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Dictyostelium discoideum chromatin capacity for endogenous and Escherichia coli RNA polymerase.

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DICTYOSTELIUM DISCOIDEUM CHROMATIN CAPACITY
FOR ENDOGENOUS AND *ESCHERICHIA COLI* RNA
POLYMERASE

by

Martha Kathleen Morris

A thesis
submitted to the Faculty of Graduate Studies
through the Department of
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of the Requirements for the Degree
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ABSTRACT

Dictyostelium discoideum Chromatin Capacity for
Endogenous and *Escherichia coli* RNA Polymerase

Differential gene expression occurs throughout the developmental cycle of
Dictyostelium discoideum. Little is known however, about the mechanisms
involved in regulation of differential gene expression. Therefore, a
preliminary study was undertaken to determine whether differences exist in
isolated chromatin from vegetative and late aggregration cells of Ax_3
with respect to template capacity of RNA polymerase binding.

Endogenous RNA polymerase activity for *D discoideum* chromatin
within intact nuclei was measured. Nuclei isolated from vegetative cells,
displayed the three forms of RNA polymerase activity in a ratio of RNA
polymerase I: RNA polymerase II: RNA polymerase III of 66:20.6:12.9,
respectively. Nuclei isolated from aggregration cells also contained all three
forms of the enzyme, however, differences occurred in the ratio of activity.
The ratio of RNA polymerase I: RNA polymerase II: RNA polymerase III in
aggregration nuclei was 20.1:70.7:8.8, respectively. This shift in activity
from RNA polymerase I to RNA polymerase II reflects the documented transition
in RNA synthesis from predominately, ribosomal RNA synthesis in vegetative
cells to predominately, messenger RNA synthesis in aggregrated cells. No
significant change was detected in the total RNA polymerase activity in
vegetative and aggregrated nuclei.

Template activity and binding capacity of *Esherichia coli* RNA
polymerase to isolated *Dictyostelium* chromatin was also measured.
Chromatin isolated from vegetative cells displayed higher template activity and
binding capacity for *E coli* RNA polymerase than chromatin isolated from
aggregration cells. It is hypothesized that the higher template capacity of
vegetative chromatin for *E coli* RNA polymerase is due to the extended
nature of *Dictyostelium* chromatin during ribosomal RNA synthesis.

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ABBREVIATIONS

ATP   Adenine triphosphate
BSA   bovine serum albumin
$CaCl_2$ calcium chloride
cAMP   cyclic adenosine monophosphate
CPM   counts per minute
CTP   cytidine triphoshate
DNA   deoxyribonucleic acid
DPM   disintergrations per minute
DTT   dithiothreitiol
EDTA   ethylenediaminetetracetic acid
GTP   guanine triphosphate
IgG   immunoglobulin G
KH_2PO_4 potassium phosphate (monobasic)
K_2HPO_4 potassium phosphate (diabasic)
$MgCl_2$ magnesium chloride
$MgSO_4$ magnesium sulphate
$MnCl_2$ manganese chloride
mRNA   messenger ribonucleic acid
$NaCl_2$ sodium chloride
Na_2HPO_4 sodium phosphate
NaOH   sodium hydroxide
$(NH_4)_2SO_4$ ammonium sulphate
PCA   perchloric acid
PEG   polyethylene glycol
psi   pounds per square inch
RNA   ribonucleic acid
rDNA   ribosomal deoxyribonucleic acid
rpm   revolutions per minute
rRNA   ribosomal ribonucleic acid
TCA   trichloroacetic acid
tRNA   transfer ribonucleic acid
UTP   uridine triphosphate

INTRODUCTION

The life cycle· of *Dictyostelium discoideum* has been extensively studied. Myxamoebae grow and reproduce in liquid axenic media, and upon starvation growth ceases and a developmental cycle is initiated. This developmental cycle consists of three stages: aggregration, pseudoplasmodial formation and culmination (Fig. 1). The process begins in response to a cAMP signal, starved amoebae will form multicellular aggregrates. These aggregrates will mound up and topple over forming a pseudoplasmodium or "slug". The pseudoplasmodia are able to migrate in search of a new food source. At the end of migration, culmination begins as the cells of the pseudoplasmodium differentiate into pre-spore and pre-stalk cells. Culmination results in the formation of a developed fruiting body containing a sorus of spores suspended upon a mass of stalk cells. Amoebae cultured in axenic media and then plated out on agar to stimulate starvation conditions will proceed through this cycle to form fruiting bodies in approximately 26 hours (Sussman, 1966).

Morphological differentiation is also accompanied by alterations in gene expression as demonstrated by the pattern of enzyme expression and the production of RNA transcripts during development. Thirteen stage-specific enzymes preferentially accumulate in *Dictyostelium* (Review, Loomis, 1975). Accumulation of each enzyme can be blocked when protein synthesis is inhibited by cycloheximide. ·Therefore, concomitant protein synthesis is required for the accumulation of stage specific enzymes. In addition, when RNA synthesis is inhibited by Actinomycin D accumulation of stage specfic enzymes continues but only for a specific period of time. This suggests that there is a limited pool of mRNA functioning for each stage specific enzyme and that when the mRNA is degraded, new· RNA synthesis is required for further

Figure 1: The life cycle of *Dictyostelium discoideum* (After Wright, 1963).

SPORE

AMOEBA

GROWTH AND
MULTIPLICATION

AGGREGATION

PSEUDOPLASMODIUM

CULMINATION

MATURE
FRUITING
BODY
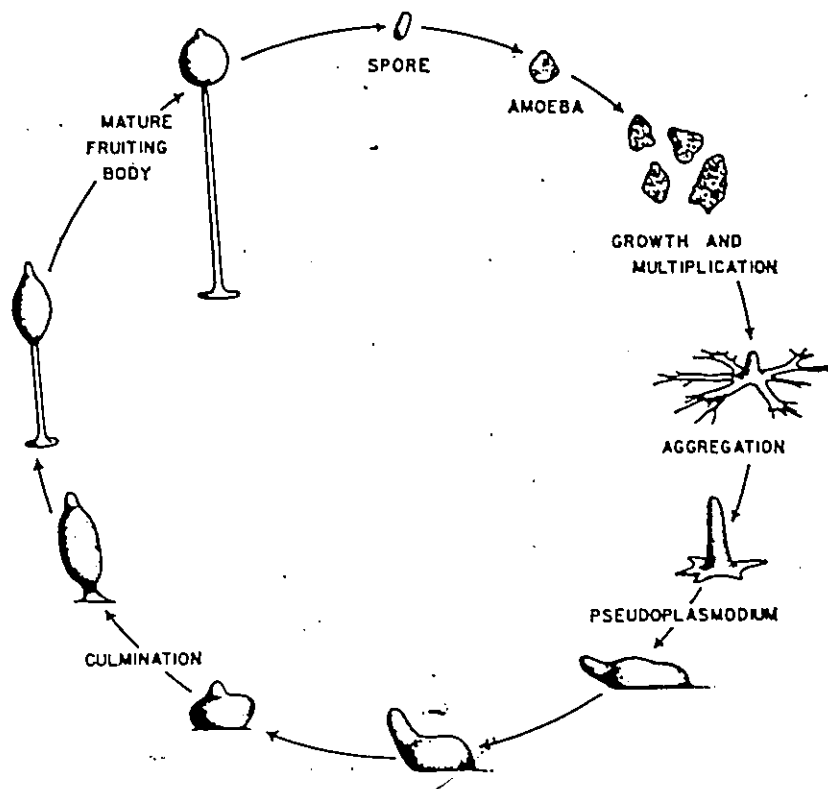
enzyme accumulation.

Firtel (1972) also demonstrated, using hybridization of extracted RNA to unique sequences of *Dictyostelium*, that only half of the RNA species present in vegetative amoebae were present in developed cells while new species of RNA consistently appeared during development. Alton and Lodish (1977a) analyzed the patterns of proteins synthesized throughout the development of *Dictyostelium* by two dimensional polyacrylamide gel electrophoresis. Messenge RNA was isolated from different stages of development and translated in a wheat germ cell free system. While continuous changes in the pattern of protein synthesis occur throughout the developmental sequence, major changes are found only in certain stages. The major changes in the pattern of protein synthesis during development take place during late aggregration which is classified as 8–12 hours after development is initiated. During this time approximately 30 proteins are newly synthesized while many others show increases in the relative rates of synthesis. In addition, about 10 proteins are either no longer synthesized or are synthesized at a reduced rate during this stage. Throughout these changes in protein synthesis, the ratio of total translatable mRNA to total cellular RNA was found to be constant during growth and differentiation. However, changes in the pattern of protein synthesis could be accounted for by parallel increases or decreases in the amounts of translatable mRNA encoding these proteins. This alteration in RNA transcripts (Firtel, 1972; Alton and Lodish, 1977) following the initiation of development suggested that a change may occur in the enzyme responsible for RNA synthesis, namely, RNA polymerase. In eukaryotic organisms, RNA polymerase is subdivided into three groups based on chromatographic analysis and α-amanitin sensitivity. RNA polymerase I, which is insensitive to α-amanitin is involved

in the synthesis of rRNA; RNA polymerase II, whose activity is inhibited by low concentrations of α-amanitin participates in the synthesis of hnRNA, the precursor of mRNA; and that RNA polymerase III, which is sensitive to relatively high concentrations of α-amanitin, is involved in the synthesis of low molecular weight RNAs including the 5S and tRNA (Roeder R.G., 1976).

Pong and Loomis (1973) electrophoretically compared RNA polymerase I and RNA polymerase II isolated from both vegetative and early aggregated cells. They found that there was no significant alteration in the subunit composition of the forms of the enzyme during development. In addition, they found that specifically, the activity of RNA polymerase II was found to decrease during development which was contrary to the necessary requirement for new mRNA synthesis (Firtel, 1972; Loomis, 1975; Alton and Lodish 1977). As techniques for the isolation procedure of RNA polymerase improved, it was determined that *Dictyostelium* possessed the three forms of the enzyme present in all eukaryotic organisms (Takiya, *et al.*, 1980; Yagura, *et al.*; 1976). Template specificities of partially purified RNA polymerase I and RNA polymerase II from different stages of development were measured using various synthetic polynucleotide templates (Takiya, *et al.*, 1980). It was found that sequences rich in pyrimidines were best utilized as templates (Takiya, *et al.*, 1980). However, minimal differences were demonstrated in the efficiency of RNA polymerase to utilize these templates during development (Takiya, *et al.*, 1980). Therefore, little evidence exists for RNA polymerase directly controlling gene transcription of developmentally regulated sequences.

Yagura, *et al.*, (1982) correlated in vivo rRNA and mRNA synthesis in *Dictyostelium* with the *in vitro* levels of RNA polymerase activity.

Sucrose gradient centrifugation of tritium labelled RNA extracted from cells at different stages of development were analyzed. It was determined that during the vegetative growth phase rRNA synthesis predominated over mRNA synthesis while during development mRNA synthesis predominated over rRNA synthesis. Likewise, this alteration in RNA synthesis was accompanied by a subsequent change in RNA polymerase activity from cleared extracts of vegetative and developing cells. High levels of RNA polymerase I activity were noted when rRNA synthesis predominated and similarily, increases in the level of RNA polymerase II activity were seen when mRNA synthesis predominated.

This distinct difference in the activity of the two forms of the RNA polymerase may be due to a number of factors. First, there may be an actual increase or decrease in the number of RNA polymerase I and RNA polymerase II molecules throughout development. As of yet there is little evidence supporting this hypothesis. Second, the actual number of molecules may not alter through development but rather there may be a change in the enzyme's ability to be transcriptionally active. It has been hypothesized that RNA polymerase may exist in two states – a bound state, which is transcriptionally active and a free state, which is not transcriptionally active. RNA polymerase from *Escherichia coli* (Chamberlin, 1976) and RNA polymerase II in human placenta (Seidman, 1980), wheat germ (Seidman, 1979) and mouse liver (Warnick, 1983) nuclei have all been found to exist in a bound and free form. Therefore, transcription may also be controlled by the ability of RNA polymerase to bind to chromatin in an active form. Finally, the chromatin structure, itself, may reflect the binding and transcriptional ability of RNA polymerase. The fundamental structural unit of chromatin is a nucleoprotein complex called a nucleosome. Nucleosomes contain two molecules each of

histones H2A, H2B, H3 and H4 in association with approximately 200 base pairs

of DNA. The nucleosome is repeated throughout the chromatin connected by a

linker region of DNA to which histone H1 is closely associated. In addition to

histone proteins, non–histone proteins are also associated with the nucleosome.

The complex nature of chromatin reveals that RNA polymerase accessibility to

the DNA may be highly regulated. There is little conclusive evidence that

either the histone and non–histone proteins, alone, regulate transcription. It

is now thought that both groups of proteins play important but unique roles in

controlling transcription.

In *Dictyostelium*, basic chromatin structure has been extensively

studied. It is established that the nucleosome has a core size of

approximately 137 base pairs which is very similar in size to that found in

other eukaryotes. The linker region is shorter than expected being only

approximately 50 base pairs (Bakke, *et al.*, 1978). Purification of the

histone proteins revealed that *Dictyostelium* has only four histones

instead of the usual five and that histone H7 is unique. The arrangement of

the histones in the nucleosome, based on molecular weight determination,

indicates that there are four molecules of H7, and two molecules each of H3 and

H4–like proteins (Bakke, 1979). The H1–like protein is found associated with

the linker region and contains fewer basic residues than expected. Because of

the arrangement and relative proportions of the histones, these differences are

not thought to affect the basic chromatin structure. Parish, *et al.*,

(1980) have found at least 5 non–histone proteins also associated with the

nucleosome.

Although chromatin structure has been elucidated, little work has been done

on chromatin as an active template in *Dictyostelium*. The ultimate goal

in studying an organism which has developmentally regulated gene sequences is
to gain insight into how gene transcription is initiated. One primary
investigation that should be first considered is whether chromatin has the same
ability to function as a template for RNA polymerase during development.
Measurement of chromatin activity as a template however is complex and can be
affected by a number of components. Template activity is detected by the
amount of RNA synthesized. Therefore, the following events may all be involved
in determining template activity: 1) the ability of RNA polymerase to bind to
chromatin 2) the ability of RNA polymerase to search for initiation sites 3)
the formation of a stable RNA polymerase–DNA initiation complex 4)
the initiation of RNA chain synthesis 5) the rate of RNA chain propagation 6)
the size of the RNA chains 7) the termination of the RNA chains and 8) the
reinitiation of the transcriptive process (Tsai, 1975).

In this study, nuclei were isolated from vegetative and late aggregration
cells of *Dictyostelium*. Endogenous levels of RNA polymerase activity
were measured in these intact nuclei. In addition, chromatin was also
isolated from the nuclei of these two cell types. Since it is known that
differential gene expression occurs throughout development, total template
activity of isolated chromatin was examined. *E coli* RNA polymerase was
used to transcribe the chromatin as successful attempts to isolate
Dictyostelium RNA polymerase yielded minimal results. Because, *E
coli* RNA polymerase is able to bind randomly and reversibily to DNA, a
distinction must be made between non–specific binding and stable initiation
complexes formed between the enzyme and chromatin. Stable initiation complexes
are detected by rifampicin challenge experiments (Tsai, 1975). Rifampicin is
an inhibitor of RNA synthesis which functions by binding to the β subunit of

RNA polymerase (Heil, *et al.*, 1970). Binding of rifampicin to RNA polymerase inhibits RNA synthesis only prior to RNA chain initiation. Therefore, *E. coli* RNA polymerase in a stable complex with DNA can initiate RNA synthesis but polymerase free in solution or randomly bound to DNA will be inhibited (Hartmann, *et al.*, 1967). This allowed the total template activity and binding capacity of chromatin for RNA polymerase to be determined during two developmental stages in *Dictyostelium*.

METHODS AND MATERIAL

Growth of the Organism

The axenic strain of *Dictyostelium discoideum* designated as Ax₃ was utilized throughout this study. Ax₃ was obtained from Dr. R. Dimond but originally was isolated by Dr. W.F. Loomis (1971). Ax₃ was grown in a modified HL5 medium consisting of 10g dextrose, 10g trypticase peptone, 5g yeast extract, 0.35g Na_2HPO_4 and 1.2g KH_2PO_4 suspended in one liter of distilled water. Cells were inoculated into 50 ml of sterile medium and grown at 23° C on a gyratory shaker to a cell density of 1 to 5 x 10⁶ cells/ml before harvesting or transferring to fresh media. For isolation of nuclei and/or chromatin, larger flasks containing 600 ml of media were inoculated with Ax₃ and harvested at the same cell density.

To study aggregrated cells, differentation was intiated according to the methods of Clark, *et al.*, (1980). Ax₃ amoebae, grown axenically, were washed twice with ice cold nutrient free buffer (NFB) containing per liter: 2.31g KH_2PO_4, 1g K_2HPO_4 and 0.5g $MgSO_4$ x $7H_2O$. Cell pellets were resuspended in cold NFB and applied at 0.2 ml cm² to nutrient free agar plates at a cell density of 10⁸ cells cm². The cells were spread evenly over the agar surface, first with the heel of a bent glass rod and then by shaking the plates. The plates were then incubated at 23° C for 10 to 12 hours. Aggregration was said to be completed when hemispherical mounds appeared on 90 percent of the agar surface. Aggregrated cells were harvested by washing the plates with cold NFB.

Isolation of Nuclei

Nuclei from vegetative and aggregrated Ax_3 amoebae were isolated according to the procedures of Charlesworth and Parish (1975) with some modifications. Harvested amoebae were washed once in phosphate buffer (0.01 M, pH 6.5) and then suspended in double distilled deionized water at a concentration of approximately 1×10^8 cells/ml. Suspended cells were shaken slowly for 30 minutes on a gyratory shaker. After shaking for the allotted time, cells were centrifuged at setting 6 on a Damon IEC centrifuge for ten minutes. The resulting pellet was suspended in cold SF buffer containing 0.05 M Tris pH 7.5, 2mM $CaCl_2$, 0.5 M sorbitol and 2.5% (w/v) ficoll. To this cell suspension was added an appropriate volume of 10% (v/v) aqueous solution of Triton-X-100 to give a final concentration of 0.1% Triton-X-100. This mixture was gently shaken at room temperature with cell lysis being constantly monitored by phase contrast microscopy. Cell lysis was normally complete within five to ten minutes. Aggregrated cells were sometimes difficult to lyze because of membrane alterations that occur when aggregrates are formed. By washing the cells once with 0.5 mM EDTA in 0.05 M Tris-HCl pH 7.5 before stirring them in water, cell lysis was accomplished using the above procedure (Charlesworth and Parish, 1977). To loosen nuclei entrapped within cellular debris, the cellular extract obtained from cell lysis was homogenized with three strokes of a homogenizer. This cell lysate was then filtered through two layers of miracloth (Calbiochem) to remove large debris. The cell filtrate was centrifuged at 1,000 g for 10 minutes at 4^0 C and the resulting pellet was suspended in cold SF buffer. This suspension was overlaid onto 25 ml of 0.5 M sucrose in 0.05 M Tris-HCl pH 7.5, 2 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and centrifuged at 1,000 g for 10 minutes. The pellet was resuspended in cold SF buffer and centrifuged through 25 ml of

0.5 M sucrose a second time. The supernatant was decanted and the pellet was suspended in 0.5 M sucrose buffer and centrifuged at 10,000 rpm for 10 minutes. The resulting pellet was air dried and then suspended in 2.3 M sucrose in 0.05 M Tris–HCl pH 7.5 and 0.01 M $MgCl_2$ by one stroke of the homogenizer. This suspension was overlaid on a 5 ml cushion of 2.3 M sucrose and centrifuged at 24,000 rpm for one hour using a SW25.1 rotor. The nuclei were collected from a ring–like pellet on the bottom of the centrifuge tube. The isolated nuclei were then washed twice with 0.25 M sucrose in 0.05 M Tris–HCl pH 7.5 and 0.01 M $MgCl_2$ (Buffer A) and then one final time in incubation buffer (62.5 mM Tris–HCl pH 7.9, 1.2 mM $MnCl_2$, 62.5 mM $(NH_4)_2SO_4$ and 2.5 mM B–mercapto–ethanol). The endogenous level of RNA polymerase activity was measured in nuclei immediately after isolation.

Attempts were also made to isolate nuclei from dormant spores of *Dictyostelium*. To break open the spore coat, numerous procedures (Appendix) were tried but little or no success was obtained in isolating intact nuclei.

Conditions for *in vitro* Incorporation

Endogenous RNA polymerase was routinely determined by measuring the incorporation of $\left[^3H\right]$–UMP into RNA (New England Nuclear 25.3 Ci/mmol) which is acid insoluble. The standard reaction mixture (250 ul total volume) contained: 150 ul of incubation buffer, 50 ul of 0.75 mM each of ATP, GTP and CTP suspended in incubation buffer, 1 uCi $\left[^3H\right]$–UTP and 50 ul of nuclear sample. Immediately before assaying, the nuclear sample was made 0.3 M with the addition of solid $(NH_4)_2SO_4$ (Cedar and Felsenfeld, 1973).

The incubation buffer and nucleotides were pre–incubated at 23.5^0 C for

10 minutes prior to the addition of nuclei. The assay was allowed to incubate an additional 15 minutes and then the reaction was terminated with 2 ml of ice cold 10% TCA containing 0.01 M pyrophosphate. The tubes were placed on ice for at least 20 minutes and then centrifuged at setting 6 of an IEC centrifuge for 10 minutes. The supernatant was decanted and the pellet was suspended in 200 ul of cold 0.2 N NaOH (Tsai, *et al.*, 1976). The acid insoluble material was reprecipitated by the addition of 2 ml of iced 10% TCA and allowed to incubate on ice for 20 minutes. The precipitate was collected on Whatman glass fiber filters (GF/C) and the filters were washed with approximately 40 ml of cold 10% TCA and then with one volume of ethanol. The filters were dried, placed in 5 ml of Scintisol (Isolab) and counted in a Beckman liquid scintillation counter.

Endogenous RNA polymerase activity in nuclei was also measured in the presence of 3 ug/ml and 33 ug/ml ∝-amanitin (Boehringer, Mannheim) (Yagura, *et al.*, 1976; Yagura, *et al.*, 1977). With these experiments, nuclei and ∝-amanitin were pre-incubated in the incubation buffer for 10 minutes. The addition of the nucleotides to each assay initiated the reaction which was terminated at the end of 15 minutes. This allowed total RNA polymerase activity to be classified into the three forms of the enzyme.

Extraction and Determination of DNA

DNA was routinely extracted from isolated nuclei. A 200 ul sample of nuclei was precipitated with an equal volume of 10% TCA containing 0.01 M pyrophosphate. This was allowed to sit on ice for 20 minutes and then centrifuged at setting 6 in a bench top centrifuge for 10 minutes. The supernatant was aspirated and the pellet was suspended in 500 ul of 0.5 M PCA. The sample was then placed in a waterbath at 80° C for 30 minutes with

constant stirring. After the 30 minute incubation, the nuclear extract was centrifuged for 10 minutes and the supernatant was removed and stored. The pellet was resuspended in 500 ul of fresh 0.5 M PCA and re-extracted a second time for 30 minutes at 80° C. The extract was again centrifuged and the resulting supernatant was pooled with the previous one obtained. DNA present in the pooled supernatants was measured with the diphenylamine reaction of Burton (1956) using an incubation of 20 hours at 30° C. A standard of calf thymus DNA was prepared by dissolving highly polymerized DNA in 5 mM NaOH. Immediately before the assay, a DNA standard was hydrolyzed with an equal volume of 1 M PCA for 15 minutes at 70° C.

Preparation of Chromatin

The nuclear pellet obtained after ultracentrifugation was washed 2 times with Buffer A at 10,000 rpm for 10 minutes. The pellet was then washed 2 more times with a 0.14 M NaCl solution. The final pellet obtained was suspended in 15 ml iced, deionized, double distilled water and placed on ice for 20 minutes. At the end of the incubation period, all nuclei had lysed as determined by phase contrast microscopy. The chromatin preparation was centrifuged at 10,000 rpm for 10 minutes and the pellet obtained was dried and suspended in an appropriate volume of incubation buffer. A 200 ul aliquot was removed to determine the amount of DNA in the sample. In addition, a 10 ul sample was diluted to 1 ml in a nuclear suspension solution (0.1 M NaCl and 0.01 M EDTA) and an $OD_{260/280}$ reading was taken to estimate DNA content (Schleif, *et al.*, 1981). This estimation of DNA allowed the RNA polymerase assay using the isolated chromatin as template to be run immediately after preparation. Values obtained were corrected for DNA content after the diphenylamine reaction had been completed.

DNA Fragmentation

Alkaline sucrose density centrifugation gradients were used to determine the degree of DNA fragmented during chromatin preparation. Gradients were prepared using the methods of Jackowski and Kim (1981). A linear gradient consisting of 5 to 20% sucrose in 0.4 M NaOH, 0.01 M EDTA and 0.1 M NaCl (Solution A) was overlaid onto a 5 ml cushion of 60% sucrose in Solution A. A lysis solution (0.05 M NaOH, 0.02 M EDTA and 0.1% Nonidet P40) was then layered over the gradient followed by a 500 ul nuclear suspension solution containing approximately 5-10 OD_{260} units of either a nuclear or chromatin preparation. After 15 minutes of lysis at 4^0 C, the gradients were centrifuged 17 hours at 24,000 rpm using a SW25.1 rotor. After ultracentrifugation, 1.2 ml fractions were collected from the gradient and an OD_{260} was measured on each fraction. Electrophoretically pure BSA (5 mg/ml) and IgG (1 mg/ml) were also used as protein markers on digesting gradients. 500 ul of both BSA and IgG were layered over a gradient and centrifuged at 24,000 rpm for 17 hours. OD_{280} readings were taken from 1.2 ml fractions collected from the gradient after ultracentrifugation.

Measurement of Template Activity and Binding Capacity

The template activity and binding capacity of prepared chromatin was measured by the incorporation of $[^3H]$-UTP into acid insoluble material by *E. coli* RNA polymerase (Boehringer Mannheim). To measure template activity, *E. coli* RNA polymerase at a concentration of 5 ug/assay was pre-incubated with 5 ug of chromatin in 200 ul of incubation buffer at 37^0 C for 40 minutes (Tsai, *et al.*, 1976). The reaction was initiated by the addition of 50 ul of a nucleotide solution containing 0.75 mM each of ATP, GTP and CTP, and 1 uCi of $[^3H]$-UTP. After a 15 minute

incubation at 37° C, 100 ug of BSA was added to each tube and the reaction was immediately terminated with 2 ml of 10% TCA containing 0.01 M pyrophosphate. The binding capacity of the chromatin preparation for *E. coli* RNA polymerase was measured using the same assay as above except rifampicin (40 ug/ml) and heparin (800 ug/ml) were included simultaneously with the nucleotide solution (Tsai, *et al.*, 1975). The reactions were terminated after a 15 minute incubation by the addition of 10% TCA. TCA insoluble material was allowed to precipitate on ice overnight. Samples were washed and filtered using the same procedure as for the measurement of endogenous RNA polymerase activity in isolated nuclei.

Isolation of RNA polymerase from *Dictyostelium discoideum*

Attempts were made to isolate and purify RNA polymerase from vegetatively growing Ax_3. Procedures utilized are discussed in the Appendix.

RESULTS

Isolation of Nuclei

Nuclei can be readily isolated from both vegetative and aggregrated Ax_3 cells. Cell lysis with a 0.1% solution of Triton-X-100 occurred within 5 to 10 minutes at 23° C with little or no visible damage to nuclei. Both the temperature and the cell concentration of Ax_3 affected the rate of cell lysis. Pederson (1977) found lysates of *Dictyostelium* amoebae to contain potent DNases and therefore, recommended that cell lysis should occur at 4° C using a 0.5% (v/v) Triton-X-100 solution. No evidence of nuclei damage by DNase was seen during preparation and attempts to lyse Ax_3 at 4° C required 25 to 30 minutes to achieve 90% lysis. In addition, nuclei isolated by a solution containing 0.5% or more of Triton-X-100 resulted in nuclei which were less active in RNA synthesis (Marzluff, W.F., *et al.*, 1973). Charlesworth and Parish (1975) reported that isolating nuclei using SF buffer and 0.1% Triton-X-100 resulted in intact nuclei free from cytoplasmic contamination. The use of this procedure found nuclei remained within the cytoplasmic skeleton or that fragments of cytoplasm became associated with nuclei as they were released from lyzing cells. Homogenization of the nuclear preparation, released nuclei from cellular debris. Microscopic examination of the nuclei with Methylene Blue stain revealed that these nuclei remained intact during homogenization. The addition of this step resulted in the isolation of clean nuclei as determined by the protein to DNA ratios (Table 1). Protein to DNA ratios from isolated nuclei and chromatin are consistent with values obtained in *Dictyostelium* (Bakke, 1977) and in other eukaryotes. In both vegetative and aggregrated cells, the amount of DNA extracted from nuclei varied from 244 ug/10^{10} to 353 ug/10^{10} cells.

Table 1: Protein/DNA ratios of isolated nuclei and chromatin from Ax$_3$.

Cell Type	Protein (ug/ml)	DNA (ug/ml)	Ratio Protein/DNA
Vegetative cell			
Nuclei	616.5	276.5	2.2
Chromatin	179.5	96.0	1.87
Aggregrated cell			
Nuclei	1038.0	293.5	3.5
Chromatin	187.5	110.7	1.69

Purification of nuclei and chromatin isolated from vegetatively growing and aggregrated cells of Ax$_3$. Protein was determined by Bradford using γ-globulin as standard (Bradford, 1976). DNA was extracted (see Methods and Materials) and then measured using the diphenylamine reaction and calf thymus DNA as standard (Burton, 1956). The data presented here is indicative of a single isolation of either nuclei or chromatin. Protein and DNA values obtained were normalized for 10^{10} Ax$_3$ cells.

Yield of nuclei isolation from whole cells was relatively low but the minimal protein contammination in the final preparation compensated for this loss.

Conditions of RNA Synthesis by Endogenous RNA polymerase Activity in Isolated Nuclei

Figure 2 demonstrates the kinetics of $[^3H]$–UTP incorporation into RNA using the assay system described in Methods and Materials. From this figure, an almost linear rate of incorporation of label into RNA occurred within the first 15 minutes of incubation, followed by a slower rate of incorporation. Therefore, all following incorporation reactions were incubated for 15 minutes at 23° C in contrast to the 40 minute incubation utilized for assaying RNA polymerase in *Dictyostelium* by Soll and Sussman (1973), Pong and Loomis (1973), Yagura, *et al.*, (1976) and Takiya, *et al.*, (1980).

The assay utilized for measuring RNA polymerase activity contained minimal salts. RNA synthesis has an absolute requirement for the presence of divalent cations. RNA polymerase assay solutions usually contain both Mn^{+2} and Mg^{+2}. However, under our conditions, RNA polymerase activity was optimal and required only the presence of 1.2 mM $MnCl_2$ (Tsai, *et al.*, 1975). Solutions containing low $MnCl_2$ concentrations, in the absence of Mg^{+2}, increase the solubility of the nuclei (Cedar and Felsenfeld, 1973).

Isolated nuclei were examined for their endogenous RNA polymerase activity during two stages of development. To isolate nuclei from aggregrated cells, cells were vigorously vortexed to disaggregrate clumps and suspend individual amoebae. Alton and Lodish (1977) demonstrated that synthesis of aggregration specific sequences required continuous cell to cell contact. Suspension of late aggregration cells for 5 hours in liquid media results in the loss of characteristic aggregration proteins. In these experiments, when isolating

Figure 2: The kinetics of endogenous RNA polymerase
activity in isolated nuclei from *D.*
discoideum.

Incorporation of $[^3H]$ –UTP into RNA was
measured in nuclei containing endogenous RNA
polymerase. Triplicate assays using 14.2 ug
DNA/assay were terminated at the various time
intervals with 10% TCA containing 0.01 M pyro-
phosphate. Assays were incubated at 0 ° C
(□) and 23 ° C (O). Incorporation of
$[^3H]$ –UTP is expressed as DPM x 10^5/
mg DNA.
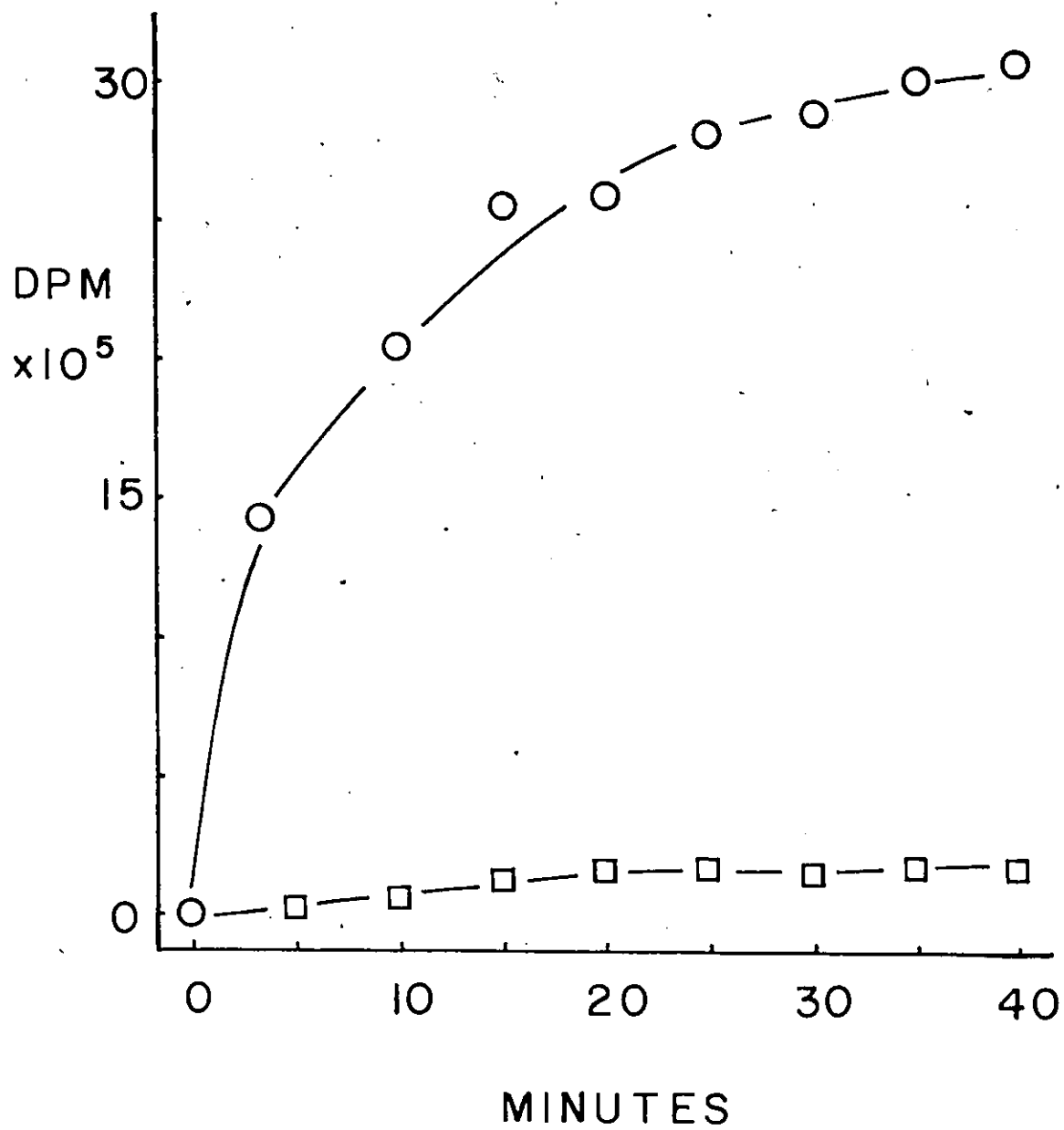
nuclei from aggregated cells, the cells were held in suspension no longer than 30 minutes. Therefore, the assumption is made that these cells still synthesize and possess aggregration specific sequences. This is supported by the fact that the average half life of mRNA during this stage of development is approximately 220 minutes (Margolskee and Lodish, 1980; Ennis, 1981). Nuclei were made 0.3 M with $(NH_4)_2SO_4$ immediately prior to assaying,

giving a final concentration of 0.362 M $(NH_4)_2SO_4$.

Increasing the salt concentration is thought to play a dual role when measuring endogenous RNA polymerase activity in nuclei. First, 0.4 M $(NH_4)_2SO_4$ concentrations in conjugation with low $MnCl_2$

further increase the solubility of the chromatin by dissociating the proteins associated with maintaining its structural conformation (Cedar and Felsenfeld, 1973). In addition, high salt concentrations removes any non-initiated RNA polymerase off the DNA. Those enzyme molecules associated with the DNA in a stable initiation complex remain intact; however, re-initiation is severly inhibited by high $(NH_4)_2SO_4$ concentrations when these sites are

later made available (Hyman and Davidson, 1970; Cedar and Felsenfeld, 1973). Table 2 shows the RNA polymerase activity obtained in isolated nuclei from vegetative and aggregated cells. In these experiments, endogenous activity of RNA polymerase was found to be higher in nuclei isolated from aggregated rather than vegetatively grown cells. This difference in endogenous RNA polymerase activity was found to be significant by one way analysis of variance with a F-ratio of 35.2. The enzyme activity was also measured in the presence of 3 ug/ml and 33 ug/ml α-amanitin. By definition, RNA polymerase I activity is resistant to α-amanitin, RNA polymerase II is sensitive to low concentrations of α-amanitin (3 ug/ml) while RNA polymerase III is sensitive to

Table 2: Endogenous RNA polymerase activity in isolated nuclei from vegetative and aggregrated cells of *Dictyostelium*.

ACTIVITY OF RNA POLYMERASE	VEGETATIVE NUCLEI (DPM/mg DNA)	AGGREGRATED NUCLEI (DPM/mg DNA)
Total RNA polymerase Activity	8.46×10^6 +/− $.27 \times 10^6$	1.20×10^6 +/− $.03 \times 10^6$
α−amanitin resistant RNA Polymerase Activity	5.62×10^5	2.42×10^5
α−amanitin sensitive (3 ug/ml) RNA Polymerase activity	1.74×10^5	8.49×10^5
α−amanitin sensitive (33 ug/ml) RNA Polymerase activity	1.10×10^5	1.06×10^5

50 ul of nuclear preparations, (containing approximately 8 to 15 ug DNA) were incubated in the presence and absence of either 3 ug/ml and 33 ug/ml α−amanitin. The nuclear preparation in the presence of α−amanitin were allowed to pre−incubated for 10 min. at 23° C with the reaction being initiated by the addition of the nucleotide triphosphates. Assays were incubated for 15 min. with the reaction being terminated with 10% TCA. The data presented here is the mean value obtained for three separate isolations of both vegetative and aggregrated nuclei. Analysis of variance of RNA polymerase activity between vegetative and aggregrated nuclei is presented in Appendix II.

relatively high concentrations of α-amanitin (33 ug/ml). According to this sensitivity, total RNA polymerase activity can be divided into the three forms of the enzyme. In vegetative nuclei, RNA polymerase I contributes approximately 66%, RNA polymerase II contributes 20.6% and RNA polymerase III contributes 12.9% of the total enzyme activity. However, in aggregrated nuclei, RNA polymerase I, II and III are 20.1%, 70.7% and 8.8% of the total enzyme activity, respectively. Statistically significant differences in RNA polymerase I and II activity occurred between vegetative growth and aggregration. This decrease in the activity of RNA polymerase I from vegetative growth to aggregration coincides with a similar decrease in rRNA synthesis (Yagura, *et al.*, 1982). Likewise, the increase in RNA polymerase II activity occuring during the same stages of development is accompanied by the simultaneous increase in messenger-like RNA (mRNA) (Yagura, *et al.*, 1982).

Isolation of Chromatin

Previous techniques to isolate chromatin from *Dictyostelium* nuclei have involved mechanical disruption either by sonication or homogenization and enzymatic digestion (Pederson, 1977; Kawashima, 1979; Bakke, *et al.*, 1979). All these procedures result in fragmentation or nicking of the isolated DNA. Fragmentation of the DNA is known to stimulate RNA polymerase activity presumably by increasing the ability of the enzyme to bind and to initiate non-specific RNA synthesis (Jackowski, *et al*, 1981).

Therefore, it was necessary to obtain a procedure which isolated pure chromatin with little or no fragmentation of DNA. Osmotic lysis of nuclei by low salt washes and then suspension in iced water was the method of choice. As seen from Table 1, protein to DNA ratios of isolated chromatin were less than 2:1,

suggesting that the chromatin obtained was relatively pure. Chromatin isolation from nuclei as measured by DNA extraction revealed only 18.1 to 29.2% yeild. The loss of DNA during chromatin isolation was due to the washing procedure as well as the "sticky" nature of the chromatin. Although all washes were done in plastic centrifuge tubes and transfers were made with plastic tips, nuclei and chromatin particulates could be seen adhering to the walls of both tubes and tips.

To determine whether the isolation of chromatin from intact nuclei resulted in variable degrees of DNA nicking; samples were denatured on alkaline surcrose density gradients. Figure 3 demonstrates the digestion profile obtained from whole nuclei and chromatin prepared from both vegetative and aggregrated cells. Whole nuclei and chromatin prepared from both vegetative and aggregration cells revealed identical profiles, suggesting that the chromatin was not subjected to excessive fragmentation during the isolation procedure. Molecular weight standards run on digesting gradients under similar conditions revealed that the bulk of DNA fragments from nuclei and chromatin had a molecular weight less than 68,000 daltons. The tailing which appeared at the end of each profile was due to the high sucrose concentration present in the final fractions.

Total Template Activity and Binding Capacity of *Dictyostelium* Chromatin

Isolated chromatin from vegetative and aggregration cells were used as template for *E. coli* RNA polymerase. To insure that RNA synthesis occurred immediately upon the addition of nucleotides, chromatin and enzyme were pre-incubated for 40 minutes. Pre-incubation allows RNA polymerase to form stable enzyme-chromatin complexes capable of supporting RNA synthesis (Tsai, 1973). Binding of RNA polymerase to the template is thought

Figure 3: The fractionation of isolated nuclei and
chromatin on alkaline sucrose digesting
gradients.

Alkaline sucrose digesting gradients of
nuclei and chromatin from vegetative (A)
and aggregration (B) cells. Over 500 ug
of nuclear preparation was placed on the
digesting gradient. Gradients were cent-
rifuged at 24,000 rpm for 17 hrs. at 4^0C
1.2 ml fractions were collected from the
gradients. and the amount of DNA (ug) in each
fraction were determined by an OD_{260}

measurement using calf thymus DNA as standard.
(O) Nuclei run on digesting gradient.
(□) Chromatin run on digesting gradient.
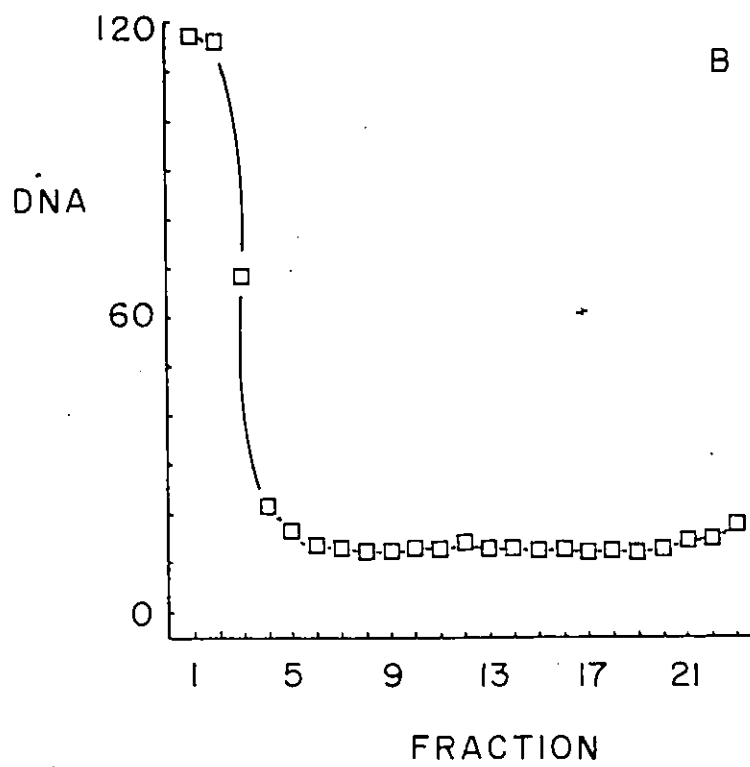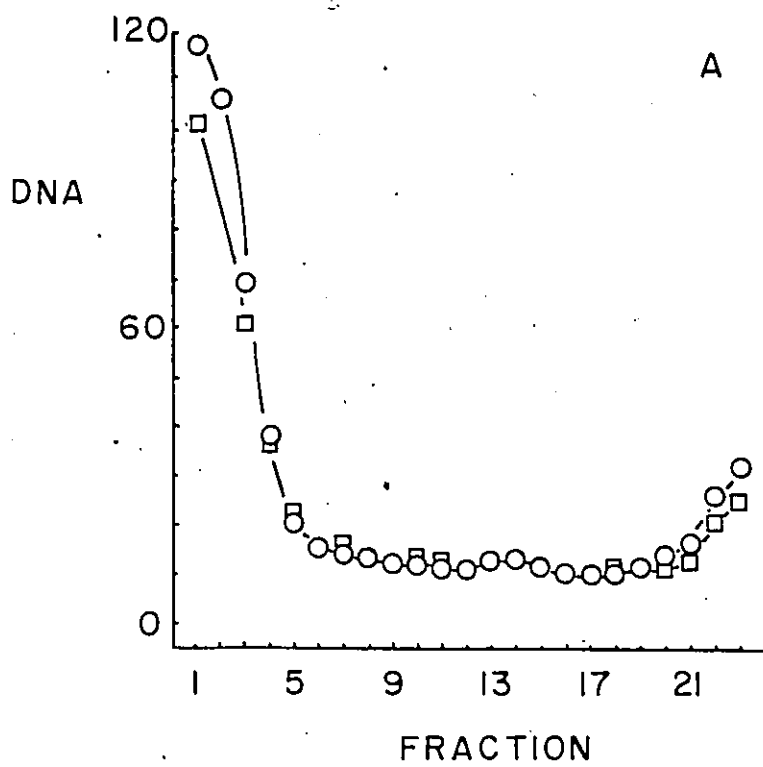
to involve a conformational change to form a stable enzyme–DNA complex. Pre-incubation times vary between 10 to 40 minutes depending upon the chromatin source (Warnick, *et al.*, 1983; Zech, *et al.*, 1981; Tomi, *et al.*, 1981; Tsai, 1973; Tsai, 1976). Since a 40 minute pre-incubation was maximal for RNA polymerase binding to the chromatin, it was utilized in these experiments. Also, template binding capacity assays were run under conditions where enzyme was capable of saturating the template. Therefore, 5 ug of RNA polymerase was pre-incubated with 2 to 5 ug of isolated chromatin. To determine whether endogenous RNA polymerase complexed with the chromatin remained active after the isolation procedure, aliquots of chromatin were assayed under identical conditions in the presence of nucleotides alone and no exogenous addition of polymerase. Incorporation of $[^3H]$–UTP under these conditions were minimal.

The incorporation of $[^3H]$–UTP into RNA by *E. coli* RNA polymerase on vegetative and aggregrated chromatin is tabulated in Table 3. As demonstrated, the total template activity as measured by the incorporation of label into RNA is significantly higher on chromatin isolated from vegetative rather than aggregrated cells (Appendix II). In addition, the incorporation of $[^3H]$–UTP into RNA was measured in the presence of rifampicin and heparin. As previously mentioned, rifampicin prevents RNA polymerase from initiating RNA synthesis unless the RNA polymerase is associated with the DNA in a stable conformation. Heparin functions to destablized enzyme that is not complexed to the DNA in the proper conformation (Wilson, 1982) and inhibit the activity of any ribonucleases present in chromatin preparations. Therefore, the incorporation of label seen to occur in the presence of rifampicin and heparin may reflect the availability of the template for stable RNA polymerase

Table 3: The template activity and binding capacity of chromatin isolated from vegetative and aggregated cells of Dictyostelium for E. coli RNA polymerase.

Chromatin	Template Activity Incorporation of [3H]-UTP into RNA DPM/mg DNA	Binding Capacity Rifampicin and Heparin resistant incorporation of [3H]-UTP into RNA DPM/mg DNA	% Incorporation resistant to Rifampicin and Heparin
Vegetative	2.76×10^6 $\pm .37 \times 10^6$	1.58×10^6 $\pm .25 \times 10^6$	57.3
Aggregration	8.70×10^5 $\pm .09 \times 10^5$	2.65×10^5 $\pm .32 \times 10^5$	30.4

The results presented here are the data obtained from four separate chromatin isolations from vegetative and aggregation nuclei. Triplicate assays containing 2-5 ug chromatin were incubated with 5 ug E. coli RNA polymerase in the presence or absence of rifampicin (40 ug/ml) and heparin (800 ug/ml). The enzyme and chromatin were pre-incubated for 40 minutes at 37°C and the reaction was initiated by the addition of either nucleotides or the simultaneous addition of nucleotides, rifampicin and heparin. Reactions were terminated after incubating an additional 15 minutes (see Methods). Endogenous RNA polymerase activity on isolated vegetative and aggregration chromatin routinely gave about 50 CPM and therefore were used as background levels. Analysis of variance of template activity and binding capacity between vegetative and aggregrated chromatin is presented in Appendix II.

binding complexes. Again, the incorporation of $[^3H]$-UTP in the presence

of rifampicin and heparin is significantly higher with vegetative-chromatin

rather than aggregrated (Appendix II). Likewise, it appears that 57.3% of the

activity represents RNA synthesized by enzyme in proper conformation vegetative

chromatin. This compares to only 30.4% of the activity representing RNA

synthesized by stable RNA polymerase–aggregration chromatin complexes. The

standard deviations obtained for the activity of incorporation of label into

RNA by vegetative and aggregration chromatin were between 13–16% and 1–12%,

respectively. Although the standard deviations were a little high using the

data obtained for vegetative chromatin, the trend demonstrated by the %

incorporated in the presence of rifampicin and heparin was consistant

throughout the separate isolation.

DISCUSSION

In recent years, in vitro studies of nuclei have been done to yield valuable information concerning in vivo controls of transcription. Solubilization of nuclei by high salt concentrations may enhance transcription by endogenous RNA polymerase. Nuclei isolated from vegetative and aggregated cells of *Dictyostelium* were examined for levels of endogenous RNA polymerase activity. Soll and Sussman (1973) found that the incorporation of $[^3H]$-UTP into RNA in vegetative nuclei was approximately twice the amount of label incorporated by nuclei isolated from cells 5 hours into the developmental sequence. However, in the present study, results showed that nuclei isolated from late aggregation cells (10 – 12 hrs.) incorporated slightly more $[^3H]$-UTP than nuclei from vegetative cells. There is two factors which support the idea that the incorporation of label into RNA should be fairly consistent in nuclei isolated from both vegetatively growing and late aggregation cells. First, the experimental conditions utilized, solubilized the nuclei for maximal transcriptional activity by RNA polymerase. However, use of such a high salt concentration does not ensure optimal expression of any form of RNA polymerase in *Dictyostelium*. Partially purified RNA polymerase I, II and III in *D. discoideum* function optimally at about 0.12 M, 0.17 M and 0.21 M $(NH_4)_2SO_4$ (Takiya, *et al.,* 1980). Since nuclei containing the enzyme from both stages of development were subjected to identical assay conditions, distinct differences obtained in the activities of the various forms of the enzyme and not in the overall activity could be demonstrated. Comparison of incorporation of label into RNA by nuclei under our assay conditions with those obtained by Soll (1973) could not be determined since their activity was reported as CPM/10^7 nuclei. Second,

although the total RNA content of *Dictyostelium* decreases during

development (White and Sussman, 1961) transitions occur in the rate of both

rRNA and mRNA synthesis. A majority of the RNA present and synthesized in

vegetative cells is rRNA (Loomis, 1975). However, as cells proceed into the

developmental cycle, rRNA content is found to decrease and although some *de*

novo synthesis of rRNA occurs, the rate of synthesis is also decreased

(Cocucci and Sussman, 1970; Yagura, *et al.*, 1982). Blumberg and Lodish

(1980) studied the polyadenylated mRNA population during differentiation. They

found that there is only one time in the developmental program when expression

of a large number of new genes is known to occur. This period occurs between 6

and 13 hours following the initiation of development, just prior to the onset

of spore—stalk cell differentiation. By cDNA hybridizations, it was determined

that over 7,000 genes are expressed at 13 hours into development, revealing a

burst of increased expression, from only approximately 4,800 genes expressed at

6 hours to a final 7,600 genes at the end of development (Blumberg, *et*

al., 1980). Cells late in aggregration (10 – 12 hrs), then, are expected to

be active in synthesizing mRNA. Therefore, the overall activity of RNA

polymerase may be similar within the isolated nuclei from vegetative and

aggregrated cells under our transcribing conditions. Changes in the activity

of the various forms of RNA polymerase throughout development is indeed more

likely. Using a concentration of 10 ug/ml —amanitin, Soll and Sussman (1973)

demonstrated alterations in the ratio of activity resistant- and sensitive to

the toxin during development. The relative proportion of incorporation

resistant and sensitive to ∝–amanitin in nuclei from vegetative cells decreased

from approximately 50:50 to 30:70 in nuclei from early aggregration cells.

However, by using one concentration of ∝–amanitin, only RNA polymerase II

activity which is sensitive to low concentrations of the toxin can be separated

from RNA polymerase I which is resistant to the toxin and RNA polymerase III

which is sensitive to only high concentrations. Incubating nuclei from

Dictyostelium in the presence and absence of 3 ug/ml and 33 ug/ml α-

amanitin, all three forms of the enzyme can be detected (Yagura, et al.,

1976; Yagura, et al., 1977). The ratio of activity of RNA polymerase

I:RNA polymerase II:RNA polymerase III in vegetative nuclei was 66:21:13,

respectively, as compared to 20:71:9, respectively, in aggregrated cells. This

demonstrates, then, that there is a major transition in RNA polymerase

activity during development; RNA polymerase I is most active during vegetative

growth while RNA polymerase II is most active during aggregation. Decreases

in the activity of RNA polymerase I with a simultaneous increase in the

activity of RNA polymerase II is also seen to occur with the activity of

partially purified enzyme isolated from high salt extracts of

Dictyostelium during the same stages of development (Yagura, et

al., 1977; Yagura, et al., 1982). This trend also parallels cellular

events where rRNA synthesis by RNA polymerase I is known to decrease during

development. Likewise, new gene expression occurring during this time would

reflect increases in RNA polymerase II activity (Firtel, 1972; Alton and

Lodish, 1977; Blumberg and Lodish, 1980). Little work has been done on the

modulation of partially purified RNA polymerase III during development because

of difficulties in detecting its activity due to the small amount present in

relative proportions to the other two forms of the enzyme. From our results,

there is a small decrease in RNA polymerase III activity between vegetative

growth and aggregration but this decrease is not significant by one way

analysis of variance (Appendix II). This is not surprising since it is thought

that RNA polymerase III is responsible for the synthesis of the 5S rRNA and tRNA. The reduction of rRNA synthesis occurring during aggregration may also decrease the requirement for the synthesis of the 5S rRNA. Therefore, although there are definite changes in the activity of the forms of RNA polymerase, the overall activity of the enzyme may be constant during development.

Chromatin was isolated from two stages of development in *Dictyostelium* where differential expression of the genome is known to occur (Blumberg and Lodish, 1980). It is widely believed that proteins associated with the chromatin play a major role in limiting the template activity of the DNA to which they are bound (Lilley, 1979). To establish whether the chromatin and associated proteins of *Dictyostelium* are capable of regulating differential gene expression, it must first be determined whether there are any alterations in template activity of the chromatin during these times of differential gene expression. *In vitro* transcription systems utilizing isolated chromatin and exogenous RNA polymerase have been developed to determine the capacity of chromatin to function as a template and synthesize RNA transcripts.

In our experiments, isolated chromatin was transcribed by *E. coli* RNA polymerase. Overall template capacity of the chromatin for *E. coli* RNA polymerase was measured. It was determined that chromatin isolated from vegetative nuclei has a higher template capacity than chromatin isolated from aggregrated nuclei. Because *E. coli* RNA polymerase can non-specifically bind to a template, stable complexing of the enzyme must be distinguished from random binding. Pre-incubation of *E. coli* RNA polymerase with *Dictyostelium* chromatin allows stable pre-initiation complexes to form. RNA synthesis is then initiated by the simultaneous addition of nucleotides and

rifampicin (Tsai, *et al.*, 1975). RNA polymerase present in a stable
initiation complex will initiate the synthesis of a RNA chain while RNA
polymerase randomly bound to the template is readily displaced. Rifampicin
prevents re-initiation of displaced polymerase (Tsai, *et al.*, 1975).
Chromatin isolated from vegetative cells was also seen to exhibit higher
binding capacity for *E. coli* RNA polymerase than chromatin isolated from
developing cells. Taken literally, this suggests that vegetative chromatin may
have more available binding sites for the enzyme than aggregration chromatin.
However, this statement should be tempered with the fact that a large amount of
controversy exists concerning the ability of *E. coli* RNA polymerase to
efficiently transcribe eukaryotic templates. Tsai (1976) compared initiation
of RNA synthesis on oviduct chromatin by hen oviduct RNA polymerase
II and *E. coli* RNA polymerase. He demonstrated that the level of
initiation sites as measured by either homologous or heterologous polymerases
followed a similar pattern. In addition, comparison of the initiation sites
utilized by these enzymes revealed that these enzymes compete with each other
for the same initiation sites (Tsai, *et al.*, 1976). Similar results
using *E. coli* RNA polymerase to transcribe mammalian chromatin have been
demonstrated (Axel, *et al.*, 1973; Gilmour and Paul, 1971; Steggles, *et
al.*, 1974). In contrast, however, Palmiter and Lee (1980) found that *E.
coli* RNA polymerase was not able to transcribe ovalbumin genes in nuclei or
chromatin as efficiently as endogenous enzyme. Likewise, comparison of
initiation of cauliflower and pea RNA polymerase II with *E. coli* RNA
polymerase on pea chromatin have revealed that the two eukaryotic enzymes
compete with each other for the same recognition sites on the chromatin whereas
the prokaryotic enzyme utilized different sites (Tomi, *et al.*, 1981).

Tomi (1981) suggests that it is the proteins associated with the chromatin which are responsible for the interaction with the eukaryotic polymerase and not with the bacterial enzyme. He concluded this since all three enzymes were able to compete for the same recognition sites on pea DNA with equal efficiency (Tomi, 1981). The basis of these studies in isolating chromatin and adding back the enzymes required for RNA synthesis is to gain insight into how the chromatin structure regulates transcription. If it is supposed that proteins on the eukaryotic chromatin can efficiently interact with only eukaryotic enzyme, the efficiency of utilizing $E.$ $coli$ RNA polymerase in these studies is greatly reduced.

In eukaryotes, transcription of active chromatin by either RNA polymerase I or II may be subjected to different control mechanisms. Active chromatin transcribed by RNA polymerase II when viewed by the electron microscope have been shown to possess a compact structure of nucleosome–like beads (Lilley and Pardon, 1979). Immunological studies demonstrated that histones and/or modified histones are contained within these nucleosome–like particles. This suggests that active genes transcribed by RNA polymerase II are continually associated with histones or modified histones (Lilley, 1979). Variable results suggesting that either nucleosome structure remains intact or that it is disrupted beyond recognition during transcription exists (Weisbrod, 1982; Levy and Noll, 1981). A most recent study by Baer and Rhodes, (1983) have confirmed that RNA polymerase II interacts with the actively transcribing nucleosome core, however, analysis of these cores suggests that histones 2A and 2B are displaced when the enzyme binds. The remaining histones present in the nucleosome core continue to be associated to the DNA during the binding of RNA polymerase II (Baer and Rhodes, 1983). Active transcription of

chromatin by RNA polymerase I, however, has demonstrated that transcribing ribosomal genes are in an extended configuration (Scheer, 1980). Labhart and Koller (1982) recently examined transcribing ribosomal chromatin in *Xenopus* oocytes using the E.M. and found that it was indistinguishable from free DNA, implying that the chromatin may be lacking all proteins not directly involved in transcription. This experiment was conducted at various ionic strengths and pH values since it has been suggested that contaminating protein may adhere to DNA during preparation for E.M. studies (Labhart, 1982). Micrococcal nuclease digestion of rDNA, however, has generated repeating patterns when electrophoresed suggesting nucleosome arrangement of DNA (Mathias, 1976; Lilley, 1979). Inactive genes in a highly reiterated gene family such as the rDNA may account for nucleosome structure detected by micrococcal nuclease (Lilley, 1979; Ness, 1983).

In *Dictyostelium* rDNA exists as extrachromosomal pallindromic dimers contained in about 90 copies within the nuclei (Cockburn, *et al.*, 1978). The number of copies of rDNA remains constant throughout development (Cockburn, *et al.*, 1978) although changes in rRNA synthesis occur. Ness *et al.*, (1983) studied the chromatin structure of the rDNA during development using the E.M. and micrococcal nuclease and restriction endonuclease digestion. All three studies revealed that active rDNA is in an extended configuration. Micrococcal nuclease digestion of rDNA from vegetative amoebae revealed that there was no regular repeating units in the coding region (Ness, 1983). While nucleosome structure is known to protect DNA against restriction endonucleases, analysis of rDNA from vegetative cells revealed extensive digestion when exposed to restriction enzymes (Ness, 1983). rDNA from *Dictyostelium* slugs, where rRNA synthesis occcurs at a low rate was

also examined. Micrococcal nuclease and restriction endonuclease digestion of this rDNA resulted in a repeating pattern which suggests that a majority of the rDNA was compacted into an inactive form (Ness, 1983).

The data obtained from this present study revealed that vegetative chromatin as compared to aggregration chromatin had a higher template capacity and binding ability for *E. coli* RNA polymerase. Even accounting for the non-specific binding which the prokaryotic enzyme may display for eukaryotic templates, the higher activity obtained with vegetative chromatin may reflect the extended nature of the chromatin. Chromatin isolated from vegetative nuclei should be capable of synthesizing abundant rRNA (Yagura, 1982) and therefore, exhibit a more extended configuration as described by Ness (1983). One characteristic distinguishing prokaryotic and eukaryotic chromatin is the nature of histones association with DNA template (Lilley, 1979). Although prokaryotic DNA is normally coated with basic proteins, this binding is very easily disrupted. In contrast, however, eukaryotic histones are usuallly tightly complexed to the DNA giving the characteristic beaded appearance. Histone depleted chromatin, such as, active rDNA in *Dictyostelium*, may readily bind *E. coli* RNA polymerase and either specifically or non-specifically initiate transcription. Although E.M. and digestion studies of rDNA in *Dictyostelium* reveal an extended configuration (Ness, *et al.*, 1983), no evidence for complete histone loss can be ascertained. However, Parish (1980) has demonstrated by electrophoretic analysis of nucleosomes from nucleoli that histone proteins are more readily lost from the DNA of nucleoli than the DNA of nuclei. This suggests that the histones in nucleoli are associated more weakly. Decrease in the template and binding capacity of prokaryotic enzyme demonstrated with aggregation chromatin may

reflect compaction of the rDNA into an inactive form. The low template and binding activity demonstrated with aggregration chromatin, however, has to be reconciled with the increase in new gene expression occurring at this time. Cockburn (1979) reports that about 17% of the nuclear DNA is rDNA while single copy DNA sequences are thought to represent 70% of the genome (Firtel, 1976) of which about 80% is being expressed during late aggregration as mRNA (Blumberg, 1981). It was expected that this large amount of synthesis occurring normally within the cell, would be reflected by the accessibility of isolated chromatin to bind RNA polymerase. As demonstrated, approximately 70% of the endogenous RNA polymerase activity in isolated nuclei was RNA polymerase II, reflecting a substantial amount of transcriptional capability for single copy sequences. However, in the transcription system using *E. coli* RNA polymerase and aggregration chromatin revealed both low template and binding capacity. Since it is known that active chromatin transcribed by RNA polymerase II requires continual association with either histones or modified histones (Tomi, 1981; Weisbrod, 1982; Lilley, 1987), we suggest that these histones play an important regulatory role in this transcription. *E. coli* RNA polymerase may lack the specificity to recognize the histones associated with the true binding sites on aggregration chromatin (Tomi, 1981). The regulation of chromatin activity by histones and the decrease in accessibility of the rDNA during late aggregration may account for the low template activity detected.

This investigation was a preliminary study to determine whether chromatin isolated from two developmental stages of *Dictyostelium* displayed any distinctions in template capacity and initiation of RNA synthesis. It can be concluded that there are indeed differences in both the template capacity and

binding of *E. coli* RNA polymerase to chromatin from vegetative and aggregrated cells. It is suggested that these apparent differences are due to regulatory mechanisms which are contained within the chromatin structure. Analysis of the size and nature of the RNA transcripts produced under our transcription conditions, would substantiate this hypothesis. Although transcription of *D. discoideum* chromatin occurred using *E. coli* RNA polymerase, an even higher efficiency of transcription would be expected using an eukaryotic enzyme. The importance of histone regulation in transcription by either aiding in RNA polymerase binding at the appropriate initiation site or by directly allowing the DNA to unwind is for future studies to ascertain.

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APPENDIX

Original attempts to isolate RNA polymerase were done to determine whether the three forms of the enzyme were present in the dormant spore of *Dictyostelium*. If the dormant spore contained the three forms of the enzyme, the activities of RNA polymerase I, RNA polymerase II and RNA polymerase III were to be monitored during the germination process. To establish that the isolation procedure yielded the three forms of the enzyme, all preliminary work was done on Ax_3, which is known to contain all forms of the enzyme. Although Ax_3 is easily cultured and harvested, cell concentrations had to remain within an employable range when routinely isolating the enzyme from dormant spores. In addition, Ax_3 had to be subjected to harsh breakage treatments. The dormant spore is contained within a rigid spore coat which is efficently broken only by three passages through a French pressure cell at 20,000 psi. The plasma membrane of Ax_3 is easily ruptured by one passage through a french pressure cell and the effects of the additional passages through the cell are unknown.

The initial isolation procedure for RNA polymerase was a modified method of Roeder *et al*. (1969). Pong and Loomis (1973), who first attempted to isolate RNA polymerase by this method obtained some success. They were able to isolate RNA polymerase I and RNA polymerase II from frozen nuclei. It is now concluded that isolation of nuclei should be avoided as the first step in purification because the yield is reduced and only a small purification advantage is achieved (Lewis, M.K. and Burgess, R.R., 1982). In addition, all attempts to isolate nuclei from dormant spores have so far been unsuccessful. Therefore, whole cell extracts of Ax_3 were used for isolating the enzyme.

The simplest procedures utilized for isolating RNA polymerase involve ammonium sulphate fractionation of the whole cell lysate and then column chromatography on DEAE–cellulose (Whatman). Two $(NH_4)_2SO_4$ fractionations were attempted a 30–70% $(NH_4)_2SO_4$ cut and a 30–50% $(NH_4)_2SO_4$ cut on whole cell lysate of Ax$_3$. The use of a high $(NH_4)_2SO_4$ concentration (greater than 30%) early in purification dissociates the enzyme–DNA–RNA complex and allows the sigma subunit to re–combine with the core enzyme (Roeder, R.G. and Rutter, W.J., 1969). This insures that functionally active RNA polymerase will be isolated. The protein precipitate resulting from the $(NH_4)_2SO_4$ fractionation was dialyzed overnight to remove the salt and then placed on a DEAE–cellulose column. The enzyme was eluted with a 0.05 M to 0.3 M $(NH_4)_2SO_4$ gradient in 0.05 M Tris–HCl pH 7.9 containing 0.01 M MgCl$_2$, 0.1 mM EDTA, 1mM DTT and 25% glycerol. One ml fractions were collected from the column. A RNA polymerase assay and a protein determination were done on each fraction. The following figure (Fig. 4) demonstrates a typical profile obtained from the DEAE column for RNA polymerase. Numerous protein peaks obtained at the end of the DEAE–cellulose profile suggest that the sample placed on the column is highly contaminated with nucleic acid. Therefore, the whole cell extract was treated by several procedures in an attempt to remove excess nucleic acid.

Polymin P (Sigma), a polycation, was used by Burgess (1975) for removing nucleic acid during purification of RNA polymerase. Polymin P precipitates DNA but few proteins. Any proteins precipitated by Polymin P can be released from precipitated DNA by increasing the salt concentration (Schleif, 1981). Because

Figure 4: The profile of RNA polymerase activity from
cell extract by DEAE–52 chromatography.

DEAE–cellulose chromatography of whole cell
lysate from Ax_3 (as prepared in appendix).

RNA polymerase activity (Tsai, 1973) and
protein concentration (Bradford, 1976) were
determined in alternate fractions. RNA
polymerase activity was measured by the CPM
obtained from incorporated $[^3H]$ –UTP
into RNA.
(□) RNA polymerase activity
(○) Protein
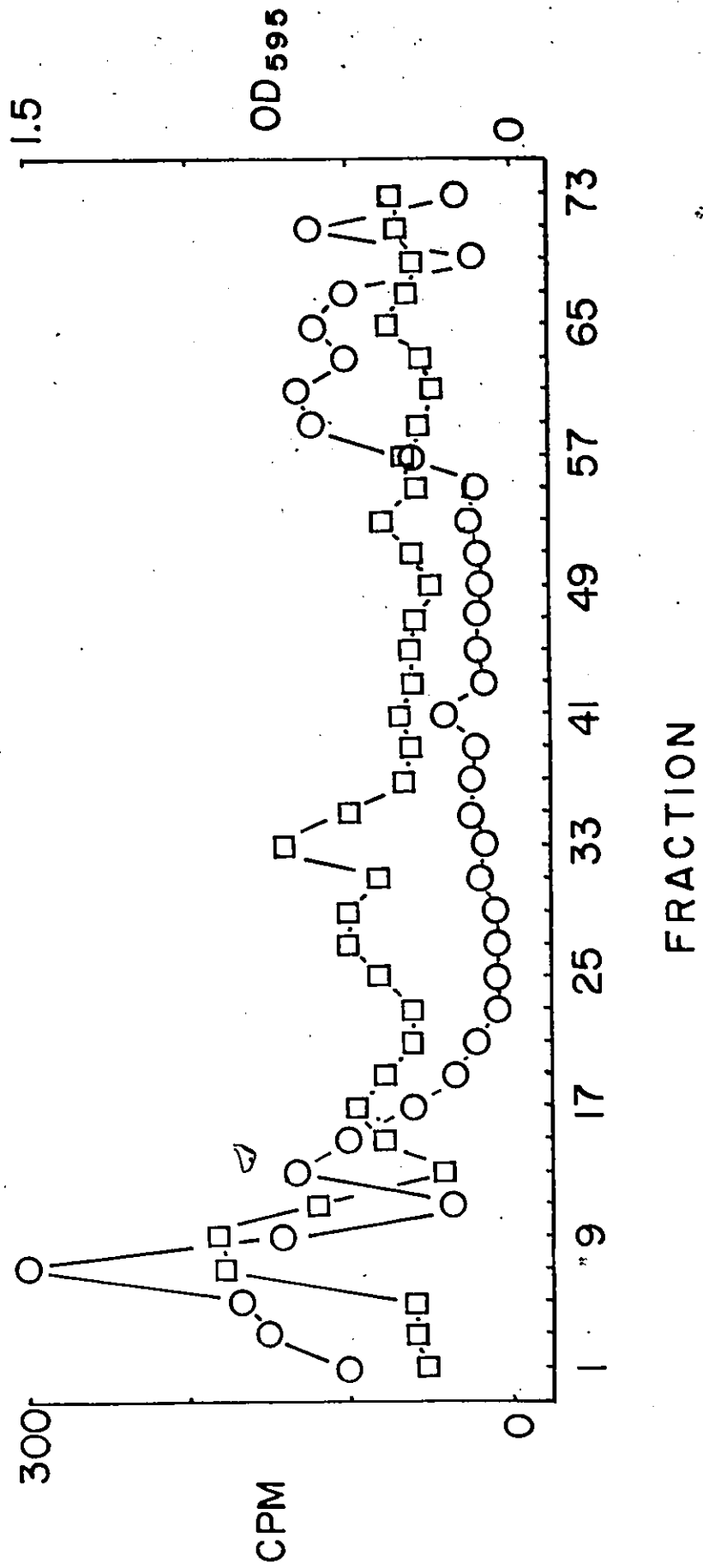
of this function, Polymin P fractionation was done on whole cell extract from Ax₃. Optimal conditions for precipitating RNA polymerase by Polymin P fractionation had to be first determined. Variable results were obtained from the percent Polymin P needed to precipitate out nucleic acid. The optimal percent Polymin P varied from 50-80%. Due to the variability in results, Polymin P fractionation was replaced with phase partitioning to remove nucleic acid. Phase partitioning can be used not only to separate DNA from proteins but also to purify DNA-bound proteins. An aqueous solution of 6.4% (w/w) of Dextran T500 (Pharmicia) and 25.6% (w/w) Polyethylene glycol (PEG) were mixed by weight with the cellular extract (Schleif, 1981). After mixing for 1 hour, the mixture is centrifuged at 5,000 x g for 10 minutes which separates it into two phases. The top phase is removed and discarded while the bottom phase is made 4 M with NaCl. Approximately 5 volumes of a 6.9% PEG solution containing 4 M NaCl is added to the bottom phase and stirred for 1 hour. Centrifugation again results in a separation of two phases. This time the top phase is removed and stored since it contains the RNA polymerase activity and the bottom phase is re-extracted with the PEG/salt solution for a second time. After centrifugation, the two top phases are pooled and fractionated with $(NH_4)_2SO_4$ to remove the PEG. 30-35% saturation with $(NH_4)_2SO_4$ will remove the PEG, having it form an oily layer on top after centrifuging for 1 hour at 10,000 x g. The lower phase obtained from this centrifugation is then saturated with 50% $(NH_4)_2SO_4$ to precipitate all proteins. Proteins are precipitated out overnight and then collected by centrifugation at 30,000 rpm for 30 minutes. The final pellet was suspended in a small volume of elution buffer and then diluted to the proper molarity as determined by conductivity measurements. Usually, the sample had

to be diluted in about 5 to 10 ml to obtain a molarity of 0.05 M

$(NH_4)_2SO_4$. The sample was then chromatographed on a Sephadex

A-25 (Pharmicia) column. A Sephadex A-25 column replaced the DEAE-

cellulose column since the Sephadex is able to separate out all three forms of

the enzyme. DEAE-cellulose only separates RNA polymerase I from RNA

polymerase II and III. The Sephadex A-25 column was eluted with a 120 ml

gradient of 0.05 M to 0.3 M $(NH_4)_2SO_4$ in elution buffer. Five

ml fractions were collected and assayed for the presence of protein and RNA

polymerase activity. The following figure (Fig. 5) demonstrates a typical

column run from a Sephadex A-25 column. Efficiency of the column usually

ranged from 60-67%[a]. The protein profile obtained is indicative of the

pattern of RNA polymerase isolation from a Sephadex A-25 column obtained by

Iwabuchi *et al.* (1979, 1980). On this column, protein peaks indicating

RNA polymerase activity are known to occur at around 0.12 M, 0.17 M and 0.21 M

$(NH_4)_2SO_4$. According to α-amanitin sensitivity and salt

requirements, RNA polymerase I elutes at 0.12 M, RNA polymerase II at 0.17 M

and RNA polymerase III elutes at 0.21 M $(NH_4)_2SO_4$ (Takiya,

et al., 1980). During all column runs, maximal activity obtained for RNA

polymerase I, RNA polymerase II and RNA polymerase III was 209.6 DPM/ug

protein, 289.1 DPM/ug protein and 20.2 DPM/ug protein, respectively.

Designation of RNA polymerase I, II and III was based only upon salt molarity

with which activity was eluted from the column.

(a) Column efficiency refers to the % of protein in all fractions

obtained from the column as compared to the total amount of protein present in

the original sample chromatographed.

Figure 5: The profile of RNA polymerase activity from
whole cell extract by Sephadex A–25 Chromato-
graphy.

Sephadex A–25 column profile for the isolation
of RNA polymerase from whole cell extract
of Ax_3. Samples to be chromatographed

were prepared as described in the Appendix.
RNA polymerase activity was detected by the
amount of $[^3H]$–UTP incorporated into RNA.
Protein was measured by Bradford (1976) using
γ–globulin as standard.
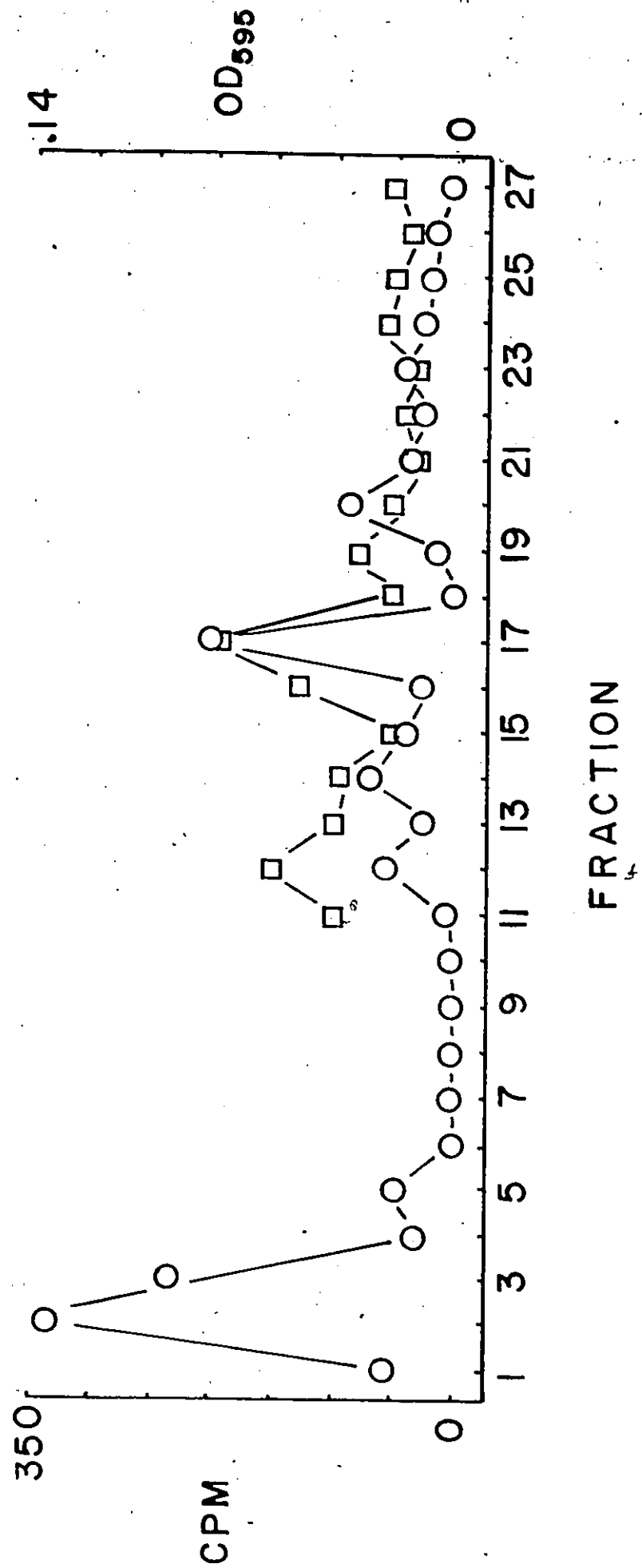(□) RNA polymerase activity
(O) Protein

Table 4 summarizes the purification of RNA polymerase for *D. discoideum*. After Sephadex chromatography, the specific activity of the enzyme greatly increases. However, this does not indicate a large amount of enzyme being present. The activity of the enzyme was only 2 to 3 times above a background level of approximately 50 CPM. One crude evaluation of enzyme activity suggests that counts should be at least 4 to 5 times above background to denote true RNA polymerase activity (Schleif, 1981).

Concentration of activity peaks from the Sephadex column was attempted by numerous procedures including vacuum dialysis, $(NH_4)_2SO_4$ precipitation, a protein concentrator (Isco) and ultra-filtration (Millipore). Many of these concentrating techniques proved ineffective. Vacuum dialysis and ultrafiltration required 48–72 hours to concentrate 10 ml down to approximately 1 ml. The extensive period of time required to concentrate was due to the presence of 25% glycerol in the isolation buffers. The high concentration of glycerol was needed to increase the stability of the enzyme. In addition, protein may be lost during dialysis by non-specific binding of proteins to the dialysis bag. Therefore, both the extensive time required to concentrate the sample as well as non-specific binding of the protein may help to explain why little increase in protein concentration was seen. Ammonium sulphate precipitation of the active fractions also yielded minimal results. Fractions were made 70% with $(NH_4)_2SO_4$ by the addition of the appropriate volume of a 100% solution of $(NH_4)_2SO_4$, stirred overnight and then centrifuged at 28,000 rpm for 1 hour. After centrifugation, no pellet or film could be detected in the centrifuge tube. This procedure was attempted three times, each time resulting in no visible pellet. It can only be suggested that

Table 4: Purification of RNA polymerase from whole cell extract
of Ax₃.

Purification Step	Protein (mg)	Activity (CPM)	Specific Activity
Supernatant from cell lysis	749	172.2	0.23
$(NH_4)_2SO_4$ Precipitation	35.98	144.0	4.0
Sephadex Chromatography			
0.12 M $(NH_4)_2SO_4$	.325	54.1	166.5
0.17 M $(NH_4)_2SO_4$	.530	136.6	257.7
0.21 M $(NH_4)_2SO_4$	.470	14.8	31.5

Summary of the purification of RNA polymerase from *D. discoideum*
Ax₃. The data is from a single representative experiment.

About 10 g (wet weight) of cells from the vegetative stage were
used as starting material. The phase partitioning step was not
included since PEG interferes with protein determination.
Activity denotes the CPM obtained for incorporation of [³H]–UTP
into RNA by 20–50 ul of sample at the various times during
purification. Protein determined by Bradford (1976).

since there was routinely less than 500 ug of protein in the total sample, ammonium sulphate may not be sensitive enough to precipitate such small quantities of protein.

One major drawback in isolating RNA polymerase is that large amounts of starting material are usually required. RNA polymerase has been isolated from whole cell extracts of a large variety of eukaryotic organisms (see Review, Lewis and Burgess, 1982) but kilogram amounts of starting material have yielded sufficient quantities of enzyme from only yeast, wheat germ amd calf thymus tissues. I was attempting to isolate the enzyme from approximately 10 grams of Ax_3 cells. Using standardized isolation procedures for RNA polymerase, I have concluded that 10 g of starting material is insufficent even to detect rather than purify the enzyme. In *Dictyostelium discoideum* spores, 10 grams of starting marterial alone would require 5,000 plates since 1 plate containing approximately 10^8 spores would have a dry weight of 2.4 mg (Jackson, 1982)).

Takiya *et al.* (1980) have isolated RNA polymerase I and II from 5 g of *Dictyostelium discoideum* nuclei. RNA polymerase III, however, could only be detected since it is present in insufficient quantitites to allow isolation. Therefore, attempts were made to detect RNA polymerase activity in nuclei isolated from approximately 1×10^{10} Ax_3 cells. Nuclei isolation utilized during this procedure was similar to that described in Methods and Materials. However, nuclei were not pelleted through 2.3 M sucrose. This increased the efficiency of nuclei isolation since nuclei free of protein contamination were not so stringently required. Nuclei were sonicated with three 10 second pulses at the maximum frequency of a Bronwill Bio-sonik sonicator. The sonicated preparation was saturated with

$(NH_4)_2SO_4$. Proteins were allowed to precipitate out

overnight and then centrifuged at 28,000 rpm for 1 hour. The pellet obtained

was suspended into a small volume of elution buffer and then diluted with the

appropriate volume of buffer to less than 0.05 M $(NH_4)_2SO_4$ as

determined by conductivity measurements. The sample was then placed on a

Sephadex A–25 column and eluted with 120 ml of elution buffer. Five ml

fractions were collected and RNA polymerase activity was measured in the

fractions eluting at 0.12 M, 0.17 M and 2.1 M $(NH_4)_2SO_4$. The

column profile obtained was identical to the profile produced from partially

purified whole cell extract of Ax_3 on Sephadex A–25. Table 5 details

the detection of RNA polymerase from isolated nuclei. Activity of the enzyme

was detected prior to placing the enzyme preparation on the Sephadex column.

Column chromatography of the enzyme preparation diluted out the activity.

To answer the original question of whether spores of *D. discoideum*

contain RNA polymerase, another approach was attempted. Since RNA

polymerase could be detected in nuclei and distinguished into the three forms

by —amanitin sensitivity, attempts were made to isolate nuclei from dormant

spores. Dormant spores contain approximately one third the DNA found in

vegetative amoebae (Sharpe, *et al.*, 1983). Therefore, since spores

contain less DNA, it would be likely that the nuclei, too, are smaller in size

than that found in vegetative amoebae. The various breakage procedures

utilized in an attempt to isolate nuclei from spores is demonstrated in Table

6. To determine if nuclei are released from spores by the various breakage

procedures, subsamples of the preparations were stained with methylene blue.

Table 5: Detection of RNA polymerase in nuclei isolated from Ax_3.

Purification Step	Protein (mg)	Activity (CPM)	Specfic Activity
Nuclei	80.55	247	3.1
Sonicated Nuclei	73.45	189	2.6
60% $(NH_4)_2SO_4$	12.59	217	17.2
Sephadex Chromatography			
0.12 M $(NH_4)_2SO_4$	.594	42	70.7
0.17 M $(NH_4)_2SO_4$	.592	45	85.1
0.21 M $(NH_4)_2SO_4$	.277	43	155.2

The detection of RNA polymerase in nuclei was isolated from approximately 1 x 10 [10] cells of Ax_3. The data presented here is from a single representative experiment. Protein was determined using Bradford (1976). Activity denotes the CPM of incorporated [^{3}H] –UTP into RNA under assay conditions (as described in Methods) by a 20 – 50 ul subsample at the various step in detection.

Table 6: Breakage Procedures Attempted for Obtaining Nuclei from
Dormant Spores.

Procedure	% Breakage	% Nuclei
French Pressure Cell		
3 Passages at 20,000 psi	100	0
1 Passage at 20,000 psi	60	0
3 Passages at 10,000 psi	75	0
Rapid Freeze Thaw		
-70^0 C to 23^0 C	25	0
-70^0 C to 80^0 C	40	0
Glass Beads		
2.5 mm bead size	65	0
5 mm bead size	67	0
Mixture of .11 mm, .17mm, 2.5 mm and 5 mm beads	64	0

Dormant spores of *D. discoideum* were disrupted by mechanical
procedures. French pressed spore preparations contained a
concentration of 1×10^8 to 1×10^9 spores/ml. Rapid freeze thaw
preparations consisted of 10^9 spores in a final pellet. Freeze
thaw preparations were also ground with a mortar and pestle to
achieve the % breakage. Spore suspensions were broken with glass
beads using a Bead–Beater (Biospec). Spore concentrations of
this preparations ranged from $1-5 \times 10^7$ spores/ml.

Following all mechanical procedures where breakage was seen to occur, no intact nuclei were discernable. Rather, small fragments which stained intensely blue were scattered throughout the preparation.

Dormant spores of *Dictyostelium* can be activated by a heat shock treatment of 45° C for 30 minutes (Cotter and Raper, 1966). Activation of a spore population results in the synchronous release of amoebae within 5 hours. Prior to releasing amoebae, spores are known to swell as the outer two layers of the spore coat ruptures (Hohl, 1969). Activated spores in the presence of cycloheximide (200 ug/ml) will swell but not release amoebae (Cotter and Raper, 1968). Therefore, activated spores in the presence of cycloheximide were treated with osmotic agents (3 M to 8 M ethylene glycol and 2.5 M sucrose) to determine if osmotic pressure would lyze the inner third layer of the spore coat (Cotter, 1977). In addition, Hohl *et al*. (1972) detailed a procedure whereby protoplasts are made from dormant spores. Activated spores in the presence of cycloheximide are incubated with cellulase (5 mg/ml) for 1 hour and then pronase (1 mg/ml) for a second hour. Protoplasts were also subjected to osmotic agents to see if they would lyze. Ethylene glycol treatment of activated spores and protoplasts resulted in cell lysis. However, methylene blue staining of the preparation revealed that nuclei had also ruptured during the osmotic treatment. Sucrose treatment of activated spores and protoplasts did not cause cells to lyse. Since the techniques attempted yielded low breakage of the dormant spore, isolation of nuclei from this developmental stage was abandoned.

APPENDIX II

TOTAL RNA POLYMERASE ACTIVITY IN NUCLEI

ANALYSIS OF VARIANCE

ONE WAY ANOVA

GROUP	MEAN	N
1	8.433	4
2	11.703	4
GRAND MEAN	10.068	8

SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F RATIO
BETWEEN	21.389	1	21.389	35.194
WITHIN	3.646	6	0.608	
TOTAL	25.036	7		

RNA POLYMERASE ACTIVITY SENSITIVE TO LOW –AMANITIN

ANALYSIS OF VARIANCE

ONE WAY ANOVA

GROUP	MEAN	N
1	1.735	4
2	8.408	4
GRAND MEAN	5.071	8

SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F RATIO
BETWEEN	89.045	1	89.045	1446.408
WITHIN	0.369	6	0.062	
TOTAL	89.414	7		

RNA POLYMERASE ACTIVITY SENSITIVE TO HIGH -AMANITIN

ANALYSIS OF VARIANCE

ONE WAY ANOVA

GROUP	MEAN	N
1	1.085	4
2	1.068	4
GRAND MEAN	1.076	8

SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F RATIO
BETWEEN	0.001	1	0.001	0.077
WITHIN	0.048	6	0.008	
TOTAL	0.049	7		

RNA POLYMERASE ACTIVITY RESISTANT TO α-AMANITIN

ANALYSIS OF VARIANCE

ONE WAY ANOVA

GROUP	MEAN	N
1	5.603	4
2	2.433	4
GRAND MEAN	4.018	8

SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F RATIO
BETWEEN	20.104	1	20.104	87.160
WITHIN	1.384	6	0.231	
TOTAL	21.488	7		

RNA POLYMERASE ACTIVITY ON CHROMATIN TEMPLATE

ANALYSIS OF VARIANCE

ONE WAY ANOVA

GROUP	MEAN	N
1	8.695	4
2	27.605	4
GRAND MEAN	18.150	8

SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F RATIO
BETWEEN	715.176	1	715.176	102.940
WITHIN	41.685	6	6.948	
TOTAL	756.861	7		

RNA POLYMERASE BINDING RESISTANT TO RIFAMPICIN AND HEPARIN

ANALYSIS OF VARIANCE

ONE WAY ANOVA

GROUP	MEAN	N
1	15.818	4
2	2.650	4
GRAND MEAN	9.234	8

SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F RATIO
BETWEEN	346.766	1	346.766	81.805
WITHIN	25.433	6	4.239	
TOTAL	372.200	7		

VITA AUCTORIS

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