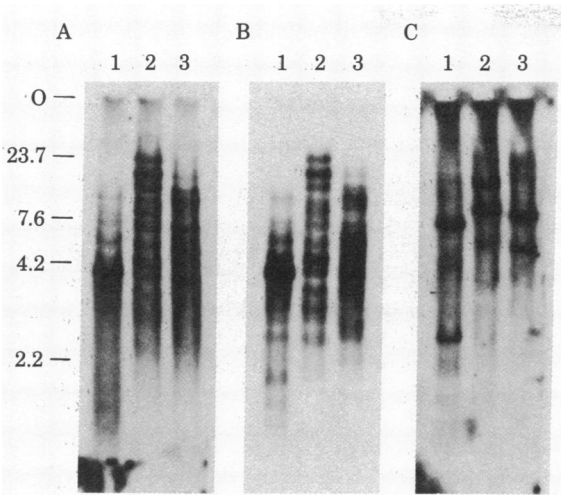

FIG. 4. Hybridization of pB41-6 (*B*) and related [pLZ-12 (*A*) and pCZ-21 (*C*)] cDNA clones to *Dictyostelium* nuclear DNA Southern blots. *Dictyostelium* nuclear DNA was digested with *Eco*RI (lanes 1), *Bgl* II (lanes 2), or *Hin*dIII (lanes 3) restriction endonuclease and fractionated on an 0.8% agarose gel. The DNA was denatured *in situ*, transferred to nitrocellulose, and prehybridized according to the method of Southern (16). Exposure was at −80°C using Kodak XR-5 film and Du Pont intensifying screens. O, origin.

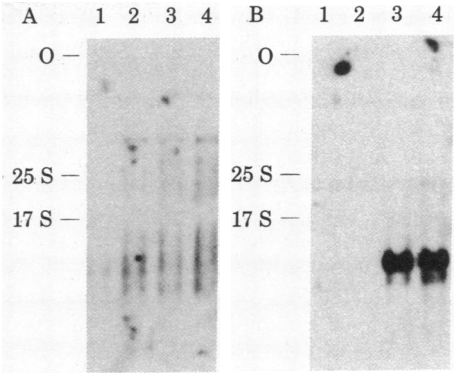

FIG. 5. mRNA accumulation patterns for cDNA clones complementary to pB41-6. (*A*) Hybridization to ^{32}P-labeled DNA of clone pLZ-12. (*B*) Hybridization to ^{32}P-labeled DNA of clone pCZ-21. Cytoplasmic polyadenylylated RNAs were size fractionated and blotted onto nitrocellulose paper. The RNA sequences observed in the vegetative RNA populations of both blots indicate hybridization to clones encoding conserved sequences. This serves as a control for the integrities of the mRNAs, a result confirmed by hybridization with other clones encoding conserved mRNAs. 25 S and 17 S refer to the migration of rRNAs; O, origin. Lanes: 1, 2, 3, 4, cytoplasmic polyadenylylated RNA from growing, 5.5-, 15-, and 22-hr cells, respectively.

There is no strong or preferential hybridization in the 2.7-kilobase region of the gel after digestion of nuclear DNA with either *Bgl* II or *Hin*dIII restriction endonucleases. Also, there is no hybridization of pB41-6 to a high molecular weight fragment derived by *Eco*RI digestion of nuclear DNA. Thus, these results show that most of the sequences complementary to pB41-6 are interspersed in the genome rather that in tandem repeats (Fig. 4; unpublished data).

Isolation and Characterization of cDNA Clones Complementary to pB41-6. On the basis of the results discussed above, we expected any cDNA clone (i.e., a cloned mRNA) having sequences complementary to pB41-6 to encode mRNA molecules that are developmentally regulated. In addition, although a given cDNA clone should encode a single transcript, we expected these cDNA clones to hybridize to many mRNA molecules—i.e., those carrying the repetitive element in the cDNA clone. Finally, these cDNA clones would be expected to hybridize to many nuclear genes, either all or a subset of the genes having sequences complementary to pB41-6. The following results demonstrate that all of these predictions are valid.

A random collection of cDNA clones prepared from 15-hr mRNAs (prepared by P. Lefebvre) was screened with nick-translated labeled pB41-6 DNA. Positive clones were picked and retested by the dot hybridization procedure (27).

The accumulation patterns for cytoplasmic polyadenylylated RNA from two cDNA clones carrying sequences complementary to pB41-6 are shown in Fig. 5. Clone pLZ-12 (Fig. 5*A*) hybridizes to a large set of different mRNA species, all of which are under developmental regulation. Moreover, these mRNA species are found as cytoplasmic polyadenylylated RNAs beginning at the 5.5-hr stage, a pattern also seen with clone pB41-6 (Fig. 1*A*). Clone pCZ-21 (Fig. 5*B*) hybridizes to a major mRNA species and several minor species that accumulate in 15- and 22-hr cells but are absent from the mRNA populations of both vegetative amoebae and 5.5-hr developing cells. There is no apparent hybridization of clone pCZ-21 to a large set of mRNA species present in 5.5-hr developing cells. Thus, we suggest that the hybridization profile observed with the original pB41-6 clone is composed of at least two sets of mRNA sequences: those expressed initially at the 5.5-hr stage and those expressed between 5 and 15 hr of development.

Hybridization of pCZ-21 to Southern DNA blots carrying *Dictyostelium* nuclear DNA digested with a set of different restriction endonucleases shows that pCZ-21 hybridizes to several discrete fragments and therefore carries sequences that are reiterated in the genome (Fig. 4). Moreover, pCZ-21 hybridizes to a subset of the genomic DNA fragments that hybridizes to pB41-6. Hybridization of pLZ-12 to blots of *Dictyostelium* nuclear DNA indicates that this clone also hybridizes to several discrete bands all of which hybridize to pB41-6 DNA (Fig. 4).

DISCUSSION

Several lines of evidence indicate that clone pB41-6 contains a specific set of repetitive DNA sequences that are found interspersed in the *Dictyostelium* genome and, when expressed, are linked only to developmentally regulated mRNAs. First, gel analysis of cytoplasmic polyadenylylated RNAs accumulated during development demonstrates the existence of a large population of mRNA species that have sequences complementary to pB41-6. All of the mRNA species complementary to pB41-6 are under developmental regulation.

Second, nuclease S1 mapping experiments indicate that clone pB41-6 carries short DNA sequences, 300–500 base pairs, that when transcribed are linked only to developmentally regulated mRNA species. The nuclease S1 map of pB41-6 does not resolve discrete DNA fragments. However, we have studied a set of cDNA clones that contain one or more DNA elements present in pB41-6. These cDNA clones hybridize to multiple restriction fragments of nuclear DNA (Fig. 4) and thus contain repetitive DNA sequences. Each of these cDNA clones contains sequences complementary to a number of developmentally regulated RNAs (Fig. 5). This analysis shows that these cDNAs and, by inference, pB41-6, contain short repetitive sequences that are expressed linked to cytoplasmic polyadenylylated RNAs that are present in 15-hr (postaggregation) but not in 5-hr (preaggregation) cells (clone pCZ-21, Fig. 5). Also, there are sequences that, when expressed, are linked to mRNAs that are induced by 5.5 hr of development but are absent or greatly reduced in growing cells (Figs. 1*A* and 5). The presence of several

repetitive elements in pB41-6 is consistent with the results of the nuclease S1 mapping in which the protection of multiple DNA fragments is observed. Note that 84% of the *Dictyostelium* DNA sequences in pB41-6 do hybridize to mRNA and thus are transcribed linked to regulated mRNAs.

Third, a set of clones that contain sequences related to pB41-6 has been isolated from a *Dictyostelium* genomic library cloned in λ Charon 28 (see below). RNA gel analysis and hybridization to these positive clones showed that all hybridized to a very large number of different mRNA species, all of which were developmentally regulated in a coordinated fashion, as is pB41-6 (R. Chisholm, personal communication). In addition, we have shown that several cDNA clones containing sequences complementary to pB41-6 hybridize to a large number of developmentally regulated cytoplasmic polyadenylylated RNAs despite the fact that a given cDNA clone should encode a single regulated mRNA species (Fig. 5; unpublished data). We note that clone pCZ-21, a cDNA clone selected by its ability to hybridize to pB41-6, hybridized to a major mRNA species that did not appear as a major component in the analysis of the mRNA species that have sequences complementary to pB41-6. We assume that this is the mRNA coded for by pCZ-21 and that most of the labeled nucleotides in the DNA hybridization probe are in the single-copy portion of this cDNA clone rather than in the short repetitive sequence(s).

The sensitivity of the techniques used in this study does not allow one to determine whether or not the genes carrying sequences complementary to pB41-6 are expressed at a very low level in growing cells.

To determine the approximate reiteration frequency of pB41-6 sequences in the *Dictyostelium* genome, Southern DNA blots carrying *Dictyostelium* DNA digested with *Eco*RI endonuclease, an enzyme known not to cut pB41-6, were hybridized with labeled pB41-6 DNA. Multiple sequences (>50) were found. Similar results were obtained with two enzymes that cut pB41-6 only once (Fig. 4). In addition, on screening a library of *Dictyostelium* nuclear DNA cloned in λ Charon 28, it was found that ≈2.5% of the clones contained sequences complementary to pB41-6 whereas other (single-copy) clones hybridized to 0.004–0.02% of the library. The average size of the inserts was 14 kilobases. Preliminary restriction mapping suggested that most of the positive clones contained more than one copy of the repetitive sequences (R. Chisholm, personal communication). Based on these results, and assuming a *Dictyostelium* genome size of 3.6×10^{10} daltons, we estimate that clone pB41-6 contains a set of sequences that are reiterated 100–200 times in the *Dictyostelium* genome.

The fact that most of the mRNA species that have sequences complementary to pB41-6 accumulate in a coordinated fashion during development suggests that these sequences may be involved in the regulatory processes leading to the concomitant induction and expression of these mRNA species. More than a decade ago Britten and Davidson (8) postulated that the association of specific repetitive sequences with single-copy DNA may be involved in the coordinate control of sets of functionally related genes during development and differentiation. Although several groups have reported the existence of short repetitive sequences linked to mRNAs in a variety of systems, there is no evidence of specific association between these repetitive sequences and differential gene expression (11). Kimmel and Firtel (10) have shown that certain *Dictyostelium* mRNAs contain reiterated sequences at their 5′ end, but these mRNAs are not developmentally regulated.

We suggest that the presence of sequences complementary to pB41-6 in a given gene defines that gene as encoding developmentally regulated mRNAs. This conclusion is consistent with the finding that all genomic clones, as well as all cDNA clones that have sequences complementary to pB41-6, encode developmentally regulated mRNAs. It should be noted, however, that during *Dictyostelium discoideum* development, several thousand genes are turned on (4, 5) and so the families of genes defined by their hybridization to pB41-6 represent a small fraction of the total mRNA species expressed during development.

We have not yet analyzed the genomic arrangement of the genes that have sequences complementary to clone pB41-6. In particular, we do not know whether they are clustered.

We believe this to be the first case in which specific repetitive sequences are linked to a set of developmentally regulated mRNAs.

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