GENE ACTIVITY DURING DEVELOPMENT OF THE CELLULAR SLIME MOLD *Dictyostelium discoideum*

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THE cellular slime mold *Dictyostelium discoideum* is an ideal eukaryotic organism with which to study the control of gene activity and development at the levels of both synthesis and translation of messenger RNA.

The strain of *Dictyostelium* (AX-3, Loomis) used is haploid and can be grown in large amounts as single amoebae in a simple axenic medium (Cocucci and Sussman 1970; Watts and Ashworth 1970). When such amoebae are removed from nutrients and deposited on the surface of a filter impregnated with a buffer, they synchronously undergo a series of morphologically defined events: groups of approximately 10^5 cells aggregate to form migrating pseudoplasmodia which subsequently differentiate into fruiting bodies containing spore and stalk cells. At specific stages during morphogenesis the specific activities of certain enzymes increase considerably and then decrease. Experiments with protein synthesis inhibitors suggest that the increase in enzyme activity is dependent on concomitant protein synthesis (see Sussman and Sussman 1969; Roth, Ashworth, and Sussman 1968; Newell 1971). This point has been firmly established for the enzyme UDP-glucose pyrophosphorylase (Franke and Sussman 1971, 1973). Recent experiments using a combination of RNA synthesis inhibitors suggests that in contrast to previous results (see Newell 1971) there is only a short lag between the periods of prior-required RNA synthesis and the appearance of developmentally regulated enzymes (Firtel, Baxter and Lodish 1973a).

When *Dictyostelium* nuclear DNA is analyzed by DNA-DNA renaturation kinetics approximately 70% of the nucleotides renature with kinetics which indicate these sequences are present only once per genome (single-copy sequence) while 30% of the sequences are repetitive with an average repetition number of approximately 150 copies per genome. The complexity of the non-reiterated DNA is approximately only 7 times the genome size of *E. coli* and the whole nuclear genome is approximately $30\text{--}35 \times 10^9$ daltons (Firtel and Bonner 1972).

In this paper we will consider the synthesis and structure of pre-mRNA in the nucleus, its processing and transport to the cytoplasm, and our present knowledge of the changes in the transcription of the *Dictyostelium* genome during the developmental cycle. We also present a model for the structure of the *Dictyostelium* chromosome.

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RESULTS

1. Synthesis of dictyostelium mRNA

Unlike that of metazoan cells (see DARNELL, JELINEK and MOLLOY 1973), the mRNA precursor in *Dictyostelium* is synthesized as a molecule only 20% longer than the mRNA. Both the mRNA and the nuclear mRNA precursor (HnRNA) but not rRNA and tRNA contain a poly(A) sequence covalently associated with the RNA. Figure 1 shows a sucrose gradient of pulse-labeled cytoplasmic RNA. There are two large radioactivity and optical density peaks of rRNA and also a peak of 5S and tRNA. In addition there is a large amount of pulse-labeled heterodisperse material sedimenting between 6 and 16S. As can be seen, 90% of this heterodisperse material binds to immobilized poly(U) and is therefore associated with at least one sequence of poly(A) (FIRTEL, JACOBSON and LODISH 1972;

FIGURE 1.—Sucrose gradient analysis of pulse-labeled cytoplasmic RNA.

A_{260} . . .

Total radioactivity ●—●

RNA radioactivity bound to poly(U) filters ○—○

Log phase cells (4×10^6 cells/ml) grown in MES-HL5 (FIRTEL and LODISH 1973) were harvested and resuspended at 3×10^7 cells/ml in 5 ml of MES-HL5 containing phosphate and 1.2 mc of ^3H -uracil. After 18 minutes, the cells were harvested and the RNA was extracted from the fractionated cytoplasm. The RNA was dissolved in 1.5 ml of 0.1% SDS and 0.5 ml was analyzed on 18 ml 15–30% SDS-sucrose gradients. Centrifugation was for 21 hours at 26,500 rpm, 22° in a Spinco SW 27.1 rotor (FIRTEL, JACOBSON and LODISH 1972). The gradient was collected from the bottom through a Gilford recording spectrophotometer and the A_{260} was monitored. An aliquot (100 μl) was counted directly and another passed through a poly(U) filter (see FIRTEL and LODISH 1973 for details).

FIGURE 2.—Sucrose gradient analysis of nuclear and cytoplasmic heterogeneous RNA.
Top panel: Total RNA radioactivity

●—● : pulse-labeled nuclear RNA
○—○ : cytoplasmic RNA — : optical density (260 nm)

Bottom panel: RNA radioactivity bound to poly(U) filters

●—● : pulse-labeled nuclear RNA ○—○ : cytoplasmic RNA

To label cytoplasmic RNA, log phase vegetative cells (3×10^6 /ml) were centrifuged and resuspended in fresh MES-HL5 medium at 3×10^7 cells/ml and treated with $3 \mu\text{g}/\text{ml}$ actinomycin D. After 15 minutes (^{32}P) PO_4 was added (2 mCi/ml) and after an additional 15 minutes the cells were diluted with 4 volumes of medium containing $125 \mu\text{g}/\text{ml}$ actinomycin D and $200 \mu\text{g}/\text{ml}$ daunomycin. After an additional incubation of 60 minutes the cells were harvested. The cells were fractionated and the RNA extracted from the cytoplasm. $3 \mu\text{g}/\text{ml}$ actinomycin D suppresses rRNA synthesis but does not affect labeling of nuclear or cytoplasmic poly(A)-containing RNA (FIRTEL and LODISH 1973) and was used to enable the cytoplasmic poly(A)-containing RNA to be visualized more readily.—To obtain pulse-labeled nuclear RNA, log phase vegetative cells (3×10^6 /ml) were harvested and resuspended in fresh MES-HL5 containing 5mM PO_4 (pH 6.5) and $200 \mu\text{Ci}/\text{ml}$ of ^3H -uracil. After 2 minutes, the cells were harvested, fractionated, and RNA extracted from the purified nuclei.—The cytoplasmic and nuclear RNA's were mixed and run on 36 ml 15 – 30% SDS-sucrose gradients. Centrifugation was in the Beckman SW 27 rotor for 20 hours at $26,000 \text{ rpm}$, 22° . The gradients were collected from the bottom through a Gilford recording spectrophotometer and the A_{260} was monitored. An aliquot was counted directly and another diluted with one volume of 0.2M Na_2HPO_4 - NaHPO_4 , 1% SDS (pH 6.8) and passed through poly(U) filters.—The optical density profile is from the cytoplasmic RNA and does not indicate the relative amount of nuclear RNA present. Centrifugation is from right to left; 28S and 18S rRNA are at fractions 9 and 16, respectively. The ^{32}P radioactivity at the top of the gradient (top panel) is not in RNA, since the material remains precipitable by trichloroacetic acid after extensive alkaline digestion (See FIRTEL and LODISH 1973 for details).

FIRTEL and LODISH 1973). Since over 90% of this RNA is polysomal we consider this material, of mean S_{20} of 13–14, to be mRNA.

Figure 2 shows the labeling pattern of pulse-labeled nuclear RNA compared with cytoplasmic RNA. The ^3H -labeled nuclear RNA exhibits a large peak of material sedimenting at 32S, smaller peaks at 22S, 18S and 4S and some heterodisperse material sedimenting between 6S and 18S. The labeled 32S species and the 22–24S RNA have the properties of precursors of rRNA (IWABUCHI, MIZUKAMI and SAMESHIMA 1971; JACOBSON, unpublished observations). In marked contrast to the case in metazoan cells, little radioactive RNA sediments faster than the 35S rRNA precursor.

Of the labeled nuclear RNA sedimenting between 6 and 18S, over 60% adsorbs to poly(U) filters indicating that it must contain at least one poly(A) sequence. The peak of nuclear poly(A)-containing RNA sediments at 15–16S, or about 2S units faster than the peak of cytoplasmic poly(A)-containing RNA.

No detectable nuclear 32S RNA binds to poly(U) filters, a result consistent with the notion that it is a precursor of rRNA. The fact that not more than 60% of the 6–18S material binds to poly(U) filters may result from a trailing of rRNA into this region. If the cells are labeled in the presence of low doses of actinomycin D which inhibits approximately 90% of the rRNA synthesis and less than 5% of the HnRNA, over 80% of the 6–18S heterodisperse radioactivity binds to poly(U) (FIRTEL and LODISH 1973).

Hence, there is good evidence that *Dictyostelium* nuclei do not contain very high-molecular-weight species of poly(A) containing RNA's akin to those found

FIGURE 3.—Dimethylsulfoxide (DMSO) gradient of nuclear and cytoplasmic heterogeneous RNA. Nuclear and cytoplasmic RNA was labeled and extracted as described in FIRTEL and LODISH 1973). Fractions (about 0.3 ml) were collected by puncturing the bottom of the tube. They were counted by adding $1\frac{1}{2}$ ml of H_2O and 10 ml of Aquasol (New England Nuclear Corp.).

in mammalian cells and which are fragmented during isolation; were this the case, one would expect much less than half of the 5–18S fragments to bind to poly(U) filters. Further evidence on this point for HnRNA from vegetative and also developing cells has been presented (FIRTEL and LODISH 1973). An analysis of the poly(A) sequences will be discussed later in this paper.

Figure 3 shows a DMSO gradient of pulse-labeled nuclear and cytoplasmic poly(A) containing RNA which was purified by adsorption to and elution from poly(U) Sepharose (FIRTEL and LODISH 1973). Using 4S, 18S, and 28S rRNA as molecular weight markers (included in a separate gradient), we determined that the number average molecular weight of the nuclear pre-mRNA is about 500,000 daltons, whereas that of cytoplasmic RNA is 20% smaller, or 400,000 daltons.

In mammalian cells a large fraction of nuclear RNA, labeled after a short pulse of radioactivity, is degraded within the nucleus, and is not transported into the cytoplasm (see DARNELL, JELINEK and MOLLOY 1973; WEINBERG 1973 for a review). In contrast, in *Dictyostelium* the vast majority (75%) of the HnRNA is transported to the cytoplasm and is present on polysomes and only 25% is degraded in the nucleus. The following experiments (data not shown) support this point: (1) if cells are pulse labeled with ^3H -uracil and "chased" with actinomycin D and daunomycin which inhibits HnRNA synthesis completely (FIRTEL and LODISH 1973), 75% of the nuclear poly(A)-containing RNA can be chased into the cytoplasm, and after 12 mins, less than 2% of the poly(A)-containing RNA remains in the nucleus (FIRTEL and LODISH 1973); (2) When labeled mRNA is hybridized to an excess of DNA complementary to mRNA (cDNA) which is synthesized using avian myeloblastosis virus DNA polymerase (an efficiency of over 90% of the mRNA copy has been obtained [VERMA *et al.* 1973]), over 90% of the RNA is incorporated into an RNA-DNA hybrid which indicates the cDNA contains sequences complementary to over 90% of the mRNA. When labeled HnRNA is hybridized to cDNA, 70% of the RNA radioactivity was incorporated into a nuclease-resistant form, which indicates that at least 70% of the sequences in nuclear RNA are in fact found in cytoplasmic mRNA. Furthermore, if the products of this hybridization are analyzed (without nuclease treatment) by chromatography on hydroxylapatite (HAP), over 90% of the nuclear RNA is found in molecules which contain at least one region of DNA-RNA hybrid (i.e., molecules which bind to HAP in 0.12 M salt) (FIRTEL, unpublished observations). Hence the regions of nuclear poly(A)-containing RNA is transported into the cytoplasm to function as mRNA (greater than 90% of cytoplasmic poly(A)-containing RNA is associated with polysomes, (FIRTEL and LODISH, manuscript in preparation), while about 30% of each molecule is not found in mRNA, and is presumably destroyed within the nucleus.

Since the poly(A) is at the 3' end of heterogenous RNA (FIRTEL and LODISH 1973; JACOBSON, FIRTEL and LODISH 1974a; FIRTEL and JACOBSON 1974), the sequences lost prior to transport must be at the 5' end as will be analyzed later in this paper.

2. Renaturation kinetics of nuclear poly(A)-containing RNA

Of the nuclear DNA of *Dictyostelium discoideum*, about 25% to 30% renatures

with kinetics indicating that these sequences are present at about 150 copies per haploid genome (reiterated DNA) and are renatured completely at a DNA $Cot = 10$. About 70% to 75% of the nuclear DNA sequences are present in only one copy per genome (single copy DNA) (FIRTEL and BONNER 1972) and renature completely by $Cot\ 3 \times 10^3$.

Figure 4 shows a hybridization of poly(A)-containing nuclear and cytoplasmic RNA to a vast excess of nuclear RNA. As has been described (FIRTEL, JACOBSON and LODISH 1972; FIRTEL and LODISH 1973) about 9–13% of the cytoplasmic RNA was in RNA–DNA hybrid by a DNA Cot of 10; hence this fraction of material is complementary to, and transcribed from, reiterated DNA. The remainder of the RNA hybridizes by a Cot of 10^4 , and hence is complementary to single-copy DNA sequences. In contrast, the nuclear RNA hybridizes with kinetics indicating that about 25–28% of the sequences are complementary to reiterated DNA, and the remainder to single copy DNA. Taken together with the fact that most of the nuclear poly(A)-containing RNA is conserved and is trans-

FIGURE 4.—Hybridization kinetics of nuclear and cytoplasmic RNA with a vast excess of DNA. 3H -cytoplasmic and ^{32}P -nuclear poly(A)-containing RNA (between 2,000 and 5,000 cpm of each per point) were hybridized together with a vast excess of DNA (50 μg per point). The experiments were performed as described previously (FIRTEL, JACOBSON and LODISH 1972) except that a 10-fold excess (5 μg) of rRNA was added.—The curve has been normalized such that the amount of hybridization at an equivalent DNA Cot of 3,000 is taken as 95%.—In this experiment 73% of the cytoplasmic and 76% of the heterogenous nuclear RNA hybridized. Irrespective of the absolute fraction of nuclear and cytoplasmic RNA which hybridizes the fraction hybridizing to repetitive DNA was always constant. The —.—.— line represents a theoretical single component curve with the same Cot as the RNA hybridizing to the single-copy DNA.

○—○ : nuclear poly(A)-containing RNA

●—● : cytoplasmic poly(A)-containing RNA

ported into the cytoplasm, the present results suggest that it is mainly transcripts of reiterated DNA present in the nuclear RNA molecules which are lost preferentially at some stage before the RNA leaves the nucleus. That these repetitive sequence transcripts are present at the 5' end is suggested by the pulse-chased experiments and by the following results. HnRNA is cleaved in half (on the average) with partial alkali treatment, and then the two halves are purified taking advantage of the fact that the 3' end containing the poly(A) bonds to poly(U) Sepharose while the presumptive 5' end does not. When the 3' end and 5' end are separately hybridized to a vast excess of DNA it is found that the 3' end contains only 16% of its sequences complementary to repetitive sequences while for whole HnRNA the value is 28%. Hence, most of the repetitive transcripts present in HnRNA are at the 5' end.

3. RNA synthesized by isolated nuclei

In the presence of the four ribonucleotide triphosphates, isolated *Dictyostelium* nuclei will synthesize both rRNA precursors and heterogeneous RNA (JACOBSON, FIRTEL and LODISH 1973b). At the optimal concentration of KCl (0.25 M), synthesis is linear for 10–20 min and continues for 40 min; there also appears to be some chain initiation as determined by ^{32}P -ATP and GTP incorporation into the RNA and isolation of labeled tetraphosphates from the RNA after RNase A, T_1 and T_2 digestion (JACOBSON, unpublished data).

Analysis by sucrose density gradient centrifugation of RNA synthesized at 0.25 M KCl (Figure 5) shows several discernible peaks of rRNA precursors at 32S, 28S, 22S, and 18S (see JACOBSON, FIRTEL and LODISH 1974b). About 30% of the RNA product will bind to poly(U) filters and hence contain at least one sequence of poly(A); the peak of poly(U)-binding material is about 15S, and the RNA has the following properties similar to that of the nuclear mRNA precursor found in pulse-labeled cells: (1) The RNA also has a base composition (23% G + C) similar to mRNA and nuclear DNA; (2) The *in vitro* heterogeneous product has exactly the same sedimentation profile on DMSO gradients as does HnRNA; (3) Its synthesis is not inhibited by low doses of actinomycin D while rRNA precursors are, however, the bulk of this RNA is inhibited specifically by α -amanitin, which does not inhibit synthesis of rRNA precursors. The *in vitro* poly(A)-containing RNA also has similar kinetics of hybridization to excess DNA as does *in vivo* RNA (JACOBSON, FIRTEL and LODISH 1974b).

4. Two types of poly(A) sequences

Polyadenylic acid [poly(A)] is resistant to RNase T_1 and A at $[\text{Na}^+]$ greater than 0.2 M and can be purified away from contaminating oligonucleotides by adsorption to and elution from immobilized poly(U) or from oligo dT cellulose. As shown in Figure 6a, *Dictyostelium* mRNA contains two discrete species of poly(A)—one migrating between 4 and 5S rRNA on 10% polyacrylamide gels (estimated size 100 nucleotides) and another peak migrating near the bromophenol blue dye (approximately 25 nucleotides). The larger poly(A) contains 99% adenylic acid and the shorter poly(A) contains 98% A. Calculations show that each molecule of mRNA has an average of 1 molecule each of poly(A)₁₀₀ and poly(A)₂₅ while, as can also be seen from Figure 6a, the *in vitro* synthesized

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RNA contains only the shorter poly(A)₂₅ (JACOBSON, FIRTEL and LODISH 1974a). Pulse-labeled nuclear HnRNA (Figure 6b) contains approximately 1 poly(A)₂₅ per molecule but only 30% of the molecules also have a larger poly(A), some of which is larger than the cytoplasmic poly(A) (FIRTEL and JACOBSON 1974). Our evidence indicates that the larger poly(A) is present at the 3' end of mRNA molecules. The poly(A)₂₅ is also present near the 3' end but must be separated by at least one nucleotide (other than A), since the two poly(A)'s are separable.

Two types of experiments also indicate that Dictyostelium DNA contains large number of sequences of poly(dA):poly(dT) of length 25 bases: (1) If Dictyostelium DNA is transcribed by *E. coli* polymerase with all 4 triphosphates, about 6% of the ³²P-ATP product is RNase T₁ and A resistant and migrates at the same position as the poly(A)₂₅ sequences. (2) Depurination of ³²P-labeled DNA yields material which binds to poly(A) Sepharose migrates at the same position on polyacrylamide gels as poly(A)₂₅, and contains over 95% T. Hybridization of ³H-poly(A) or poly(U) to Dictyostelium DNA indicates there are approximately 14–15,000 sequences each of (dT)₂₅ and (dA)₂₅. This is sufficient to code for 1 poly(A)₂₅ per gene (FIRTEL 1972; see below). Melting profiles of the ³H-poly(A)–DNA hybrids is consistent with hybrids being short in length (HAYES *et al.*, 1971). Further experiments indicate that these sequences are interspersed within the genome and are not preferentially associated with single-copy or repetitive sequences (FIRTEL, unpublished observation).

All the data taken together indicates that the poly(A)₂₅ is transcribed from the nuclear DNA while the larger poly(A) sequence is added after transcription some time prior to transport into the cytoplasm (JACOBSON, FIRTEL and LODISH 1974a; FIRTEL and JACOBSON 1974).

5. Expression of the single-copy DNA during development

In order to determine the qualitative and quantitative changes in the transcription of single-copy sequences during development, hybridization has been performed with RNA from four stages of Dictyostelium developing to isolated single-copy DNA (FIRTEL 1972). In these experiments large quantities of unlabeled whole-cell RNA was hybridized to a small quantity of labeled purified single-copy sequences (RNA excess hybridization). Under the experimental con-

FIGURE 5.—RNA synthesis is isolated by repeated differential centrifugation of detergent lysed cells by a modification (JACOBSON, FIRTEL and LODISH 1973b) of the procedure of COCUCCHI and SUSSMAN (1970). Reactions contained, in a final volume of 100 μ l: 0.04M Tris-HCl, pH 7.9, 0.01M MgCl₂, 0.25M KCl, 0.16mM GTP, CTP and UTP, 0.16mM α ³²P-ATP (5 C/mm), 0.1mM dithiothreitol, 5% (v/v) glycerol and nuclei from 0.8–4.0 $\times$ 10⁶ cells. After 10 minutes of incubation at 22°, reactions (100 μ l) were terminated by addition of SDS to 0.2% and layered on 15–30% sucrose gradients in 0.1M Tris-HCl, pH 7.3, 0.1M NaCl, and 0.5% SDS. Centrifugation was in the Spinco SW 50.1 rotor for 2.75 hours at 46,000 rpm and 22°. Aliquots were assayed for acid-precipitable radioactivity and for binding to poly(U) filters (JACOBSON, FIRTEL and LODISH 1973b). A. No additions to reaction. B. Incubation in the presence of 2 μ g/ml Actinomycin D. C. Incubation in the presence of 10 μ g/ml α -amanitin. D. Incubation in the present of 2 μ g/ml Actinomycin D and 10 μ g/ml α -amanitin.

FIGURE 6.—Analysis of poly(A) on polyacrylamide gels. A. mRNA labeled in whole cells with ^3H -adenine (FIRTEL and LODISH, 1973) was mixed with mRNA precursor labeled in isolated nuclei (at 0.25M KCl) with α ^{32}P -ATP (JACOBSON, FIRTEL and LODISH 1974b) and digested with RNases A and T_1 in 2XSSC for 30 minutes at 37°. After deproteinization, poly(A) was separated from oligonucleotides by poly(U) Sepharose chromatography (FIRTEL *et al.*, 1973; FIRTEL and LODISH 1973) and electrophoresed in a 10% polyacrylamide gel as described by BISHOP *et al.* (1967). After electrophoresis, gels were sliced and solubilized in scintillation fluid containing 3.5% (v/v) Amersham NCS. Dictyostelium 4S and 5S RNAs were electrophoresed in a parallel gel with poly(A) synthesized in isolated nuclei. BPB: position of Bromphenol blue tracking dye.

ditions there is essentially no DNA-DNA renaturation (low DNA Cot) and the rate of the reaction is determined by the concentration of the input RNA (pseudo-first order reaction). The amount of single-copy DNA transcribed at any stage is determined by the fraction of the single-copy DNA which is present in a hybrid at high Rots determined by hydroxylapatite chromatography (Rot is a term equivalent to Cot equal to the product [initial RNA concentration in moles/l] [seconds]). The small fraction of DNA which renatures as a DNA-DNA duplex is subtracted as described previously (FIRTEL 1972).

In order to determine the changes between different stages in the transcription of the non-reiterated sequence, RNA addition hybridizations were performed. In this case saturating amounts of RNA from 2 stages are hybridized and the fraction of DNA in hybrid is determined. If the RNA sequences from the two stages are the same, there will be no change in the level of hybridization. If the sequences are totally different, the hybridization value will be the sum of the saturating values of both preparations. If some sequences are the same and some are different, an intermediate level will be obtained (see FIRTEL 1972 for details). The experimental results are given in Table 1 and Figure 7. As can be seen there are both qualitative and quantitative changes in the portion of the single-copy sequences present at the various developmental stages. Tm's of the hybrids showed there was little if any base mismatching as expected for single-copy DNA (FIRTEL 1972). The largest difference is obtained with RNA from vegetative cells and early culminating cells (20 hr). A combined hybridization value of approximately 25% of the single-copy DNA is obtained. Of the RNA from 20 hr cells approximately 40% of the sequences are in common with vegetative RNA while 60% represent new sequences transcribed since vegetative growth and present in 20 hr cells. If RNA's from all 4 stages are hybridized together, 28% of the single-copy DNA is found in hybrid. Assuming transcription of only 1 strand, this represents 56% of the single-copy sequences (equivalent to approximately 18×10^6 nucleotide pairs). These results indicate that there are major changes in the expression of the single-copy DNA and that regulation of differentiation must occur at least in part at the level of transcription.

Initial experiments using cDNA synthesized from mRNA isolated from early culminating (18 hr) cells support this data (FIRTEL, unpublished observations). While mRNA from 18 hr cells protects 94% of this cDNA, mRNA from vegetative cells protects only 53%. This result indicates that vegetative RNA contains

●—● : ^3H -labeled poly(A) of mRNA
X—X—X : poly(A) labeled with ^{32}P in isolated nuclei.

B. ^{14}C -nuclear and ^3H -cytoplasmic adenine labeled heterogenous RNA (see FIRTEL and LODISH 1973) were isolated by poly(U) Sepharose chromatography (FIRTEL and LODISH 1973) and poly(A) was subsequently isolated as described in FIRTEL, JACOBSON and LODISH 1972. Poly(A) was further purified by adsorption to and elution from oligo dT cellulose and subjected to electrophoresis in 10% polyacrylamide gels as described in part A.

○—○ : ^3H -labeled poly(A) of mRNA
●—● : ^{14}C -labeled poly(A) of HnRNA

TABLE 1

Hybridization of RNA from several developmental stages with unique DNA

Developmental stage of RNA	Concn. (mg/ml)	Hours incubation	Percent DNA in duplex	Percent DNA in DNA/RNA hybrid	Percent DNA in DNA/DNA duplex
Log phase cells	16	24	17.3 ± 0.2	14.4 ± 0.2	2.9 ± 0.1
(0 hr)		48	18.0 ± 0.2	15.1 ± 0.2	2.9 ± 0.1
Pre-aggregation	16	24	15.1 ± 0.3	12.6 ± 0.6	2.5 ± 0.2
(6 hr)		48	16.2 ± 0.3	13.7 ± 0.3	2.5 ± 0.2
Late-aggregate	16	24	18.3 ± 0.3	15.8 ± 0.3	2.5 ± 0.2
Early slug		48	19.6 ± 0.3	17.1 ± 0.3	2.5 ± 0.2
(12 hr)					
Early culmination	16	24	21.5 ± 0.2	18.9 ± 0.2	2.6 ± 0.1
(20 hr)		48	21.9 ± 0.2	19.3 ± 0.2	2.6 ± 0.1
0 hr—RNase* treated					
before incubation	16	24	4.0 ± 0.2		
None		24	5.0 ± 0.2		
Ascites RNA	15	24	4.0		

Conditions: 0.48 M-phosphate buffer, 0.002 M-TES, 0.1% sodium dodecyl sulfate, 0.3 g $^{32}\text{PO}_4$ -labeled DNA, 80 to 120,000 cts/min/g, 62°. Volume for 24-hr incubation 0.3 ml; 0.6 ml for 48-hr incubation. Duplicate points were run with each batch of RNA and DNA. Two batches of RNA and 3 batches of DNA were used to determine average for 6 and 12 hour RNA; 4 batches of RNA and DNA were used for 0 and 20 hours. The errors shown represent the ranges of the data used.

* Experiment was performed with all RNA samples with the same results ± 5%.

Data was taken from FIRTEL 1972.

sequences which represent 56% (53%/94%) of the total mRNA sequences at 18 hr. (It is important to note that cDNA represents total sequences and reflects the relative amounts of different mRNA's.) The difference between this value and the value obtained by hybridization-addition studies may be due to certain mRNA's being present at higher concentrations than other messengers (see VERMA *et al.* 1974) and it is these messengers from 18 hr cells which may be in common between the two stages.

6. Protein changes during development

Another method of analyzing regulation of development in *Dictyostelium* would be to look for changes in major cell proteins with the goal of isolating a mRNA for a specific developmentally regulated protein. If this could be done it would enable one to study the transcriptional and post-transcriptional regulation of a specific gene and gene product.

In order to approach this question, cells were pulse-labeled for 2 hr periods during development with ^{35}S -methionine. The proteins were subjected to electrophoresis on polyacrylamide gels and visualized by autoradiography. Figure 8 shows the results. There are proteins which appear and disappear during development. The most prevalent change is in the heavy band (approximately 48,000 mw) which represents as much as 20% of the newly synthesized proteins in early developing cells. By late aggregation, it represents only a small portion of the newly synthesized protein. Experiments have indicated that this protein is Dicty-

FIGURE 7.—Histograms of amount of DNA hybridized to RNA at or near saturation. Histogram shows per cent of DNA in hybrid for RNA from individual development stages and from 2 or 4 stages combined. R_0 of the RNA from each stage was maintained at 22×10^3 . 4.8 mg of RNA from each stage were incubated at 62° , 0.48 M-phosphate buffer in 0.3 ml for 24 hr. For experiments in which RNA from 2 stages were combined, the incubation was in 0.6 ml for 48 hr and for RNA from 4 stages 1.2 ml and 96 hr were used.

□ : Estimation of fraction of complexity of DNA sequences present at transcripts in both stages. (Refers only to histograms of hybridization of RNA from a single stage.)

▒ : Estimates of fraction of complexity of the RNA sequences from the earlier of the 2 stages no longer present in later stage.

▒ : Estimates of fraction of complexity of RNA from later stage which represents transcripts of new sequences; i.e., sequences synthesized and accumulated during the relevant period of development.

Data was taken from FIRTEL 1972.

ostelium actin (TUCHMAN and LODISH, manuscript in preparation). It should be pointed out that although many bands can be seen on gels these represent proteins present at relatively high concentrations. From the hybridization results (see DISCUSSION; FIRTEL 1972) we would expect the total number of different proteins to be on the order of several thousand at any one stage.

Toward the end of isolating this message, mRNA from early developing cells has been added to a reticulocyte cell-free protein synthesizing system. A new protein can be seen on polyacrylamide gels which has the same mobility as Dictyostelium *in vivo*-labeled actin (LODISH, FIRTEL, ALTON and DESALU, unpublished observation). Tryptic peptides indicate that the proteins are identical.

DISCUSSION

In metazoans the HnRNA is approximately 10–20 times larger than mRNA and approximately 90% of the sequences are rapidly turned over in the nucleus

[³⁵S] METHIONINE PROTEIN SYNTHESIS DURING DICTYOSTELIUM DEVELOPMENT

FIGURE 8.—Changes in the patterns of protein synthesis during slime mold growth and development. 10 ml of vegetative cells at a concentration of 2×10^6 /ml were labeled for 6 hours with 400 μ Ci of ³⁵S-methionine (100 Ci/mmole). The cells were then harvested by centrifugation and washed twice.—Developing cells were labeled for the interval indicated in the figure. ³⁵S-methionine (100 Ci/mole) was diluted in PDF to a concentration of 75 μ Ci/ml. 75 μ l of the label was deposited on one half of a millipore pad barely saturated in PDF. Immediately thereafter, one half of a filter supporting 2.5×10^7 cells at the desired stage of development was lifted from its pad, briefly touched to a dry pad and transferred to the radioactive pad. After the desired interval, the cells were washed off the filter in 3 ml of PDF and harvested by centrifugation.

The vegetative and developing cell pellets were taken up in 50 μ l of 2X gel sample buffer (FAIRBANKS, STECK and WALLACH 1971) and lysed by vigorous mixing and heating to 70° in this buffer. Gel samples were equalized to 1×10^5 cpm/gel and heated to 100° for 3 min before electrophoresis. 5.5% acrylamide gels were run, fixed and stained according to the procedures of FAIRBANKS, STECK and WALLACH 1971. Shown are radioautographs of the gels.

while the remaining sequences, probably at the 3' end associated with the poly(A), are transported into the cytoplasm (see DARNELL, JELINEK and MOLLOY 1973; WEINBERG 1973).

In Dictyostelium, a eukaryotic protist, the nuclear mRNA precursor weighs approximately 500,000 daltons (15S in a neutral sucrose gradient) and is only 20% larger than the mRNA. Pulse-chase experiments and hybridizations with cDNA to mRNA indicate approximately 75% of the HnRNA sequences are con-

STRUCTURE AND TRANSCRIPTION OF DICTYOSTELIUM DNA

FIGURE 9.—A model of Dictyostelium nuclear DNA. The primary genetic unit in Dictyostelium contains a repetitive DNA sequence (R) of average 300–350 nucleotides at the 5' end; a sequence of non-reiterated or single-copy (SC) DNA of 1100–1200 nucleotides; and a sequence of 25 adenylic acid residues (A₂₅) at the 3' end. This unit is transcribed by RNA polymerase and then poly(A) of 100–150 residues is added after transcription. Upon transport of the heterogeneous RNA to the cytoplasm, the majority of the 5' repetitive sequences are removed, leaving a short repetitive sequence. The evidence for the presence of a portion of the repetitive sequence transcript (r) on the majority of mRNA molecules is given in FIRTEL and LODISH 1973. Since the poly A₂₅ and poly A₁₀₀ are separable on polyacrylamide gels after T₁ and RNase A, at least one other nucleotide (X) must be present between the two poly(A) sequences at the 3' end of the short poly A₂₅ and the 5' end of the larger poly A₁₀₀.

served and transported to the cytoplasm. DNA-driven hybridizations indicate that 25% of the nuclear poly(A) containing RNA is transcribed from reiterated sequences and that most of these sequences are preferentially located at the 5' end of the HnRNA molecule.

The mechanism of synthesis of mRNA in Dictyostelium is summarized in Figure 9. As previously described the poly(A)₂₅ appeared to be encoded in the DNA and transcribed as an integral part of the HnRNA. The poly(A)₁₀₀ is not encoded by the DNA and is added after transcription but prior to transport to the cytoplasm (JACOBSON, FIRTEL and LODISH 1974a; FIRTEL and JACOBSON 1974). From labeling kinetics and appearance of the poly(A)₁₀₀ on HnRNA, the poly(A)₁₀₀ is probably added prior to the removal of the 5' repetitive sequences (FIRTEL and JACOBSON 1974). The results indicate that the majority of the pulse-labeled nuclear and cytoplasmic heterogeneous RNA has a poly(A) sequence associated with it and that mRNA is approximately 90% single-copy sequences. (Other results [FIRTEL and LODISH 1973] indicate that the majority of

mRNA molecules contain short and repetitive sequences associated with them and exclude the notion that 90% of the molecules are transcribed solely from single-copy sequence and 10% from repetitive sequences.) We assume that the majority of the single-copy sequences in the HnRNA are transported to the cytoplasm and appear on polysomes (although this has not yet been definitely proven by hybridization experiments).

Figure 9 also indicates the structure of the Dictyostelium nuclear DNA. We conclude that a large fraction of the repetitive and single-copy sequences are interspersed throughout the genome. Repetitive sequences (R) of about 300–350 nucleotides are associated with single-copy (SC) sequences of average 1,000–1,100 nucleotides. If 56% of the Dictyostelium genome is transcribed into poly(A)-containing molecules (mRNA precursors) of average size 500,000 daltons, then the Dictyostelium genome would contain about 10,000–20,000 “genes”. We have shown that the Dictyostelium genome contains 14–15,000 sequences of poly(dT)₂₅ and that these sequences are also interspersed in the genome (JACOBSON, FIRTEL and LODISH 1974a). We believe that they correspond to the 3' end of the transcribed mRNA and that dT₂₅ may be associated with a transcription termination signal. Preliminary experiments which examine the fraction of DNA molecules of given lengths which contain both repetitive and single-copy sequences support this model (FIRTEL, unpublished observations).

The unit R·SC·A₂₅ is the primary transcription unit in Dictyostelium. Since less than 100% of the genome appears to be transcribed, we believe that there is a spacer unit in between the primary transcription units but there is as yet no direct demonstration of spacers. This chromosome is very similar to the known size and organization of the interspersed repetitive and single-copy sequences in *Xenopus* (DAVIDSON *et al.*, 1973).

From polyacrylamide gel studies of proteins pulse-labeled during development it is apparent that there are changes in the relative rates of synthesis of certain major protein bands. Hybridization experiments with whole cell RNA have shown that at 20 hr of development, RNA sequences are present which are complementary to 38% of the DNA. Of this, approximately 17% represent sequences present at all stages. If all of the transcribed sequences are present on polysomes then approximately 11,000 proteins containing 330 amino acids are being coded for at 20 hr of which approximately 6,000 are new relative to vegetative cells. These data indicate that regulation at the level of transcription represents one of the primary control points in Dictyostelium development.

Presently, we are trying to isolate mRNA for specific developmentally regulated proteins. The ability to do this along with the ability to synthesize DNA complementary to it will enable us to examine the regulation of gene activation of specific genes, the processing of specific mRNA precursors, and the translation of this message. We are hoping that this approach may help to answer the function of the repetitive sequences associated with the 5' end of the HnRNA. It is possible that there are regulatory sequences of the kind reported by BRITTEN and DAVIDSON (1969). If this is the case, no evidence exists as to whether the tran-

scribed repetitive sequences serve any further function or whether they represent the transcription of an operator-type region.

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