1983

Sequence and Functional Structures of the Extrachromosomal rDNA Minichromosome of Physrum Polycephalum

Gerald Ray Campbell

SEQUENCE AND FUNCTIONAL STRUCTURES OF THE EXTRACHROMOSOMAL rDNA MINICHROMOSOME OF PHYSARUM POLYCEPHALUM

Gerald R. Campbell

Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
at the Rockefeller University

April 28, 1983

PREFACE

As in any learning endeavor, the people and the environment played a very major role in the completion of this work and in my continued education. I would like to thank the Rockefeller University community at large for the openness and support they have shown during the past several years.

It would be impossible for me to properly acknowledge all of the people who contributed material or advice during my research, as there was at least one person from every laboratory involved in the study of molecular biology who should be included in such a list. Each of these people, and of course their colleagues and support staff, made my work here a little easier, or in some cases made it possible. To all of the personnel I have sought advice, information or material from in the laboratories of Drs. Chua, Darnell, Hanafusa, Model, Reich, Robertson, Strickland, Young and Zinder, a warm thanks. To those who contributed more substantially, including Drs. Carl Baker, Susan Haynes, Warren Jelenik, Ken Krauter, Peter Model, and Ed Ziff, many thanks for your patience and generosity.

I would like to thank Dr. Peter Melera of the Walker Laboratories of Sloan-Kettering for his assistance, and for the 5.8s RNA which he supplied.

Of course, my greatest debt is to those who have worked with me in the laboratory of Dr. Vincent Allfrey. Without their support, patience, and hard work none of my own work would have been possible. I owe a great deal to Dr. Allfrey, who got me interested in this unique and interesting system during my first months here at Rockefeller, and who has provided information and support ever since. Four students from the Kettering Institute, Peter Bergold, Margaret Cheung, Rick Mickitch, and Kathy Scotto, each contributed results used in this dissertation. Others in the laboratory without whom this work would have been difficult or impossible include Dimitrius Drivas, Larry Kenyon, Susan Kozak, Len

Lothstein, and Drs. Christopher Prior and Irene Sun. Special thanks go to those working directly on various aspects of this project, including Drs. Virginia Littau for her excellent electron microscopy, and Yu-Fei Shen, who worked with me on the S1 mapping and *in vitro* transcription experiments. But I owe the most to Dr. Edward Johnson, my mentor and coworker through the sometimes difficult years that followed. Without him, none of my work here would have been remotely possible.

I would also like to acknowledge my wife Sharon for her unflagging support and extreme patience throughout the endless hours of experiment and writing.

SUMMARY

The ribosomal RNA genes of Physarum polycephalum are encoded on multiple copies of linear, palindromic extrachromosomal DNA molecules (rDNA), which have been isolated both as a satellite DNA molecule, and as a "minichromosome" with associated chromatin proteins.

The genes coding for the 19 S, 26 S, and 5.8 S rRNAs are transcribed as a single, large precursor molecule, beginning nearer the center of the palindrome and transcribing outward. The genes are in the order 19 - 5.8 - 26, and the 26 S gene contains two intervening sequences. The intervening sequences are transcribed, and later excised out.

The region of the rDNA previously identified as the transcription initiation site was cloned in Charon 28 bacteriophage. The initial large clone was subcloned in M13mp9, and a small clone containing the initiation site was isolated. The transcription initiation site was mapped within this clone using S1 nuclease protection experiments, and in vitro transcription run-off synthesis. The DNA sequence of over 700 nucleotides of this clone has been determined, including the putative transcription initiation site. Comparison of this sequence to other rRNA transcription initiation sites finds no consistent region of homology with any other set of initiator sequences. A consistent structural feature, an inverted repeat at approximately -30 to -50 nucleotides, was identified in this and other rRNA transcription initiation sites examined.

The transcribed regions of this rDNA minichromosome were found to be packaged into an altered, extended nucleosomal arrangement during active stages of the Physarum life cycle. This altered conformation is specific to transcribed areas, is more sensitive to nuclease digestion, and is separable as a slowly sedimenting monomer peak on sucrose gradients. This altered monomer nucleosome or "lexosome" contains, after

staphylococcal nuclease digestion, a monomeric length of DNA (144 bp), and has been shown subsequently to contain a full complement of core histones, along with two specific associated proteins (Prior et. al., 1983 and Prior, 1982).

A model for the activation of the transcription unit, based on a combination of the conserved inverted repeat and the chromatin structural studies, is proposed.

The ends of the linear molecule have been investigated, and shown to contain inverted repeats, length and sequence heterogeneities, single-strand one-nucleotide gaps in one or both strands at variable intervals, and a covalently bound protein. The rDNA terminal Eco RI fragment was cloned in Charon 13, and a large, representative clone was restriction mapped, subcloned into M13 vectors, and partially sequenced. The mapping and sequence data confirm the presence of inverted repeats, and identify several components of these repeats. A model is proposed to explain how these features may function to allow the replication of the 5' end of the linear rDNA molecule.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

ABBREVIATIONS

bp	base pair(s) of DNA
BPB	bromophenol blue (a dye)
BSA	bovine serum albumin
dNTP	any deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid (a chelating agent)
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid (a calcium-specific chelating agent)
EPNP	1,2 epoxy-3-(p-nitro-phenoxy)propane (a protease inhibitor)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (a buffer, pK 7.5)
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs of DNA
LT	low Tris buffer: 10 mM Tris pH 7.4, 10 mM EDTA, 50 mM NaCl
MW	molecular weight in daltons
PMSF	phenylmethylsulfonyl fluoride (a protease inhibitor)
rDNA	ribosomal DNA
rRNA	ribosomal RNA, including 19 S, 26 S, and 5.8 S species encoded on the rDNA
RF	replicative form, double stranded closed circular DNA
SDS	sodium dodecyl sulfate
SSC	standard saline citrate: 0.9 M NaCl, 0.09 M Na_3citrate, pH 7.0
SSCP	SSC plus 15 mM K_2HPO_4 and 1 mM EDTA, pH 7.2
TCA	trichloroacetic acid
TE	Tris-EDTA buffer: 10 mM Tris, 1 mM EDTA at the specified pH
TES	[N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (a buffer, pI 7.5)
TPES	36 mM Tris-Cl pH 7.2, 30 mM NaH_2PO_4, 1 mM EDTA, 0.2% SDS
Tris	Tris(hydroxymethyl)aminomethane (a buffer, pI 8.2)
XCFF	xylene cyanol FF (a dye)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

INTRODUCTION

The ribosomal RNA genes of Physarum polycephalum, a myxomycete, are a useful model system for studying the molecular structures associated with a single gene during its various functions, such as transcription and replication, because they are easily isolatable in biochemical amounts in nearly pure form. (For review see Allfrey et. al., 1977, Goodman, 1980, Holt, 1980, and Melera, 1980). In addition, these genes and their associated flanking sequences contain several unique features which allow their experimental manipulation, and which should prove to be informative in their own right.

The Presence of Extrachromosomal, Palindromic, Amplified rDNA in Physarum: Previous work has shown that the rRNA genes in Physarum polycephalum reside on multiple copies of linear, extrachromosomal, palindromic rDNA molecules (Allfrey et. al., 1978, Bradbury et. al., 1975, Hall and Braun, 1977, Molgaard et. al., 1976 and Vogt and Braun, 1976a). There are approximately 150 copies of this palindromic rDNA per haploid genome at all times throughout the life cycle (Affolter and Braun, 1978, Braun et. al., 1977, Hall et. al., 1978, Hall et. al., 1975). These rDNA molecules are located exclusively in the nucleolus (Bradbury et. al., 1973 and Ryser et. al., 1973), making up the greater portion of the DNA in that subnuclear organelle.

The presence of multiple copies of the ribosomal RNA genes is common throughout all living organisms (c.f. Bird, 1980 and Lewin, 1980). In many organisms, including large numbers of amphibians and insects, the rDNA is extrachromosomal at specific times during the life cycle (c.f. Gall, 1969, Birnstiel et. al., 1971 and Bird, 1980). In most of these cases, the extrachromosomal rDNA exists as tandem arrays on circular DNA molecules. In Physarum however, these rDNA molecules are present in all stages of the life cycle of (Affolter and Braun, 1978, Braun et. al., 1977, Hall et. al., 1978, Hall et. al., 1975), and are apparently linear at all times.

Linear extrachromosomal rDNA molecules are known in several organisms including Paramecium (Findley and Gall, 1978), numerous hypotrichs (c.f. Klobutcher et. al., 1981), and Dictyostelium (Cockburn et. al., 1978). In most of the hypotrichs (including all of the Euplotes, Oxytricha, Stylonichia and Tetrahymena species that have been studies), all genes in the macronucleus are apparently contained on extrachromosomal, linear palindromic molecules, and it seems that the rDNA is treated the same as any other gene. In Paramecium all of the genes in the macronucleus are again extrachromosomal, but the rRNA genes exist as arrays of tandem repeats, rather than palindromic dimers. And in the case of Dictyostelium the extrachromosomal rDNA molecules may or may not be present at all stages in the life cycle (Emery and Weiner, 1981). Physarum rDNA may therefore be unique, or nearly unique, in being present as a linear "minichromosome" throughout all stages of mitosis and meiosis.

The reasons for the extrachromosomal location of these genes is unknown, but provides fertile ground for speculation. The extrachromosomal nature of the rDNA molecules may enable them to be moved easily from one part of the nucleus to another, as happens during mitosis. Having repeated genes located on extrachromosomal molecules may provide chromosomal single-copy genes with some protection from unwanted crossing-over events. Large palindromic arrays may be unstable if they are contained in a chromosome, and the palindromic arrangement of rRNA genes may be more efficient than tandem arrays for a variety of reasons. Or this arrangement may merely reflect an evolutionary experiment which was neither selected for or against, or a holdover from a time in evolution when extrachromosomal palindromes were the norm. But regardless of the reasons for their existence, the extrachromosomal rDNA molecules are an easily accessible system for study.

Physarum rDNA is also different from the rDNAs of many other species in that it does not appear to have any regions with unusual sequence composition (Matthews et. al., 1978 and Steer et. al., 1978), but rather is homogeneous throughout its length. The rDNA of Xenopus laevis, for example, contains a region of A-T rich sequences in the untranscribed spacer, and is very G-C rich throughout most of the

transcribed region (Moss et. al., 1980 and c.f. Lewin, 1980). However, this pattern is not conserved throughout animals, or even amphibia, since the closely related species Xenopus borealis shows a different pattern of base composition in its untranscribed spacer regions (c.f. Lewin, 1980). This would seem to indicate that base composition is irrelevant, or nearly so, to transcription and transcription initiation.

Functional Domains of the rDNA: The extrachromosomal rDNA molecule of Physarum must perform two prime functions: it must be transcribed into rRNA, and it must replicate itself so that later generations will be provided with sufficient rDNA to allow transcription of the necessary rRNA. Since the sequences involved in these two functions do not overlap, the rDNA may be divided conveniently into several functional domains as shown in Fig. 1.

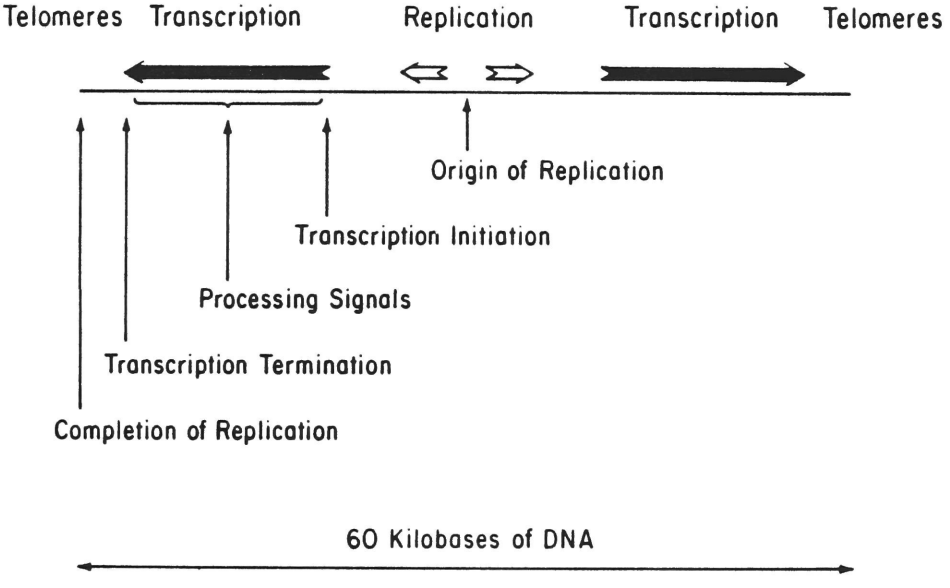


Figure 1: Locations of Various Control Regions on the Palindromic rDNA. A map of the various regions involved in the major functions of the rDNA palindrome in Physarum. See text for discussion.

Replication

Replication: Origin: The extrachromosomal rDNA is replicated throughout the cell cycle, in a random, unscheduled fashion (Braun and Evans, 1969 and unpublished observations), beginning at an origin near but not at the center of the palindrome (Vogt and Braun, 1977). This region contains not only the center of symmetry of the palindrome, but many tandem inverted repeat sequences (Vogt and Braun, 1976 and Ferris and Vogt, 1982). A previous report of rolling circle replication (Bohnert et. al., 1975) has proven to be unfounded.

There is no master copy or chromosomal copy which is amplified, as is the case for organisms which differentially amplify their rDNA during some developmental stage (c.f. Gall, 1969, Bird, 1980 and Lewin, 1980), but rather rDNA molecules from the extrachromosomal pool are replicated at random (Braun and Evans, 1969 and Vogt and Braun, 1977). This is similar to the replication of Tetrahymena rDNA during the vegetative stage (Truett and Gall, 1977 and Cech and Brehm, 1981) which has only one origin at the center of its rDNA palindrome. However, Tetrahymena amplifies its rDNA during the period immediately after sexual conjugation, during the process of rebuilding the macronucleus. The amplified rDNA is initially copied from a single, chromosomally integrated half-palindromic copy (Yao and Gall, 1977), through a half-palindromic extrachromosomal intermediate (Pan and Blackburn, 1981). This amplification process involves the addition of terminal simple sequences (Blackburn and Gall, 1978, Pan and Blackburn, 1981 and Yao and Gall, 1977), and probably the breakdown of one copy of the micronuclear chromosome (Yao, 1981). A similar process is probably used in other hypotrichs (Klobutcher et. al., 1981).

The random replication of molecules in the total pool is similar to the replication of bacterial plasmids and mitochondrial DNAs (Lewin, 1980). This replication must be controlled by some mechanism, to ensure

that the population of rDNA molecules maintains a nearly constant size. This control may be similar to the feedback mechanism which operates for bacterial plasmids. The sequences required for this copy number regulation are in all probability located very near the origin of replication, in the center of the rDNA molecule.

Replication: Termini: As is widely known, replication of a linear molecule cannot complete the 5' end of either strand, due to the need for an RNA primer to allow the DNA polymerase to begin strand synthesis. Since the Physarum rDNA has linear ends, and can be isolated in large amounts, it offers a system for discovering the exact mechanism whereby linear ends can be completed. While this is only one of many systems that can be studied, it may be one of the best model systems for replications at chromosome telomeres since it apparently does not involve a special amplification mechanism at any time in its life cycle.

In many systems, Nature has solved the problem of replicating ends by eliminating them. Bacterial chromosomes, plasmids, many bacteriophage and viruses, and mitochondrial and chloroplast DNA are all circular (see Lewin, 1980 and 1977 for discussion and references). Several other bacteriophage and viruses can become circular during the replication process. The circles can then replicate either as a θ structure, or by a rolling circle mechanism.

Other systems have evolved to complete the replication of termini. Adenovirus, and probably all of the parvoviruses, replicate their linear DNA starting at one end, using a protein as a primer (Rekosh et. al., 1977 and Robinson and Bellett, 1977). A similar mechanism exists in the linear bacteriophage ϕ29, and other viruses (see Wimmer, 1982 for review). Other primers, such as tRNAs, are also used for this purpose (c.f. Varmus and Swanstrom, 1982).

Tetrahymena, and presumably other hypotrichs, have another mechanism to complete their linear extrachromosomal DNA replication. While the mechanism is not yet completely understood, it seems to depend on the template-independant synthesis of the terminal hexanucleotide repeat sequence (King and Yao, 1982), or its movement en bloc, (probably as a

double stranded sequence) from an unconnected site to the ends of the extrachromosomal DNAs. If this template-independant synthesis and joining to the extrachromosomal DNA continues during its replication, then replication of the termini could simply be a matter of balancing the replication rate and the primer-caused deletion of sequence at each replication against the template-independant synthesis of replacement sequences. While this may be a highly specialized mechanism, only functioning in the macronucleus of hypotrichs, it is of extreme interest that the same hexanucleotide sequence may be capable of supporting replication at DNA ends in yeast (Szostak and Blackburn, 1982). This sequence is, by its nature, incapable of forming a base-paired foldback.

The yeast telomeres, which have also been investigated in this system, have a much different sequence, and may have a different structure. Neither the sequence nor the structure has been determined to date.

Another strategy employed by some viruses and bacteriophage to complete the replication of the ends of a linear DNA molecule is the use of site-specific recombination. One example of this is the use of the LTR as a movable primer in retrovirus replication (reviewed by Temin, 1981, see also Varmus and Swanstrom, 1982).

Other mechanisms for end completion have been proposed, based on the presence of terminal inverted repeats (Bateman, 1975 and Cavalier-Smith, 1974). The common feature of these models is the use of an inverted repeat sequence as a second-strand primer, something which is known to occur in retrovirus second-strand synthesis (Temin, 1981 and 1982).

Which, if any, of these mechanisms is used in Physarum has been one of the prime focuses of our research. To answer this question, we have investigated the structures found on rDNA termini in vivo, and cloned them to determine their sequence. These results are reported later in this thesis.

Transcription

Transcription: Initiation: Another focus of our research is transcription initiation. Transcription initiates at approximately 17.2 kb from each end of the rDNA, and proceeds outward on the palindrome (Sun et. al., 1979 and Grainger and Ogle, 1978). This is similar to the pattern in Dictyostelium (Grainger and Maizels, 1982), and the hypotrichs which have been examined, notably including Tetrahymena (Engberg et. al., 1976 and Karrer and Gall, 1976).

Transcription initiation occurs at different rates in various portions of the mitotic cycle and life cycle, varying from almost complete saturation with polymerase in the active plasmodial stage S and G2 phases, to virtual absence in spherulating or sporulating forms (Hall and Turnock, 1976 and Sauer, 1978). This regulation may reflect complex changes in the macromolecular structure of the rDNA chromatin complex (Wille and Steffens, 1979 and LeSturgeon and Rusch, 1973), or a simpler, more immediate effect of a small molecule (Hildebrandt and Sauer, 1977 and Keuhn et. al., 1979). No evidence has been found to date of any regulatory step in the transcription process after initiation, or any processing step.

Transcription of the rRNA genes is a function of RNA polymerase 1, which has been isolated and studied in other laboratories (Burgess and Burgess, 1974 and Gornicki et. al., 1974). RNA polymerase 1 activity can be distinguished from polymerase 2 or 3 activity on the basis of its insensitivity to high doses of the drug α-amanitin (Grant, 1972).

In order to assay which specific regions of the rDNA are required for transcription initiation, various parts of the rDNA molecule have been cloned using recombinant DNA techniques, and the transcription initiation point localized. These results are reported in this thesis. Further investigation using these cloned rDNA fragments will be required before the exact sequences involved can be identified.

Transcription: Termination: Transcription does not continue until the template is exhausted, but terminates at a definite site(s) immediately downstream from the 26 S gene (Kukita et. al., 1981). The sequences immediately around the termination point have been identified, but thus far no report of any functional assay using these sequences has appeared. What sequence(s) or structure(s) are required for accurate termination have also not yet been determined.

Transcription: Chromatin Structure: The rDNA does not exist in the nucleolus as naked DNA, but is complexed with a large number of chromatin proteins (see Walker et. al., 1980 for review). Previous results demonstrated that the bulk of Physarum chromatin is packaged into regular nucleosomal repeating units (Jockusch and Walker, 1974, Stalder and Braun, 1978 and Vogt and Braun, 1976a). Our work has recently shown that the packaging of active, transcribed regions is not exactly the same as that of untranscribed regions. This will be discussed later in this thesis.

METHODS

Culture of Physarum polycephalum: Physarum polycephalum strain a x i, was grown in suspension culture as microplasmodia, as described by Daniel and Baldwin (1964). For cell cycle dependant experiments, microplasmodia were fused on paper supports to form macroplasmodia approximately 3-5 cm in diameter, using the same medium, or on 1.0% agar plates made with the same medium (Rusch, 1969).

Recombinant DNA Vectors and Hosts: Charon bacteriophage and their associated host strains DP50 sup f and K802 were purchased from the laboratory of Dr. Fred Blattner, and used according to his recommendations (Blattner, 1978 and Blattner at. al., 1977).

Lambda packaging strains BHB2688 and BHB2690 were obtained from the laboratory of Dr. Peter Melera, and used as instructed (Hohn, 1982).

Lambda phage 1059, and associated bacterial hosts Q358, Q359, and D91 were obtained from Dr. Jonathan Karn (Karn et. al., 1980).

Plasmid pBR322 was a gift from Peter Bergold.

M13 cloning vectors mp7, mp8, and mp9 were obtained from Dr. Ken Krauter. M13mp10 and mp11 were obtained from the laboratory of Dr. Nam Hai Chua. All M13 vectors were used according to the protocols set out by Messing (1980) unless otherwise stated.

All bacterial strains were grown on NZY medium (c.f. Blattner, 1978) unless growth on minimal medium was required, in which case M9 (c.f. Hohn, 1982) with appropriate supplementation was used. Transformation with recombinant DNAs was done using in vitro packaging (Hohn, 1982) for Charon and 1059 phage, and by the modified calcium shock procedure (Mandel and Higa, 1970, and Messing, 1980) for M13 vectors.

Isolation of Nuclei, Nucleoli, and Ribosomal DNA: Nuclei were isolated from mass cultures of Physarum by the method of Mohberg and Rusch (1971). Nucleoli were prepared from nuclei by passage through a French

pressure cell at 8000 psi at a concentration of approximately 10^{10} nuclei/ml in 0.25 M sucrose, 10 mM Tris-Cl, pH 7.2, followed by centrifugation as described by Bradbury et. al. (1973).

DNA was prepared from nuclei or nucleoli according to Gross-Bellard et. al. (1973). Ribosomal DNA was further purified from nucleolar preparations by cesium chloride density gradient centrifugation (Bradbury et. al., 1973).

Restriction Enzyme Digestions: Restriction enzymes were obtained variously from Bethesda Research Labs, New England Biolabs, or Boehringer Mannheim Biochemicals. Each was used under the conditions recommended by the manufacturer unless otherwise specified. Double digests were done serially, with the first restriction enzyme being inactivated or removed before addition of the second, unless the buffers were compatible. When stated 'restricted to completion', the digest was carried out with an amount of enzyme that would result in a greater than two-fold overdigestion, as corrected for site density, within two hours. Amounts of enzyme for partial digestions were corrected for site density, and proceeded for several minutes to one hour. In most cases, partial digests were aliquoted from the reaction into stop buffer to achieve a range of digestion conditions.

Other Enzyme Reactions: DNA polymerase 1 (E. coli), Klenow fragment, terminal transferase, polynucleotide kinase, bacterial alkaline phosphatase, nuclease BAL-31, nuclease S1, calf alkaline phosphatase, DNAse 1, RNAses A and T1, proteinase K, and β-galactosidase were obtained at various times from one of the above-mentioned suppliers, Sigma Chemicals, or Worthington. All were used according to manufacturers specifications or standard conditions unless otherwise stated.

Preparation of rRNA Species: 19 S and 26 S rRNAs were prepared from in vivo labeled Physarum microplasmodia using the first two steps of the three step phenol extraction procedure described by Melera and Rusch (1973). Specific activities were approximately 6×10^5 cpm/μg. Cold 19 S and 26 S rRNAs were prepared by the same method.

5.8 S rRNA was prepared from a ^{3}H-uridine labeled microplasmodial culture using the same method. The 26 S peak from the first gradient was collected, precipitated, and redissolved in gradient buffer containing 20% formamide. After heating at 69°C for 2 minutes, the rRNA was cooled, and rerun on a sucrose gradient. All of the RNA smaller than 10 S was pooled, precipitated, and electrophoresed thhrough a preparative 5% acrylamide gel. The only detectable RNA peak was eluted by soaking the gel slice in 0.1 M NaCl at 30°C for 18 hours. The resulting RNA was found to coelectrophorese with authentic 5.8 S marker RNAs.

Gel Electrophoresis: Agarose gels varied in concentration from 1.0% to 1.6% as indicated. (See McDonald et. al., 1977 for appropriate gel concentrations for various size ranges). For most uses 1.4% was satisfactory. Gel and reservoir buffers were Tris-acetate buffer (Allet et. al., 1973). DNA samples were loaded in buffer containing 10% glycerol, 0.05% each xylene cyanol FF and bromophenol blue, 0.1% SDS and 10 mM EDTA.

Polyacrylamide gels were used in various percentages ranging from 3.8% acrylamide-0.2% bis to 12.08% acrylamide-0.42% bis in Tris-borate buffer (Sanger et. al., 1977), either with urea (usually 7 M) or without.

Gel forms used were usually vertical slab gels, 3 mm thick with pocket-forming combs, or 1.5 mm thick. Elecrophoresis was for the specified time at 100 V unless otherwise stated. Occasionally a horizontal gel was run, using a BRL gel apparatus. Electrophoresis in these cases was at 150 V.

Electroelution: Excised agarose gel fragments containing a single DNA band were electroeluted using an ISCO Model 1750 Sample Concentrator. Electroelution was for 50 minutes, in 1/2X TA buffer. Samples were recovered from the eluate by ethanol precipitation. Recoveries ranged from 20 to 90%.

R-Loop Mapping: R-loop hybridization (Davis et. al., 1971) was carried out in 50 mM TES pH 7.0, 80% formamide, 0.3 M NaCl, 10 mM EDTA,

using 10-20 µg rDNA and 10-25 µg purified 19 S and/or 26 S rRNA, for 4 hours at 45°C (Campbell et al., 1979). Hybridized samples were diluted into 0.1 M Tris-Cl pH 8.5, 50% formamide, 10 mM EDTA. Cytochrome c was added to a final concentration of 50 µg/ml, and the samples were spread onto a hypophase of 10 mM Tris-Cl pH 8.5, 20% formamide. Grids were stained with uranyl acetate and rotary shadowed with platinum-palladium. Length measurements were made by comparison with included ϕX174 single-stranded or replicative form DNA.

Partial Denaturation Studies: Partial denaturation of rDNA or clone 229b insert molecules was carried out as described for R-loop mapping except that no RNA was included.

Strategies for Cloning rDNA Fragments: Since the restriction map of the rDNA was already known, precise strategies for cloning given restriction fragments could be followed. Since the two control regions of interest were the transcription initiation region, and the termini of the linear rDNA molecules, two such strategies were developed.

Cloning rDNA Fragments Containing the Transcription Initiation Site: The first strategy, to clone large fragments which included the initiator region, began with restricting isolated rDNA with Bam HI to completion. This DNA was then digested to approximately 1/2 completion with Bgl II, which yields the same cohesive end as Bam HI. Following extraction of the restriction enzymes, the DNA was mixed with Bam HI-cut Charon 30 DNA in a 1:2.5 weight ratio, and ligated at 6-8°C overnight (Blattner, 1978). The ligation mix was then transformed into K802 and plated, with recombinant plaques being picked (Maniatis et. al., 1978), and their DNA restriction mapped to identify which of the several potential donor fragments they represented.

Using this method, a recombinant was obtained representing the region from the central Bam site at 23.9 kb to the Bgl II site at 9.0 kb. This clone was designated PrD100.

Restriction mapping showed several restriction site differences between this clone and the rDNA restriction map. The Bgl II site (12.5

kb in the rDNA) was missing, as was one Xho I site (15.8 kb). In addition, a Sal I site was found at 18.1 kb on the rDNA map, and during subsequent large-scale amplifications a new Eco RI site appeared at 17.9 kb. This variant has since completely taken over the stock. These variant sites, with the exception of the Eco RI site, may represent sequence heterogeneity in the Physarum rDNA in vivo, or may be mutations such as that which gave rise to the Eco RI site.

Since the new Sal site created a 0.9 kb fragment which contained the initiation site (Sun et. al., 1979), further subcloning was done with Sal I digested PrD100 DNA, and the Sal I site in M13mp9. It was reasoned that the use of the M13 vector would favor small fragments, such as the two very near the initiator region. In fact this was the case, as several independant isolates of the 0.9 kb initiator fragment were found, while other fragments were not. Two of these isolates, designated Prd100S24 and PrD100S35 (hereafter S24 and S35, respectively), were restriction mapped in greater detail and subjected to DNA sequence determination.

Cloning of rDNA Terminal Fragments: In order to isolate fragments from the termini of the rDNA, a different cloning strategy was employed, as shown in Fig. 2. Since the structure at the extreme terminus of the linear rDNA molecule is not known, it is possible that some structure such a hairpin or extended single-strand region (see Emery and Weiner, 1981) could exist that would block ligation of this end to a vector molecule. To circumvent this potential problem, rDNA was first briefly digested with S1 nuclease to remove any potential terminal hairpins or single-stranded ends, and then Eco RI linkers were attached to the newly-formed blunt ends. The rDNA was then restricted with Eco RI, and ligated to Eco RI cut Charon 13. Several potential recombinants were identified by screening with a nick-translated rDNA terminal fragment, and these were picked and replated. Those plaques positive for hybridization with a second nick-translated rDNA terminal fragment were picked and studied further.

One of these clones, PrD229, was determined to be 5.3 kb in length,

CLONING rDNA TERMINI IN PHAGE CHARON 13

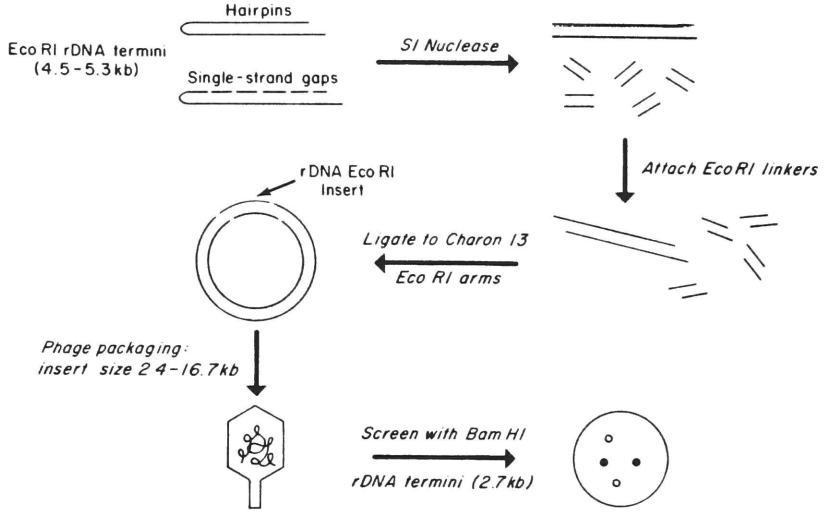


Figure 2: Cloning rDNA Termini in Phage Charon 13. Flowchart of steps for rDNA terminal restriction fragment cloning. Exact methods are given in text. rDNA Eco RI terminal restriction fragments were isolated, and treated briefly with S1 nuclease to remove any potential terminal hairpin. Breakage at gaps will also result. Blunt-ended fragments were then ligated to Eco R1 linkers, cleaved with Eco R1 to generate sticky ends, and ligated to Eco R1 cut Charon 13 DNA. Phage libraries were transformed by in vitro packaging, and screened by hybridization with purified nick-translated rDNA terminal restriction fragments.

and was chosen for further study because it was the size expected for a complete rDNA terminal Eco RI fragment. Restriction mapping of this insert revealed that the expected Bam HI site at 2.7 kb was missing in the majority (but not all) of the molecules in any given preparation, and two or three Hind III sites were discovered, one or two very close to one end and the other in the center of the insert. Another Bam HI site may exist, near the opposite end of the insert. Further hybridization results indicated that the end of the insert without the Hind III site was the end which hybridized to the rDNA Bam c fragment.

The insert fragment was subcloned *in toto* into M13mp7, and sequencing performed on several clones. Two distinct sequences were obtained, presumably from either end of the insert, but in no case was the insert found to be full length, so that deletions could be affecting the sequences obtained.

The insert was further subcloned by partial Hae III restriction of the intact PrD229 DNA, and ligation to Sma I cut M13mp9. Recombinants which hybridized with the Bam c fragment of rDNA were picked and sequenced. A similar strategy was employed with the enzyme Sau 3A, ligated into the Bam HI site in M13mp9.

An alternative cloning procedure was also used. PrD229 DNA was digested with Eco RI, and fragments separated on an agarose gel. The insert fragment was electroeluted and treated with bacterial alkaline phosphatase and polynucleotide kinase using ^{32}P-ATP to label the ends. The DNA was then cut with Hind III, and again electrophoresed, and the radiolabeled band(s) recovered by electroelution. This DNA was then Hae III digested and ligated to Sma I cut M13mp9.

Nuclease BAL-31 Subcloning: BAL-31 digestions of Bam H1 cut S24 and S35 DNAs were carried out as described by Maniatis et. al. (1982, see also Poncz et. al., 1982), using 5 U BAL-31 for 25 µg of S24 DNA in the initial subcloning, and 0.1 U BAL-31 for 5 µg S24d1.5 DNA for the secondary deletions.

Initial subcloning reactions were aliquotted into an equal volume of 40 mM EGTA at 1,2,3 and 5 minutes for one pool, and 7,10,12 and 15 minutes for the second pool. Pools were then made to 0.2 mM each dNTP, and 5 mM DTT by addition of concentrated stock solutions, and 1 U Klenow enzyme was added. After incubation at 37°C for 30 minutes, the polymerase was inactivated by heating to 70°C for 10 minutes, then cooling on ice. Hind III (12 U) was added to each pool, and incubated at 37°C for 1 hour. Each reaction was then diluted with 1 volume of Chambon B (500 mM Tris-Cl, pH 8.0, 10 mM EDTA, 10 mM NaCl) (Gross-Bellard et. al., 1973), and extracted once each with phenol and phenol/chloroform (1:1) and twice with ether before ethanol precipitation. Each sample was then

redissolved in ligase buffer with Sma I and Hind III cut mp9 DNA, and ligated. Ligation mixes were transformed as described previously.

Secondary deletion reactions were all pooled into 15 µl of 0.5 M EGTA, diluted to 90 µl with water, and then made 2 mM dNTPs and 20 mM DTT by addition of concentrated stocks. 2 U Klenow enzyme were added, and the reaction mix incubated at 37° for 1 hour. The reaction was stopped by addition of 20 µl 0.25 M EDTA pH 8.0 and 80 µl 5 M Na-acetate, followed by precipitation with three volumes of ethanol. Precipitated nucleic acids were dissolved in ligase buffer, and incubated with 5 U ligase overnight at room temperature. DNA was then transfected into competent JM103 cells as usual.

Preparation of Nucleosomes Using Staphylococcal Nuclease: Nucleolar preparations containing 40 to 80% rDNA were treated with 40 U/ml staphylococcal nuclease in buffer containing 20 mM Tris-Cl pH 7.8, 1 mM $CaCl_2$, 60 mM KCl, 15 mM NaCl, and 1.0 mM PMSF for various times at 37°C, and digested DNA prepared by phase extraction as previously described (Johnson et. al., 1978b). Samples containing 5-10 µg DNA were electrophoresed in agarose gels as described above, or separated on a 10-40% linear sucrose gradient (Johnson et. al., 1978a).

Restriction fragment DNA was labeled by nick translation, and electrophoresed on agarose gels. DNA was eluted from these gels by incubating gel slices in 0.5 ml of 25 mM TES pH 7.0, 80% formamide, 0.1% SDS, 0.3 M NaCl, 1.0 mM EDTA for 12 hours at 25°C, and aliquotted for use in hybridization experiments.

Treatment of Nucleoli or Nuclei with DNAse I: Nuclei were disrupted by 5 strokes of a loose-fitting dounce prior to digestion. Nuclear homogenate or nucleoli were pelleted at 500 g for 10 minutes, and resuspended in digestion buffer consisting of 10 mM Tris-Cl pH 7.0, 0.32 M sucrose, 50 mM NaCl, 5 mM $MgCl_2$ at a concentration of 200-300 µg DNA/ml (Johnson et. al., 1978b). This solution was incubated at 37°C for various times with 20 U/ml DNAse I, and reaction was then stopped by adding EDTA to 20 mM and SDS to 1.0%. The DNA was then prepared as described above for staphylococcal nuclease.

Filter Hybridization Methods: Ribosomal DNA was restricted and electrophoresed through agarose gels as previously described. The DNA was then transferred to nitrocellulose filters by the method of Southern (1975). The filters were dried at 80°C in vacuum, and stored dessicated until use.

For DNA-RNA hybridizations, filters were immersed in a minimal volume of hybridization buffer (25 mM TES pH 7.0, 49% formamide, 0.9 M NaCl, 0.1% SDS, 1.0 mM EDTA), and 1-2 x 10^5 cpm of ^{32}P labeled RNA (> 10^6 cpm/µg) were added per 1 cm width of filter. The filter was then sealed and incubated at 50°C for 18 hours. After incubation the filters were washed once with 50 ml of 50° hybridization buffer, and 3 times with 2x SSC. The filters were then treated with ribonucleases A (10 µg/ml) and T1 (5 U/ml) for 1 hour at room temperature in 2x SSC, rinsed several more times with 2x SSC, dried and autoradiographed (Campbell et. al., 1979).

For DNA-DNA hybridizations, the filter was first incubated with prehybridization buffer (0.02% polyvinylpyrrolidone [average MW 360,000] 0.02% Ficoll [average MW 400,000], 1.0% glycine, 6x SSC, 1.0 mM EDTA, 0.2 mg/ml BSA, 10 µg/ml denatured salmon sperm DNA) for more than 4 hours at 68°C. After binding sites on the filter were saturated, the prehybridization buffer was poured off, and prewarmed hybridization buffer (as above, except 1 µg/ml salmon sperm DNA) was added. Probe DNA (^{32}P labeled to greater than 10^7 cpm/µg) was denatured by addition of an equal volume of 0.2 M NaOH for 10 minutes, followed by neutralization with 1 initial volume of 0.2 M HCl, and one-half initial volume of Tris-Cl pH 7.0. The denatured probe was added to the 68° hybridization mix, and the filter was sealed and incubated for greater than 16 hours at 68°C.

After hybridization, the filters were washed once with warmed hybridization buffer without BSA or DNA, twice with 2x SSC - 0.5% SDS - 10 mM EDTA, and several times with 2x SSC, and then dried and autoradiographed.

Plaque lifts were performed essentially as described by Benton and Davis (1977), with occasional modification intended to reduce backgrounds. The use of SSCP rather than SSC to wash the filter before drying is recommended, to reduce nonspecific backgrounds and spotting.

Solution Hybridization Methods: To determine whether the different types of chromatin subunits were derived from coding regions of the rRNA genes, solution hybridizations were carried out as previously described (Johnson et. al., 1978a). Excess in vivo ^{32}P-phosphate-labeled rRNAs (2-7 x 10^5 cpm/µg) were mixed with 1-10 µg chromatin fragment-derived DNA in 200 µl of 49% formamide, 25 mM TES pH 7.0, 0.9 M MaCl, 1 mM EDTA, 0.1% SDS, and incubated at 60°C for 16 hours or longer. Following hybridization, the samples were treated with 500 U RNAse A and 5 U of RNAse T1 in 1.3 ml of 10 mM Tris-Cl pH 8.0, 0.4 M NaCl, 2 mM EDTA, 0.02% Nonidet P-40 at 37°C for 2 hours. Following treatment with RNAses, undigested RNA was precipitated with a final concentration of 10% TCA, using 150 µg of yeast carrier RNA. Precipitated RNAs were collected on nitrocellulose filters, and assayed by scintillation counting.

To define which regions of the rDNA molecule are associated with the altered nucleosomal conformation, rDNA restriction fragments were hybridized to chromatin fragment-derived DNA, as previously described (Johnson et. al., 1979). DNA isolated from the various types of chromatin subunits (see above) was denatured and partially degraded by boiling in 0.3 M NaOH for 10 minutes. The average DNA length in each type of sample was approximately 300 nucleotides following this treatment. Denatured chromatin DNA (0.5 µg) was hybridized with nick-translated rDNA restriction fragments Hin c, Eco c, or Bam b (5 x 10^{-4} µg; 7 x 10^7 cpm/µg) in 50 µl of 0.14 M sodium phosphate buffer pH 6.8, 1.0 M NaCl, 1.0 mM EDTA at 68°C for various times ranging from 5 minutes to 30 hours (Gallimore et. al., 1974 and Sharp et. al., 1974). The extent of hybridization was determined by digesting single-stranded nucleic acids with S1 nuclease, using conditions analogous to those used for S1 protection mapping (see S1 Mapping, below). Radioactivity remaining after precipitation was determined by scintillation counting.

The rate of hybridization was determined by linearizing the data (Gallimore et. al., 1974). The reciprocal of the fraction of unannealed DNA (1 minus the the fraction of annealed DNA) is plotted against the ratio of the time elapsed and the half-time for the annealing of the probe alone,

$$1 / (1 - fA) \text{ versus } t / t_{1/2p}$$

A steeper slope indicates more hybridization, while a slope of two indicates no hybridization with the chromatin-derived DNA. Using this linearization allows computation of the fraction of probe DNA represented in the chromatin sample, and quantitative comparison between representation of the various chromatin species in a given restriction fragment.

S1 Mapping: 1.5 - 20 pmoles of the DNA fragment were digested with bacterial alkaline phosphatase, extracted twice with phenol and three times with ether, and ethanol precipitated as described previously. The DNA was then end labeled with γ-^{32}P-ATP and polynucleotide kinase according to the suppliers specifications.

Total Physarum RNA was extracted either as previously described (Melera and Rusch, 1973), or by the guanidine thiocyanate method of Miller and Sollner-Webb (1981).

1.5 to 5 pmoles of DNA was combined with 5-10 µg total RNA, and ethanol precipitated. Pellets were washed with 95% ethanol and vacuum dried, and redissolved in 20 µl of S1 hybridization buffer (40 mM PIPES pH 6.4, 1.0 mM EDTA, 0.4 M NaCl, 80% formamide). After dissolution was complete the samples were heated to 75°C to denature the DNA, and then incubated at 56°C for 3 hours.

Hybridized samples were diluted into 10 volumes of ice-cold S1 nuclease buffer (50 mM Na-acetate pH 4.6, 0.28 M NaCl, 4.5 mM $ZnSO_4$, containing 20 µg/ml denatured salmon sperm DNA), and 100 U S1 nuclease added, followed by incubation at room temperature for 60 minutes. The

samples were then extracted once with phenol and once with ether, and ethanol precipitated. They were redissolved in formamide gel loading buffer, and electrophoresed on 4% acrylamide gels at 100 V for 4-6 hours.

Preparation of Recombinant Phage and Plasmid DNA: DNA was prepared from bacteriophage λ cloning vectors and recombinants by the procedure of Blattner (1978, and Blattner et. al., 1977), based on the density gradient purification of intact phage particles.

Plasmid DNAs, and replicative form DNA from M13 phage and recombinants, were prepared by a variety of methods during my work. These methods include the cleared lysate procedure (Greenberg, 1981, c.f. Kahn et. al., 1979), the rapid boiling method (Holms and Quigley, 1981), and most successfully the alkaline lysis method of Birnboim and Doly (1979). After lysis, the closed circular DNA was purified on a cesium chloride - ethidium bromide gradient as described (c.f. Maniatis et. al., 1982).

Template Preparation for M13 sequencing: Template was prepared by a modification of the method described by Messing (1980). Single clear plaques were picked from M13 cloning plates using sterile pasteur pipets. Each plaque was resuspended in 1 ml of LT, and may be stored at 4°C for several weeks. The titer goes down sharply initially, but plateaus at a workable level.

0.1 ml of the plaque storage buffer was inoculated into 5 ml of NZY medium with 0.1 ml of late log or early stationary phase cells, and shaken at 37°C for 6 to 10 hours. At this point the culture should be very turbid but not at stationary phase.

The cultures (usually 24 at a time) were spun at 6000 x g for 5 minutes, and the supernatant poured into clean centrifuge tubes. 1 ml of 20% PEG in 2.5 M NaCl was added, and the mixture vortexed and allowed to stand at 4°C for several hours (up to several days).

The PEG precipitate was centrifuged at 12,000 rpm in a Sorvall SAE600 rotor for 10 minutes, and the supernatant carefully poured off and discarded (after decontamination). Each pellet was then redissolved

in 0.5 ml of LT, and transferred to microfuge tubes. Each sample was then extracted once each with phenol, three times with phenol/chloroform 1:1, and once with chloroform, removing the aqueous phase to a fresh microfuge tube each time. The samples were then extracted with ether, and the DNA precipitated by addition of 50 µl of 0.1 M spermine tetrahydrochloride and incubation at 4°C for several hours (Hoopes and McClure, 1981).

Spermine precipitates were spun down for 5 minutes in the microfuge, and redissolved in 100 µl of 5 M ammonium acetate. Vigorous vortexing is required, especially for large pellets. After dissolution was complete, the DNA was diluted with one volume of TE, and reprecipitated by the addition of 3 volumes of ethanol. Samples were chilled at -70°C for 10-15 minutes, and then spun down for 5 minutes. The pellet was then redissolved in 100 µl of TE, made to 0.1M NaCl, and reprecipitated with 2.5 volumes of ethanol. Samples were kept in ethanol until needed. One night before use, samples were spun down, rinsed with 95% ethanol, and vacuum dried overnight.

Sequencing: The methods used are based on those of Sanger et. al. (1977) as outlined by Messing (1980). The following modifications have been found to improve the length and quality of the readable sequence ladder.

One-half of the template from a preparation is used for each set of reactions. This is about 10-50 times the recommended level. The template is redissolved in 10 µl of TE, rather than LT.

The amount of primer used is 1 µl, one-quarter the amount recommended. Increasing the amount of primer does not result in increased signal, and eventually leads to false priming and hence useless sequence ladders.

Annealing is performed as though the primer were double-stranded, with boiling for 3 minutes and heating at 55-60°C for 20 minutes. The annealing reactions are then transferred to 40°C, and kept at this temperature until use. Allowing the reaction to cool to room temperature

seems too allow secondary structure to form, which causes artifact bands at certain positions in all tracks, and leads to a shorter readable sequence.

The concentrations of the nucleotides not involved in the dideoxy/deoxy-nucleotide balance are doubled. The concentrations of all nucleotides in the chase solution are 20 mM.

The amount of polymerase used is approximately 1.0 U for each reaction. (Boehringer/Mannheim Klenow enzyme is preferred, although BRL works. NEN enzyme is very unstable upon storage, and up to 5 U are necessary after a few weeks of use.) No new enzyme is added with the chase.

The reaction tubes and nucleotide/dideoxy mixes are prewarmed to 37°C before the reaction is started. The initial reaction is 15-20 minutes at 37°C, and the chase is at least 15 minutes.

Reactions are stopped with 15 µl of formamide stop buffer, and kept on ice until being heated immediately prior to use. Samples are used within two hours of stopping.

The gel buffer is 90 mM Tris-borate, 2 mM EDTA (acid form), not pH adjusted. The two short gels are 7.6% acrylamide, 0.4% bis, while the long gel is 5.7% acrylamide, 0.3% bis. All gels are 60% (10M) urea. The high urea concentration is to increase denaturation even at lower temperatures (see tips, below). This high urea concentration necessitates some special precautions in handling the gel solutions to keep the urea in solution, but simple warming of the solution and the plates seems to be sufficient. The following tips have been found to be helpful for the timing of the reactions and/or the final results.

1. Gel forms and the gel solution are prepared the night before. The gel forms are made of 1/4-inch Pyrex plates to increase heat resistance and decrease smiling. The thicker plates also decrease bowing during polymerization, obviating the need for a bottom shim.

2. Templates are spun down, ethanol rinsed and vacuum dried the night before.

3. Gels are poured before or during the incubations. The gel plates need to be warm but not hot, and the combs must be scrubbed with ethanol before use. Gels should not polymerize for more than 1.5-2 hours before use, or else the teeth may stick to the comb.

4. Gels are prewarmed at 34-36 watts, and run at 32-34 watts constant power if a constant power supply is available (approximately 1500 volts if a constant voltage supply is used). Each gel is run on an independant power supply, because each gel will pull different amounts of current as it rewarms after loading. The gels are run warm but not hot to the touch, about 50-55^{o}C. Hotter temperatures do not increase resolution and cause increased smiling.

The first gel is stopped after the BPB has run off the bottom, about 2.5 hours. The intermediate gel is run until the XCFF would have traveled 1.4 gel lengths, about 6 hours. The long gel is run until the XCFF would have traveled about 1.75 gel lengths, usually about 6-7 hours.

Gels are fixed for 10 minutes in 10% acetic acid - 10% glycerol, and transferred to filter paper before drying for 1.5 hours in a BioRad gel dryer (80^{o}C under vacuum). Dried gels are autoradiographed at room temperature without intensifying screens, using Fuji X-ray film. Typically the first gel shows about 20,000 cpm to the Geiger counter at the XCFF dye band, and requires about 12 hours to expose properly. Longer exposures are necessitated if some of the tracks are faint and/or not balanced properly so that they fade out at one end.

Sequence Homology Identification: Sequence homologies were identified by using the "seqfit" program of Staden (1980). Windows of 15 nucleotides in length, beginning every ten nucleotides, were searched against the entire comparison sequence, and 60% homologous sequences were flagged. Homologies were identified by consecutive windows being homologous to consecutive sequences in the comparison sequence.

Secondary Structure Analysis: Potential secondary structures were found by creation of a matrix of possible complementarity between each nucleotide of a sequence and every other nucleotide, using pairing values as indicated by Tinoco (Tinoco et. al., 1971). After several potential secondary structures had been identified, each was drawn out, and the energy calculated (Tinoco et. al., 1973). The most stable in any given area was retained for comparison.

Computer Data Analysis of Sequence Information: Computer programs for sequence data handling and analysis were obtained from the Rockefeller University Computer Services network. These programs included the initial Staden programs (Staden, 1980), and several programs which were devised and/or adapted to use on the system by Peter Model and Warren Jelenik. Additional programs were devised for matrix analysis of possible secondary structures according to Tinoco's rules (Tinoco et. al., 1971), as were shell scripts for use of the Staden programs in an automatic fashion. Documentation of these programs is available on request either from Computing Services, Peter Model, or myself as applicable.

In Vitro Transcription: An S-100 fraction was prepared from Physarum plasmodia by a modification of the method described by Grummt (1981b). 25 ml of settled plasmodia from a three day growth were packed by centrifugation at 2000 rpm (750 x g) for 5 minutes, washed once with ice cold 1x SSCP, and repelleted. Packed volume was approximately 15 ml. The pellet was washed once with 20 ml 1/10x hypotonic buffer (1 mM HEPES pH 7.9, 1 mM KCl, 0.15 mM $MgCl_2$, 0.05 mM DTT), repacked, and then allowed to stand in 1/10x hypotonic buffer for 10 minutes at $4^{o}C$. When swelling was completed, 1/10 volume of isotonic adjusting buffer (0.3 M HEPES pH 7.9, 1.4 M KCl, 0.03 M $MgCl_2$) was added, and the mixture allowed to stand for a further thirty minutes before centrifugation at 100,000 g for 60 minutes. The supernatant was dialyzed against 20 mM HEPES pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 1 mM DTT, 0.1 mM EPNP and 0.1 mM PMSF overnight at $4^{o}C$, and stored frozen at $-80^{o}C$ until use.

In vitro transcription reactions were carried out with 50 µl of the S-100 mixture. 10.3 µl of reaction buffer (50 mM each of three cold NTPs, 500 mM creatine phosphate, 1 M $MgCl_2$, 1 M KCl, and 10 µCi/µl α-^{32}P-NTP with 10 mM corresponding cold NTP), 1 µg DNA, 200 µg/ml α-amanitin, 50 µg/ml Actinomycin D, or 10 µg of RNAse A were added as indicated, and the volume adjusted to 100 µl with H_2O. Reactions were incubated at 25°C for 30 minutes, extracted once with phenol, once with phenol-chloroform 1:1, and once with chloroform before precipitation with 3 volumes of 95% ethanol - 0.2 M Na-acetate.

Precipitated nucleic acids were redissolved in 14 µl of formamide loading buffer plus 2 µl of 50% glycerol, and loaded onto a 4% polyacrylamide gel (in TPES and 7 M urea) for electrophoresis at 100 V for 6 hours.

Statement of Compliance with Recombinant DNA Guidelines: All experiments involving recombinant DNA were done in compliance with the National Institutes of Health Guidelines for Research Involving Recombinant DNA that were in effect at the time of the experiment.

RESULTS

Restriction Enzyme Mapping of the rDNA: Previous results had already indicated that the restriction enzymes Eco RI and Hind III cleaved twice at each end of the palindromic rDNA molecule (Bradbury et. al., 1975). Utilizing these two enzymes in double digestion experiments, and other restriction sites as they became known, the data shown in Table 1 were generated. Using these data, and the knowledge that the rDNA molecule was palindromic, it was possible to unambiguously map the restriction enzyme cleavage sites for Bam HI, Pst I, Bgl II, Xho I, Sal I, and Pvu II (Table 2). In addition, a map was constructed of the cleavage sites of Hpa I which indicated that there was a region of very extensive digestion by this enzyme. The fragments resulting from a region of DNA nearly 3.3 kb in length were too small to visualize on the agarose gels we used, indicating that they were less than 0.3 kb in length. These fragments are collectively considered to be the Hpa h fragment in the discussion that follows.

During these restriction digestion analyses, it was consistently noted that the terminal restriction fragment appeared as a broad, heterogeneous band. A size variation of $\pm$ 0.4 kb was found, and was localized to the terminal 0.5 kb of the rDNA molecule. The size of the terminal fragments presented in Tables 1 and 2 are averages, as indicated in footnote 1 to Table 1.

Restriction Fragments Resulting from the Digestion of rDNA with Two Enzymes

Enzymes		Fragments & their assigned origin										
Eco RI	Hin III	34.6[1] Hin a	5.3[2] Eco b	5.0 Hin c	2.3 Eco c	0.4[3] (Eco a (Hin b						
Eco RI	Bam HI	16.3[1] (Eco a (Bam a	12.8[1] Bam b	2.7[2] Bam c	2.6 (Eco b (Bam a	2.3 Eco c						
Hin III	Bam HI	12.8[1] Bam b	10.9 (Hin a (Bam a	5.3 (Hin b (Bam a	5.0 Hin c	2.7[2] Bam c						
Eco RI	Bgl II[4]	35.6[1] Bg2 a	5.3[2] Eco b	3.3 Bg2 c	2.3 Eco c	1.8 (Eco a (Bg2 b						
Hin III	Bgl II[4]	34.6[1] Hin a	8.0[2] Hin b	3.3 Bg2 c	1.4 (Hin c (Bg2 b	0.4[3] (Hin c (Bg2 a						
Bam HI	Bgl II	12.8[1] Bam b	11.2 (Bam a (Bg2 a	6.3 (Bam a (Bg2 b	3.3 Bg2 c	2.7[2] Bam c						
Eco RI	Pst I	28.7[1] Pst a	8.3 (Eco a (Pst b	3.0[2] Pst c	2.3 (Eco c (Pst b	2.2 (Eco b (Pst d	0.1[3] (Eco c (Pst d					
Hin III	Pst I	28.7[1] Pst a	5.0 Hin c	3.0[2] Pst c	2.9 (Hin a (Pst b	2.7[5] (Hin b (Pst b	2.3 Pst d					
Bam HI	Pst I	12.8[1] Bam b	10.6 Pst b	8.0 (Bam a (Pst a	2.7[2] Bam c	2.3 Pst d	0.3[3] (Bam a (Pst c					
Bgl II	Pst I	28.7[1] Pst a	4.1 (Bg2 b (Pst b	3.2[6] Bg2 c	3.2[6] (Bg2 a (Pst b	3.0[2] Pst c	2.3 Pst d					
Eco RI	Hpa I	9.9 Hpa a	5.9 Hpa b	3.2 Hpa c	3.0[2] Hpa d	1.7 Hpa f	1.5 (Eco c (Hpa e	0.8 (Eco c (Hpa g	0.6[3] (Eco b (Hpa g	0.4[3] (Eco a (Hpa e	ə[7] Hpa h	
Hin III	Hpa I	5.9 Hpa b	5.0 Hin b	4.9 (Hin a Hpa a	3.2 Hpa c	3.0[2] Hpa d	1.9 Hpa e	1.7 Hpa f	1.4 Hpa g	ə[7] Hpa h	0.0[3,8] (Hin b (Hpa a	
Bam HI	Hpa I	9.9 Hpa a	3.2 (Bam b (Hpa b	3.2 Hpa c	2.7 (Bam a (Hpa b	2.7[2] Bam c	1.9 Hpa e	1.7 Hpa f	1.4 Hpa g	ə[7] Hpa h	0.3[3] (Bam a (Hpa d	
Bgl II	Hpa I	5.9[6] Hpa b	5.3[6] (Bg2 a (Hpa a	3.3[6] Bg2 c	3.2[6] Hpa c	3.0[2] Hpa d	1.9 Hpa e	1.7 Hpa f	1.4 Hpa g	1.3 (Bg2 b	ə[7] Hpa h	
Pst I	Hpa I	7.9 (Pst b (Hpa a	5.9 Hpa b	3.2 Hpa c	3.0[2] Pst c	2.0 (Pst a (Hpa a	1.9 Hpa e	1.7 Hpa f	0.8 (Pst b (Hpa g	0.6[3] (Pst d (Hpa g	ə[7] Hpa h	0.0[8] (Pst c (Hpa d

Hin III	Sal I	25.9[1] Sal a	5.9[2] Sal c	4.2 (Hin c (Sal b	2.1 (Hin b (Sal b	2.0[6] (Hin a (Sal d	2.0[6] Sal e	0.8 (Hin c (Sal d	0.3[3] Sal f					
Bam HI	Sal I	12.8[1] Bam b	6.6[6] (Bam a (Sal a	6.3[6] Sal b	3.2 (Bam a (Sal c	2.8 Sal d	2.7[2] Bam c	2.0 Sal e	0.3[3] Sal f					
Pst I	Sal I	25.9[1] Sal a	6.3 Sal b	3.0[2] Pst c	2.8 Sal d	2.3 Pst d	1.1 (Pst a (Sal e	0.9 (Pst b (Sal e	0.6[3] (Pst a (Sal c	0.3[3] Sal f				
Hpa I	Sal I	5.9 Hpa b	4.2 (Hpa a (Sal b	3.2 Hpa c	3.0[2] Hpa d	2.8 Sal d	2.0 Sal e	1.9 Hpa e	1.7 Hpa f	1.2 (hpa g (Sal c	0.6[3] (Hpa a (Sal a	0.3[3] Sal f	a[7] Hpa h	0.2[3] (Hpa g (Sal b
Hin III	Xho I	26.5[1] Xho a	5.9[2] Xho b	3.1 Xho c	2.0 (Hin c (Xho e	1.8 (Hin a (Xho d	1.5 Xho f	1.2 Xho g	1.1 Xho h	0.3[3] (Hin c (Xho d	0.1[3] (Hin b (Xho e			
Bam HI	Xho I	12.8[1] Bam b	6.9 (Bam a (Xho a	3.2 (Bam a (Xho b	3.1 Xho c	2.7[2] Bam b	2.1 Xho d	2.1 Xho e	1.5 Xho f	1.2 Xho g	1.1 Xho h			
Pst I	Xho I	26.5[1] Xho a	3.1 Xho c	3.0[2] Pst c	2.3 Pst d	2.1 Xho d	2.1 Xho e	1.5 Xho f	1.2 (Pst a (Xho g	1.1 Xho h	0.6[3] (Pst b (Xho b	0.1[3] (Pst b (Xho g		
Hpa I	Xho I	5.9 Hpa b	3.2 Hpa c	3.1 (Hpa a (Xho c	3.0[2] Hpa d	2.1 Xho d	1.9 Hpa e	1.6 Hpa f	1.5 Xho f	1.2 Xho g	1.2 (Hpa g (Xho b	1.1 Xho h	0.9 (Hpa a (Xho a	a[7] Hpa h	0.2[3] (Hpa g (Xho c	0.0[3,8] (Hpa e (Xho c
Pst I	Pvu II	28.7[1] Pst a	3.5 (Pst b (Pv2 a	3.2 Pv2 c	3.0 (Pst b (Pv2 b	2.5 (Pst c (Pv2 b	2.3 Pst d	0.9 Pv2 d	0.5[2,3] Pv2 e					
Hpa I	Pvu II	5.9 Hpa b	5.6[7] (Hpa a (Pv2 a	3.2 Hpa c	2.5 (Hpa d (Pv2 b	1.9 Hpa e	1.7 Hpa f	1.4 Hpa g	0.9 Pv2 d	0.5[2,3] Pv2 e	0.3[3] (Hpa a (Pv2 b	a[7] Hpa h		
Xho I	Pvu II	26.5[1] Xho a	5.4 (Xho b (Pv2 b	2.1 Xho d	2.1 Xho e	1.9 (Xho c (Pv2 c	1.3 (Xho f (Pv2 d	1.2 Xho g	1.1 Xho h	0.9 Pv2 d	0.5[2,3] Pv2 e	0.3[3] (Xho e (Pv2 b	0.2[3] (Xho f (Pv2 d	

1 Fragment is too large to determine the size directly - size stated is determined by summing subfragments.

2 Size represents an average value for a heterogeneous band.

3 Fragment is too small to be seen in our gel system.

4 The Bgl II site at 9.4 kb may be a closely spaced doublet, such as 9.0 and 9.4 kb.

5 The fragment runs as if it was a smaller size. This may indicate that one of the flanking sites is is actually a closely spaced doublet of sites.

6 These pairs of fragments run as an indistinguishable doublet.

7 Represents frogments of less than 400 nucleotides in length, which collectively span 3.3 kb and are Hpah.

8 These two restriction sites are indistinguishable on the basis of the current data. Fragment origin assigned is provisional.

Sizes and Coordinates of Physarum rDNA Restriction Fragments

Enzyme	a	b	c	d	e	f	g	h	i
Eco RI	7.6-53.0 (45.4)	0 -5.3 (5.3*)	5.3-7.6 (2.3)						
Hind III	13.0-47.6 (34.6)	0 -8.0 (8.0*)	8.0-13.0 (5.0)						
Bam HI	2.7-23.9 (21.2)	23.9-36.7 (12.8)	0 -2.7 (2.7*)						
Bgl II	12.5-48.1 (35.6)	0 -9.4 (9.4*)	9.4-12.5 (3.1)						
Pst I	15.9-44.7 (28.8)	5.3-15.9 (10.6)	0 -3.0 (3.0*)	3.0-5.3 (2.3)					
Hpa I	8.0-17.9 (9.9)	21.2-27.1 (5.9)	27.1-30.3 (3.2)	0 -3.0 (3.0*)	6.1-8.0 (1.9)	3.0-4.7 (1.7)	4.7-6.1 (1.4)		
Sal I	17.3-43.3 (26.0)	5.9-12.2 (6.3)	0 -5.9 (5.9*)	12.2-15.0 (2.8)	15.0-17.0 (2.0)	17.0-17.3 (0.3)			
Xho I	17.0-43.6 (26.6)	0 -5.8 (5.8*)	8.0-11.1 (3.1)	12.6-14.7 (2.1)	6.0-8.0 (2.0)	11.1-12.6 (1.5)	15.8-17.0 (1.2)	14.7-15.8 (1.1)	5.8-6.0 (0.2)
Pvu II	12.4-48.2 (35.8)	0.5-8.3 (7.8)	9.2-12.4 (3.2)	8.3-9.0 (0.9)	0 -0.5 (0.5*)				

* Hetergeneous band, corresponding to the rDNA terminal fragment. Size given is an average, the band spans ± 400 bp.

Table 1: (previous two pages) Sizes of Restriction Fragments Resulting from Digestion of Physarum rDNA with Two Restriction Enzymes. Fragment sizes are given in kilobase pairs.

Table 2: Sizes and Coordinates of Physarum rDNA Restriction Fragments. Fragment sizes are given in kilobase pairs; coordinates are given in kilobase pairs beginning at an "average" end. Terminal fragments are heterogeneous in size, varying by ±400 base pairs from the average end at 0.0 kilobase pairs.

The overall restriction map presented in Fig. 6 and Table 2 may be considered a good overall guide to the location of the large restriction fragments present in rDNA restriction digestions, with the possible exceptions noted in the footnotes to Table 1. However, any given restriction site may in fact be present as a tightly spaced doublet of sites, as underscored by the discovery that the Sal I single site originally presented (Campbell et. al., 1979) at 17.3 kb was in fact such a doublet (Table 2), as was the single Xho I site originally presented at 5.9 kb (Kukita et. al., 1981).

In addition, Ferris and Vogt (1982) claim that the two closely spaced Hpa I cuts in the center of the molecule on that map are not present, with the Hpa c fragment spanning the center of the molecule and being represented only once per molecule. This would result in a Bam b fragment of only 9.6 kb in length. Our information clearly indicates that the Bam b fragment is in fact substantially longer than 10 kb, and that the Hpa c fragment is present in equimolar quantities with several other fragments which are represented twice per molecule. We therefore believe that this difference represents an acquired strain difference between the a x i and M_3C VIII strains.

We have considered the possibility that some restriction sites may be protected by DNA modification. Information from another laboratory (Harold R. Matthews, personal communication) indicates that the rDNA of Physarum is modified very rarely if at all, indicating that all restriction sites should in fact be cut. This is of some interest given the great variability in the amount of methylation which occurs on the rDNAs of various species (Bird and Taggert, 1980 and Rae and Steele, 1979).

It appears from the map presented here that there is a relative absence of restriction sites in the central spacer region. This is due to the presence of large blocks of repeated sequences, as indicated by the restriction patterns of Hpa I (Table 1) and Kpn I (Ferris and Vogt, 1982), which cut very frequently in a portion of the central spacer. Any restriction site not represented in the fairly short repeat sequence would therefore not cleave (or cleave only very rarely) in the large 28

kb central spacer region, whereas those enzymes which do cleave in the repeat would produce extremely small fragments, and therefore be difficult or impossible to map. This would result in a selection of the data, causing it to indicate a lack of restriction sites such as seen in Table 2 and Fig. 6.

In some cases, notably with the restriction enzyme Xho I, the heterogeneous terminal fragment appears to be cut at a second restriction site in a subpopulation of the rDNA molecules. This is most clearly demonstrated in the 26 S rRNA hybridization shown in Fig. 3, where two sharp bands, slightly smaller than the fuzzy terminal fragment of 5.9 kb, also hybridize with the labeled rRNA. Whether this restriction site heterogeneity could be generated by some means which does not involve sequence heterogeneity is unknown, but the simplest assumption is that subpopulations of rDNA termini are present, some of which contain a restriction site that is not present in the sequence of other populations of rDNA termini. This point will be considered in detail in the section dealing with terminal sequences and their possible mode of replication.

Sequence heterogeneity is not prominent in other areas of the rDNA, although the region immediately upstream of the promoter (in the Hpa I repeats) may be heterogeneous in length (Vogt and Braun, 1976b). Also, strain differences are known to occur (compare present results and Ferris and Vogt, 1981). This observation suggests the presence of some mechanism which maintains the rDNA population as a homogeneous family. This mechanism may be nothing more than ordinary homologous recombination (c.f. Bird, 1980 and Dover, 1982), occurring at a higher frequency than normal because of the high concentration of identical sequences present in close proximity, and the lack of any constraints on such recombination which would result from problems in resolving structures distal to the recombination site. Such recombination has been observed in chromosomal rRNA genes in primates (Arnheim et. al., 1980), and is presumably fairly common, as evidenced by the dispersion of rDNA spacer sequences throughout the genome (Arnheim et. al., 1980, Dawid et. al., 1981 and Yao et. al., 1981). If in fact this is the case, the addition

of a large number of altered rDNA molecules may result in the creation of an unstable population which would resolve itself by eliminating one or the other of the two alternative rDNA forms. Experiments of this type may be possible, using two different Physarum strains which differ in their rDNA restriction patterns. Alternatively, such a difference could be created by recombinant DNA technology, and artificially altered rDNAs inserted into growing Physarum plasmodia. Such experiments should be carried out when the rDNA is used as a recombinant DNA vector.

Mapping of the Major rRNA Coding Regions

Mapping of rRNA Genes by Southern Hybridization: Purified rDNA was digested to completion with each of the restriction enzymes listed in the preceeding section, and transferred to nitrocellulose filters by the method of Southern (1975). Filters or filter strips were then hybridized separately with 19 S or 26 S rRNA labeled in vivo to a specific activity of approximately 5×10^5 cpm/µg, in the presence of a 3-5-fold molar excess of the cold, heterologous rRNA. The filters were then treated and autoradiographed as described.

5.8 S rRNA was isolated as described, and labeled by polynucleotide kinase and γ-^{32}P-ATP after partial alkaline hydrolysis. This RNA was then hybridized to filter replicas of Hind III or Xho I digested rDNA as described.

The results of these experiments are shown in Fig. 3. 19 S rRNA hybridizes to Hind III a b, and Xho I d and f but not c or h, and Sal I b and d, indicating that this gene spans a region from less than 12.2 kb but greater than 11.1 kb, to greater than 13.0 but less than 14.7 kb on the rDNA map. It was assumed from the very slight hybridization with Hind III a that the end point was closer to 13.0 than 14 kb. Since this gene is expected to occupy 2.1 kb of DNA (Melera and Rusch, 1973), these results indicate that the gene is not much larger than the minimum coding length. Other hybridizations are consistent with this interpretation.

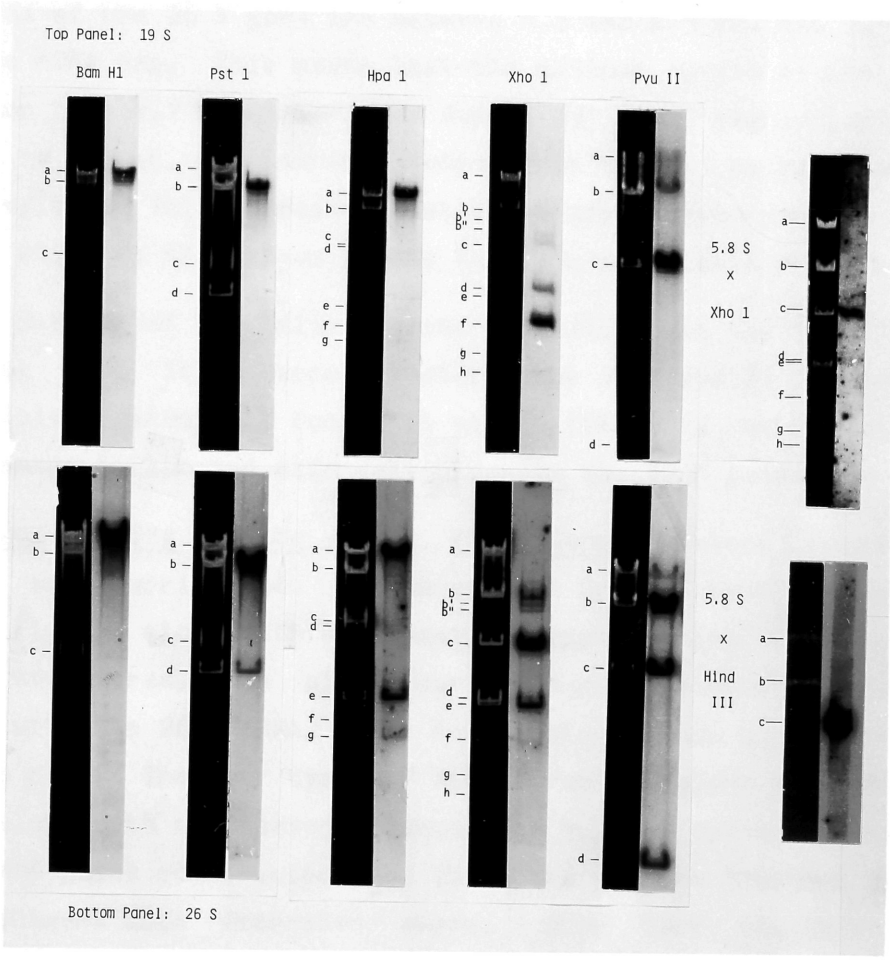


Figure 3: Southern Hybridization of rRNA species to rDNA Restriction Fragments. ^{32}P labeled 19 S (upper panel), 26 S (lower panel) and 5.8 S (right) rRNAs were hybridized to Southern transfers of various rDNA restriction digests as indicated.

The 26 S rRNA hybridizes to Bgl II b and c, Hpa I a, e and g but not f, Xho I b, c and e but not f, and Pst b and d, indicating than the boundaries of the 26 S gene are between 4.7 and 5.3 kb, and 9.4 and 11.1 kb on the rDNA map. This means that the minimum length of the 26 S gene is greater than 4.1 kb (Melera and Rusch, 1973) and the length could be as much as 6.4 kb, considerably longer than the 4.1 kb required for the coding regions. This indicates that there may be intervening sequences present, which is also shown by the R-loop mapping data shown below.

The 5.8 S rRNA hybridizes to the Hind III c and Xho I c fragments, indicating that it is located between the 19 S and 26 S genes. It is not possible to precisely locate it within this area using this data, but it cannot be located extremely close to the 19 S gene.

R-Loop Mapping of the Major rRNA Genes: R-loop formation and analysis was carried out as described. Several hybrid molecules are shown in Fig. 4, along with a schematic representation of one of the looping structures. In all, four types of R-looping structures were observed with the 26 S rRNA, while only a single loop was ever observed with 19 S rRNA. The four types of structures are shown schematically in Fig. 5, along with the measured lengths of each of the units, and a map of the rRNA genes constructed from this data and the Southern hybridization experiment data described above. Note that the hybridization results and the R-loop mapping data are consistent with each other, and together give a very complete and precise picture of the structure and location of the rRNA genes. These results were subsequently confirmed by other workers (Gubler and Braun, 1979). More precise results should become available soon as the DNA sequence of this region is determined.

Figure 6 presents a summary restriction map showing the surmised positions of each of the rRNA genes.

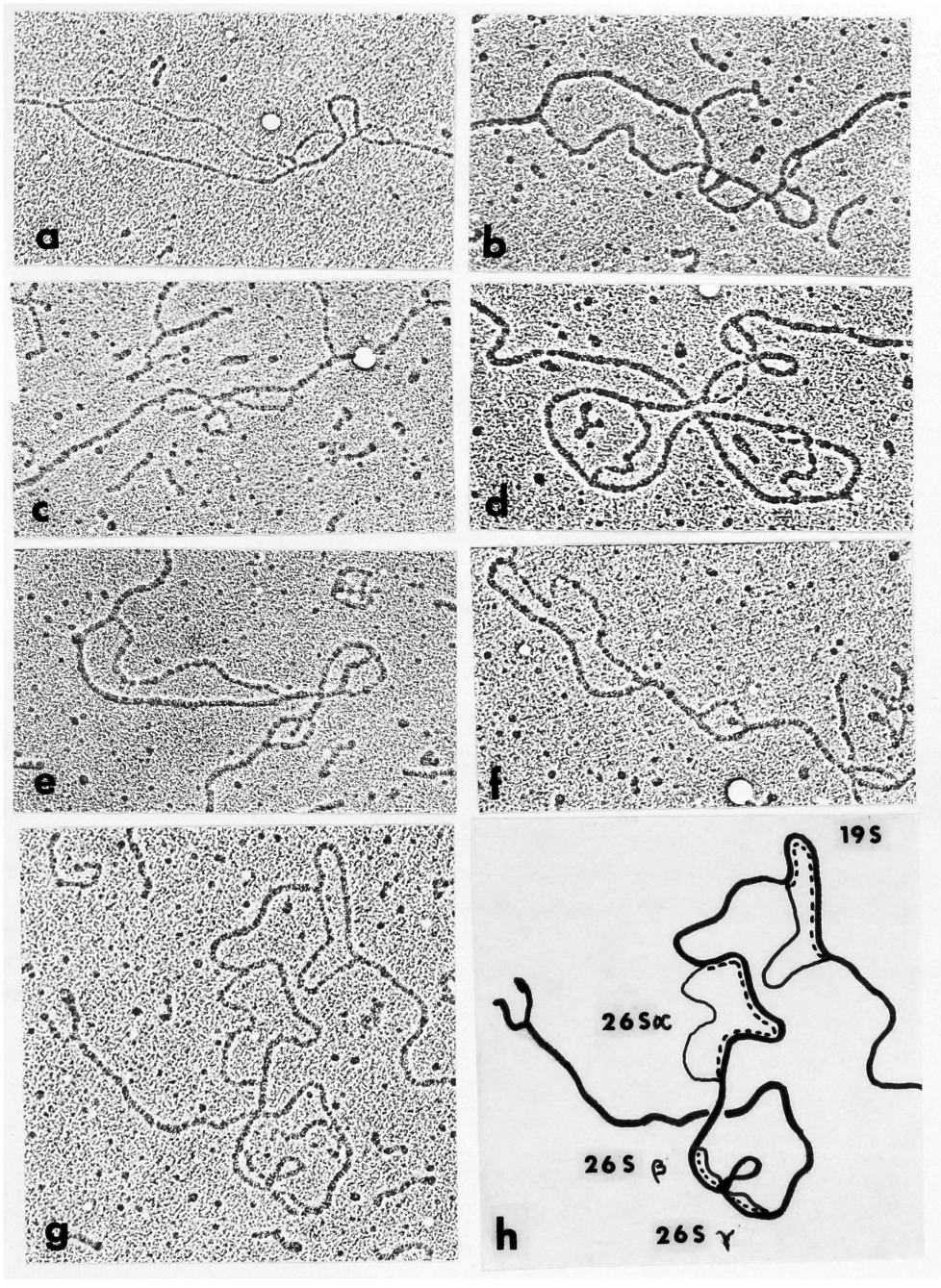
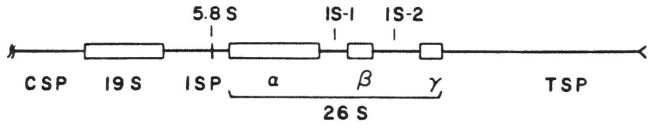


Figure 4: R-Loop Hybridization of 19 S and 26 S rRNA to rDNA.

a,b - Five-loop structure seen with 26 S rRNA (24%).
c,d - Separate α-loop and β,γ-triple-loop seen with 26 S rRNA (37%) (d also includes a 19 S loop)
e,f - Three separate loops seen with 26 S rRNA (19%)
g 19 S rRNA loop plus separate 26 S α and β,γ loops
h Schematic drawing of g



	CSP	19 S	ISP	α	IS-1	β	IS-2	γ	TSP
kb	35.64 ±2.08	2.10 ±0.21	1.66 ±0.12	2.41 ±0.33	0.68 ±0.13	0.70 ±0.11	1.21 ±0.14	0.59 ±0.14	5.37 ±0.88

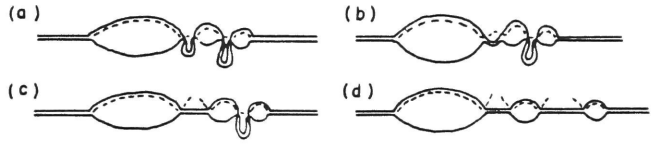


Figure 5: R-Looping Structures and rRNA Coding Region Map. Upper: Map of the rRNA coding regions. CSP = central spacer, ISP = internal (transcribed) spacer which includes the 5.8 S gene, IS-1 and 2 = intervening sequence 1 and 2, TSP = terminal spacer.
Center: Sizes of the various components of the rRNA transcription unit, in kb, with standard deviations, as determined from R-looping.
Lower: Schematic drawings of the common 26 S rRNA R-loop structures.

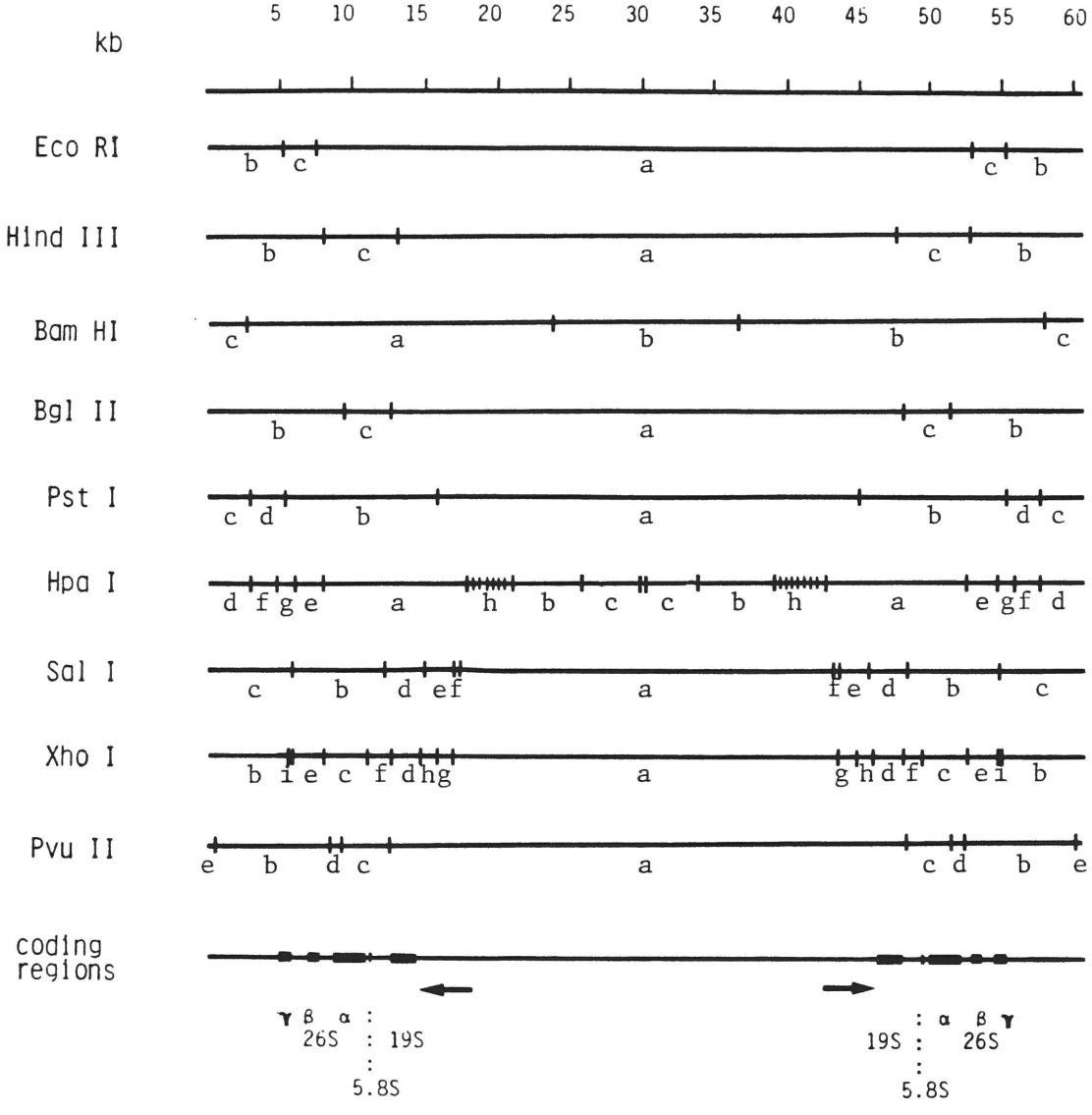


Figure 6: Restriction Site and Transcription Unit Map of rDNA. Cut sites of the indicated restriction enzymes are shown along an rDNA molecule with "average" length terminal restriction fragments (see text). Fragment names are indicated. The Hpa I h fragment represents a region of 3.3 kb which is cleaved into an unknown number of small (less than 0.3 kb) fragments.
Lower: Coding regions are indicated by thickened lines. The arrow indicated start site and direction of transcription.

Transcription Initiation Region

Cloning of rDNA Regions Containing the Transcription Initiation Site: Previous results (Grainger and Ogle, 1978) had indicated that the direction of transcription on the rDNA palindrome was from the central spacer region outward toward the termini. Mapping of the transcription initiation site by Sun (Sun et. al., 1979), using γ-S-nucleotide triphosphates, indicated that the initiation site was located central to, and close to the center-most Xho I site at 17.0 kb.

As can be seen from the restriction map in Fig. 6, this site is in the large central spacer region which is not cleaved by most restriction enzymes. This severely limits the enzymes which may be used to generate clonable fragments from this region - in fact the only enzyme which gives cohesive ends which cleaves further upstream is Bam HI. Since Bam HI had to be one of the enzymes used to clone the initiator fragment, is was decided to use as the other end a Bgl II site, which would leave the same cohesive ends. In addition, it was expected that this combination of enzymes would generate clones containing large fragments of the rDNA that spanned its over 90% of the molecule, from the Bam HI site at 2.7 kb to the corresponding site at the other end of the molecule.

A restriction mixture containing 2 μg of rDNA which had been restricted to completion with Bam HI, and then digested in two aliquots to either approximately 1/3 or 2/3 completion with Bgl II, was prepared. The two aliquots were combined, and added to 5 μg of Bam HI-cut Charon 28 DNA. The mixture was ligated overnight at 4°C, and transformed into K802 competent cells. Resultant plaques were picked for further analysis. The number of recombinants was extremely low, in comparison to a mock ligation mixture containing no rDNA. This indicates that something in the rDNA preparation interfered with the ligation reaction or transformation, or that the rDNA fragments gave rise to nonviable recombinants. This was assumed to be caused by contamination of the rDNA preparations with mucopolysaccharide, which inhibits DNA ligase,

and was not further investigated.

One recombinant was found to contain the initiation region. This clone, named PrD100, spanned the rDNA molecule from the Bam HI cut at 23.9 kb to the Bgl II site at 9.4 kb, and did not contain any major deletions as determined by restriction mapping. However, there were a few restriction enzyme sites present on the rDNA restriction map that were not present in the clone, and vice versa. The Bgl II site at 12.5 kb on the rDNA map had disappeared, as had the Xho I site at 15.8 kb. One additional Sal I site was found at 18.1 kb. In addition, during the period of culture of this clone, an additional Eco RI site appeared at approximately 17.9 kb. This site was not present in the first large-scale DNA preparation, but was present in the fourth preparation and all subsequent ones. No recloning had occurred during this period. It is interesting to note that three of the four restriction enzyme site changes are within less than one kilobase of the transcription initiation point. This region is in an area that has been identified as having length heterogeneity in the rDNA (Vogt and Braun, 1976a). These variant sites may therefore simply reflect restriction site heterogeneity in the rDNA population. Alternately, they may be the result of mutations during or after the initial cloning event. This is certainly the case for at least one of the restriction site changes, which appeared during the period of large-scale amplification and subsequently overgrew the culture, apparently due to a significant selective advantage during growth. This mutation, and any others, have not kept this cloned fragment from being active in an <u>in vitro</u> transcription assay, nor have they interfered with the S1 protection mapping experiments (see below). The changes are therefore extremely minor (probably a single base-pair transition).

Since the Sal I sites produced two very small fragments, one of which must contain the transcription initiation site, PrD100 DNA was subcloned by Sal I digestion and ligation into Sal I-cut M13mp9. Several recombinant plaques were identified by the lack of color reaction with X-gal, and picked for further analysis. Many of these plaques, upon sequencing, showed vector sequences with single nucleotide

deletions at the Sal I site, indicating that a small amount of exonuclease activity was present in the restriction or ligation reaction. However, several showed new sequences, and were saved for further testing.

Minipreps of the RFs from these clones were Sal I restricted and analyzed by gel electrophoresis. In addition, the DNA fragments were transferred to nitrocellulose and hybridized with authentic nick-translated rDNA to unambiguously identify those clones with an rDNA origin. Two clones were found to originate from the 0.9 kb fragment of PrD100, between the Sal I site at 17.3 kb on the rDNA map, to the new Sal I site at 18.2 kb. Both of these clones were found to contain the new Eco RI site, and both were otherwise identical in all restriction sites and in their sequence beginning at the primer-proximal Sal I site through the Nru I site. Both are therefore considered to be identical, and were used interchangeably in the following experiments and subclonings.

During the Sal I subcloning, none of the other Sal I fragments of PrD100 were cloned, or were cloned but underwent extensive deletion. In the case of the larger fragments, the latter possibility is likely since M13 is known to delete large inserts (Messing, 1980). The lack of the 0.3 kb Sal g fragment is unusual, and may reflect some interaction between insert sequences and host or vector sequences or functions.

Subcloning of the S24 clone is diagrammed in Fig. 7. During the restriction enzyme subcloning procedures using Ava II/Sal I, Eco RI/Sal I, Nru I/Bam HI, and Sma I, the indicated restriction enzyme cuts were made in purified S24 DNA. In the case of Bam HI, the restriction was followed by nick translation with Klenow fragment, to make the cohesive end blunt. The restriction mixtures were then precipitated, redissolved in ligase buffer, and ligated without added vector. Ligation mixes were transformed into competent JM103, and several plaques were picked. These clones are designated with names beginning with S24A, S24E, S24N, and S24Sm, respectively, for each type of subcloning. In the Ava II/Sal I and Eco RI/Sal I subcloning, parental (blue) plaques were generated,

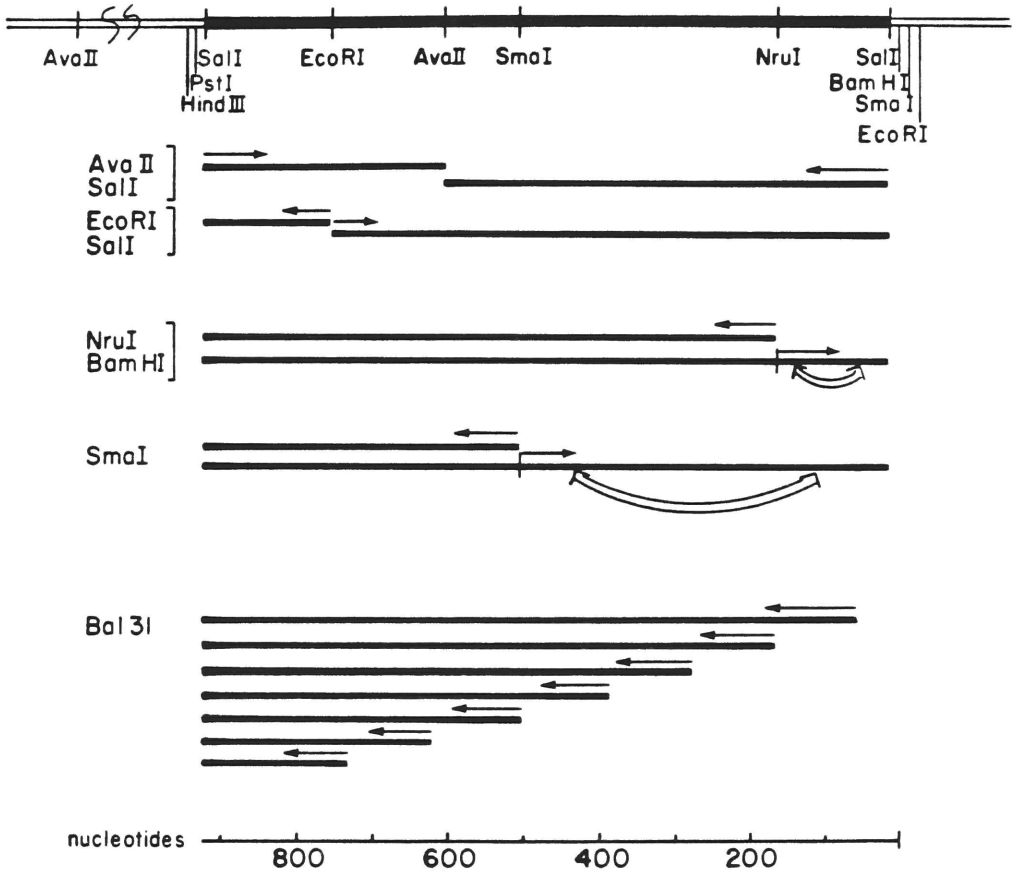


Figure 7: PrD100S35 Subcloning and Sequencing Strategy. a: Restriction map of the PrD100S35 (and PrD100S24) insert fragment and flanking regions. Scale (below) in nucleotides is numbered with 0 at the Sal I site to the right, near the sequencing primer site. Other lines indicate the possible subclone fragments obtainable from each type of subcloning procedure (see text). Arrows indicate the end of the subclone which would be near the priming site. Open two-ended arrows indicate that the fragment indicated would be inverted.
b: Actual start sites and directions for sequencing are indicated by arrows. Sequencing from these sites allowed a determination of the major part of the sequence for this clone, presented in Fig. 8.

as expected. The ratio of clear to blue plaques was extremely high in the Eco RI/Sal I mixture, again as expected since the polylinker fragment between the Eco RI and Sal I sites is extremely short, and would

not precipitate as efficiently as the longer insert fragments. The ratio of clear to blue plaques in the Ava II/Sal I subcloning was approximately 2 to 1, as would be expected from the number of potential subclones which could be generated.

In each type of subcloning, three clear plaques were picked for template preparation and sequencing. The start sites and direction of sequencing are also shown in Fig. 7. In addition, in the Ava II/Sal I and Eco RI/Sal I subclonings 24 plaques were picked for phage DNA preparation, and tested for complementarity to S24 phage DNA.

The first two types of subcloning would be expected to yield each end of the insert, one of which would be in an inverted orientation relative to the S24 clone. (In these two cases the M13mp9 parental type should also be regenerated.) In addition to these types of clones, multiple fragments could be inserted tandemly. However, in no case was any portion of the original insert found in a reversed orientation. Since some 48 clones were tested for complementarity to the S24 clone, this finding indicates that some orientation- or strand-specific interaction is interfering with the cloning procedure. Similar phenomena have been noted previously in the f1 and M13 cloning systems (Phyllis Moses and Gian Paolo Dotto, personal communications).

In the latter two cases, a fragment between the two indicated restriction sites would be expected to be deleted in the majority of subclones; however this fragment may also reinsert in the original orientation (regenerating the parental S24 clone), or in the opposite orientation.

In the small number of these subclones which have been sequenced, there is no instance of the small restriction fragment reinserting in the opposite orientation. However, neither is there an example of it reinserting in the same orientation, so this may merely reflect a propensity for circle formation in the ligation reaction.

In addition to subcloning from restriction fragments, deletions from the end nearest the primer site were created by cleaving S24 DNA

with Bam HI, digesting for a wide range of times with BAL31 nuclease as described in the Methods section, nick translating with Klenow fragment to generate blunt ends, and cleaving the opposite end of the insert with Hind III. In order to prevent the original vector from ligating to the digested insert molecules and forming viable phage which lack the priming site (which would be useless for sequence determination), the S24 DNA was cut with Bgl II in addition to Bam HI. Bgl II cuts once within an M13 gene, so that BAL31 digestion beginning at this site should delete information required for formation of a viable recombinant. The shortened insert fragments with one Hind III cohesive end were then ligated, along with the original vector DNA from the BAL31 digest, to Sma I and Hind III cut-M13mp9, and transformed into competent JM103 as described. Clear plaques were picked, replaqued for purification, and grown as described in Methods for sequencing template preparation. These clones are designated as the S24d1 and S24d2 series, for the first and second pools from the BAL31 digestion.

One of these deletion mutants, S24d1.5, contained approximately 180 nucleotides of sequence from the "far" end (away from the primer site), which contained the transcription initiation site. In order to determine more exactly which sequences are necessary for the accurate in vitro transcription of this sequence, further BAL31 deletion mutants were constructed using this clone as the starting point. Deletions were created starting at the upstream (from the initiation site) end by Sal I restriction and BAL31 digestion, followed by nick translation and ligation without added vector. Several such deletions have been isolated, and will be sequenced and used as substrates in in vitro transcription reactions.

Sequence of the S24 Subclone: The sequences obtained from these various subcloning procedures were combined to produce the partial sequence of the initial S24 clone, as shown in Fig. 8. Note that each restriction site has been sequenced through, ruling out the possibility that any given restriction site is a tandem doublet of sites that would not be detectable by restriction fragment analysis. Most portions of the sequence have been sequenced at least twice from two different start

```
****          10          20          30          40          50
   0 gtcgactccc ggccgttcga atggccgaat gtcacgaaac cacgtttacc
  50 cgccagacgc tcactacagg atcggaatct gcgttgtgcc caactgattc
 100 gcacggagga gagaggtctc tacactggtg ttttgagaac gtgtttcgcg
 150 atccgacaga atccaagcgg taccccacct ccccccaacca cgccggaacc
 200 ggttaaagtc aacggggaaa agaaagaaga gatacgcgct tgcttcacgg
 250 ttgccgacag aaaggggttc cgtcattgtt ttgtccgagc agacaatgcg
 300 gttcgaagcg ccccggatgg tgcatagcca cacgaacgag ttccatacgc
 350 ctccatccac cttaaattat atctaatcgg cctgacacac cggttggctc
 400 aagattcgcc cagggctccc ctaagataat ccgattccca aga-taattt
 450 agcac
 500 cccggggatg cacagcggcc aacagtaata tagggcggcg aagggcgata
 550 ggaaaggaga acaccgccgt taagtggcgt ctcccccca cctccacatc
 600 gggtcccgtt taggggtata gagtgtttat ggcttggatt gaacaaacgt
 650 ttttgtccca cc
 700
 750 gcgtggattg tggaattcca aatgtgtcct gcttgcttga tgccgtagag
 800 gctcttgttc agccttgagc acgatatccc tagttctcta ctgtatccgt
 850 cgtcccatag tgccaccttg aggtggacag tacag-ctgt atctatgtct
 900 cgtgaggtac gctaagtcta tgtcgac
```

Figure 8: Sequence of PrD100S24. The sequence of portion of the PrD100S24 (equivalent to PrD100S35) clone. Blanks represent areas of undetermined sequence. The numbering of the last two portions of DNA is tenative, pending determination of the complete sequence. Sequence starts at the Sal I site to the right of the restriction map in Fig. 7.

sites, so the possibility of any artifact is minimal. However, it has not been possible to confirm this sequence by sequencing the complementary strand since clones in the opposite orientation have not been found.

S1 Protection Mapping of the Transcription Initiation Region: In order to determine the exact location of the transcription initiation site for the 42 S pre-rRNA, S1 protection mapping was carried out as described.

Initial attempts to perform S1 mapping with isolated total nuclear RNA were unsuccessful. This is not surprising, however, since Physarum is known to process RNA precursor molecules very quickly (unpublished observations, and Gubler et. al., 1980), and nuclear isolation requires several hours before the first phenol extraction which would unequivocally stop protein-mediated processing. Since nuclear RNA seems to be

processed too quickly to use, total RNA had to be substituted.

In order to determine which restriction fragment the initiator was located in, DNA from clone PrD100 was digested with Sal I, treated with bacterial alkaline phosphatase, and end labeled with polynucleotide kinase and γ-^{32}P-ATP. The labeled fragments were then separated on an agarose gel, and recovered by electroelution. Each purified fragment was then hybridized with a vast excess of total Physarum RNA, followed by digestion with S1 nuclease to destroy any unhybridized fragments. The resultant mixture was reprecipitated, redissolved in gel loading buffer and electrophoresed on an acrylamide-urea gel. Radioactive bands were located by autoradiography. Sizes of these bands were calculated by comparison with a photograph of a co-electrophoresed λ DNA Hind III digest.

Only one Sal I fragment gave a consistent protection fragment. This fragment is the 0.92 kb fragment that lies between 17.3 and 18.2 kb on the rDNA map. This is the correct region for initiation to occur, from the results of Sun et. al. (1979). This fragment was subcloned into M13mp9 as described above, and additional S1 mapping was carried out.

When Sal I cut S35 DNA is hybridized with total Physarum RNA and S1 protection mapped, a large band of either approximately 830 or 690 nucleotides was found, as shown in Fig. 9. The sizes of these fragments are not accurately determined in this gel system.

When S35 DNA is Eco RI cut and S1 protection mapped, a protection fragment of approximately 55 nucleotides was obtained. This would correspond to 805 nucleotides from the Sal I site. These results are diagrammed in Fig. 11.

Using one particular preparation, a protection fragment with Eco RI cut S24 DNA was not found. An Ava II protection fragment of approximately 80 bp was obtained, corresponding to approximately 680 nucleotides from the Sal I site. As the two results each seem to be reproducable, the results seem to indicate that there are in fact different

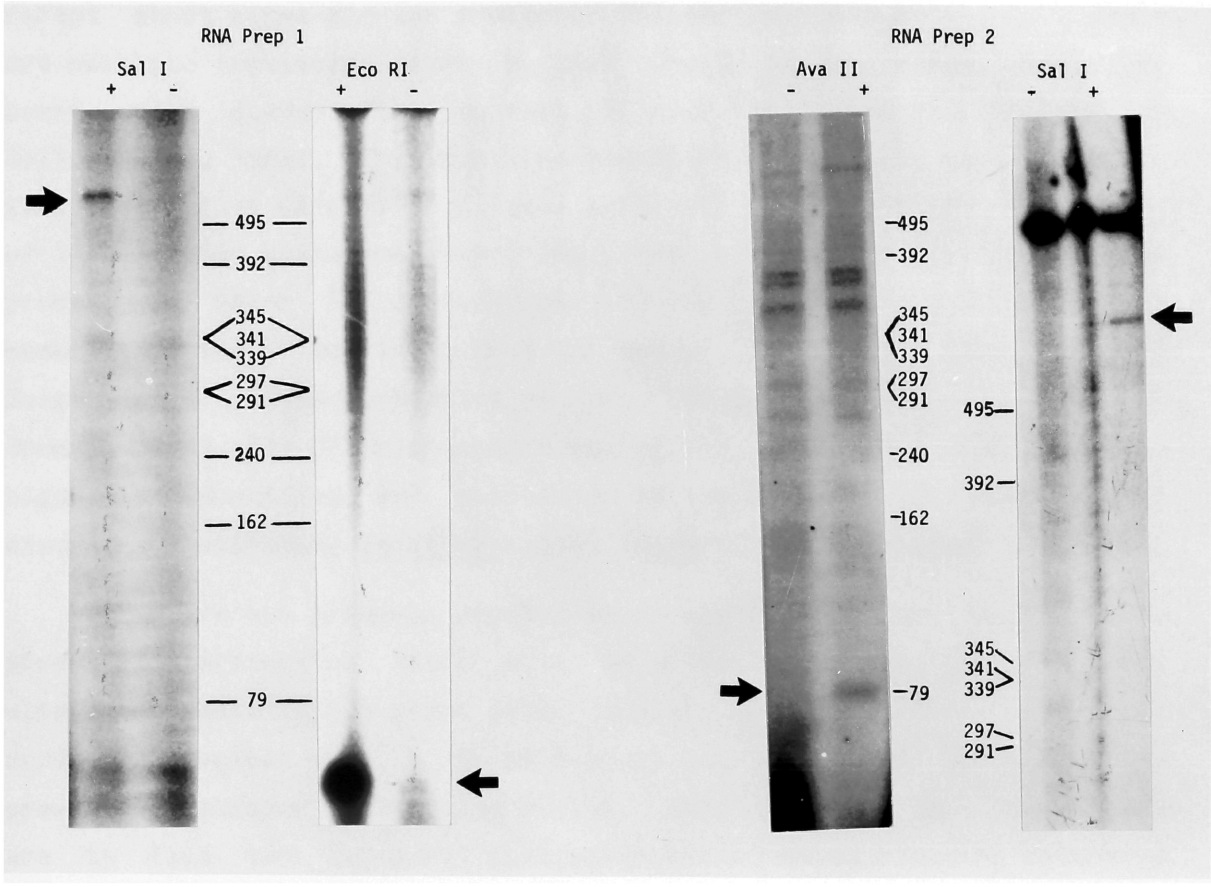


Figure 9: S1 Nuclease Protection Mapping of the pre-rRNA Transcript on PrD100S35 Cloned DNA Fragments. Two distinct types of results are shown. The first type (left) gives a Sal I fragment protected region of approximately 820 nucleotides, and an Eco R1 fragment protected region of 55 nucleotides, both indicating an RNA start site at 815 nucleotides on the PrD100S35 map. The second type (right) gave a Sal I fragment protected region of 680 nucleotides and an Ava II fragment protected region of 80 nucleotides, both mapping to nucleotide 680 on the PrD100S35 map. All sizes are ± 10 nucleotides or 5%, whichever is larger.

lengths of rRNA precursors which hybridize to this region of DNA. Different start sites for the precursor RNA can indicate either that there are multiple initiation sites, or that there is an early processing event which cleaves the nascent RNA near the 5' end (c.f. Miller and Sollner-Webb, 1981). They can also result from artifacts such as localized melting at oligo-T or oligo-A stretches (c.f. Bach et. al., 1981), or 1-nucleotide mismatches resulting from a mutation in the cloned probe. In order to distinguish between these alternatives, and to ensure that the 5' end is in fact the actual initiation site (compare Saiga et. al., 1982 with Niles et. al., 1981b; Urano et. al., 1980 with Grummt, 1982), the 5' triphosphate end of the nascent RNA must be unambiguously identified and positioned on the map. To this end, further experiments utilizing in vitro capped Physarum RNA are being undertaken.

There are two previous results which may indicate whether the downstream S1 protection start site is actually a processing site or an alternate transcription start site. Sun et. al. (1979) found that transcription begins with a G in a small but significant percent of the pre-rRNAs initiated in isolated nuclei. This would indicate that there are in fact two (or more) initiation sites, which could in theory be separated by several hundred nucleotides.

Results in other systems which have two initiation sites (Niles et. al., 1981b and Young and Steitz, 1979), however, usually show the two sites to be within 20 to 30 nucleotides of each other. In only one case (Bayev et. al., 1980) has the separation been as much as 100 nucleotides, and they are often within a very few nucleotides of each other (Baker et. al., 1979, Benoist and Chambon, 1981, Grosschedl and Birnstiel, 1980 and Martin and Chambon, 1981). Transcription initiation further downstream may be inhibited by the transcribing polymerases from the upstream site. On the other hand, processing sites may appear at any distance from the transcription initiation site. These analogies would indicate that the downstream site is probably a processing site.

The other results which speak to this point are the in vitro transcription experiments reported herein. These results are considered

further in the following discussion.

In Vitro Transcription: Utilizing the S24 and S35 clones (both represent the new Sal I fragment which contains the S1 protection start^U points), in vitro transcription was attempted using modifications of both major reported systems (Weil et. al., 1979b and Manley et. al., 1980). The S100 system of Weil et. al. gave positive results, but to date we have not had success with the Manley extract system. Whether this is due to the sensitivity of some component to the ammonium sulfate precipitation steps or pH changes, or due to the loss of a required component in the initial extraction is not known.

Sal I cleaved clone S35 DNA was used in an in vitro transcription system based on the S100 supernatant preparation described by Grummt (1981b). When labeled nucleotides are incubated with S100 in the absence of added DNA, label is only incorporated into small RNAs (less than 100 nucleotides). When S35 DNA is added, a single large RNA molecule (approximately 830 $\pm$ 40 nucleotides) is also seen, as shown in Fig. 10. A small band (approximately 100 nucleotides) is also seen, as is a very high background of small RNA fragments. Both of these bands are seen when the transcription is carried out in the presence of 200 µg/ml α-amanitin, indicating that these bands are not transcribed by RNA polymerase II or III. Addition of 50 µg/ml Actinomycin D, however, abolishes transcription of the large RNA band, in keeping with the Actinomycin D sensitivity of RNA polymerase I. The 100 nucleotide band still appears, indicating that it results from some non-RNA polymerase-mediated mechanism. That the large band is actually RNA is shown by the fact that it can be completely destroyed by addition of RNAse A, as can the remainder of the labeled bands. If sonicated calf thymus DNA is used as template, random lengths of runoff products are seen as expected from the random nature of the primer.

The Sal I run-off transcription product is too long to accurately determine its length. It is not even possible to state unequivocally that it is a single band, as any RNAs which are within a few nucleotides of each other will coelectrophorese in this range and gel system.

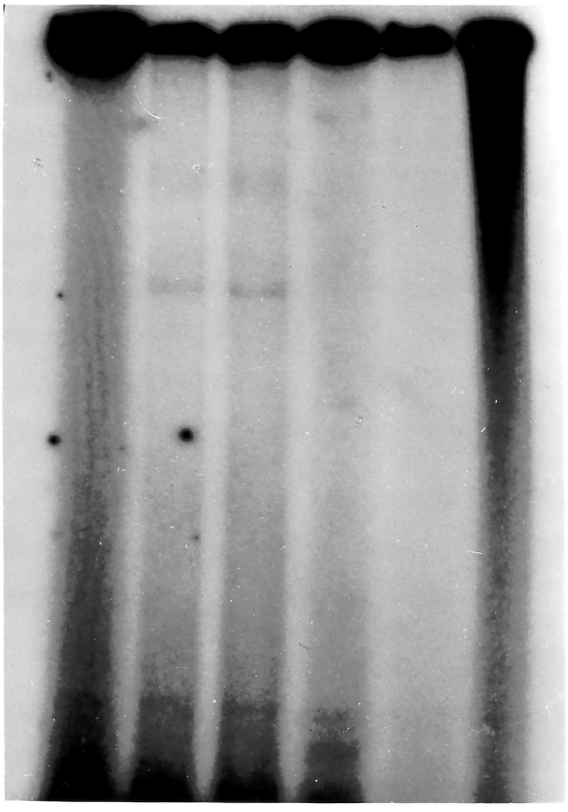


Figure 10: In Vitro Transcription of PrD100S35 Cloned DNA. Purified PrD100S35 DNA was in vitro transcribed using the S100 system (Weil et. al., 1979b).

a. S100 with no DNA added
b. a. + PrD100S35 DNA
c. b. + 200 µg/ml α-amanitin
d. b. + 50 µg/ml Actinomycin D
e. b. + 10 µg RNAse A
f. a. + calf thymus DNA

However, a band corresponding to the shorter Sal I S1 protection fragment (720 nucleotides) was never found. Thus the downstream S1 site is almost certainly not an initiation site.

Further determination of the *in vitro* initiation sites would be greatly facilitated by the use of smaller fragments, giving small and more easily sized run-off products. This would also determine if there are in fact two initiation site, as found by Sun et. al. (1979), and how close they are to each other. Some polymerase II promoters which lack the TATA box show multiple initiation sites (Baker et. al., 1979, Benoist and Chambon, 1981, Grosschedl and Birnstiel, 1980 and Martin and Chambon, 1981), usually at any purine within a tightly defined region of less than ten nucleotides. This is probably similar to what is happening in the *Physarum* rDNA, and is contrasted to other results (Bayev et. al., 1980, Niles et. al., 1981a and Young and Steitz, 1979) in which two different initiation sites are separated by 20 up to 100 nucleotides.

However, there is a very large background of labeled RNA species in the smaller region of the gels, which would preclude the use of smaller fragments at the present time.

This background is not sensitive to inhibition by α-amanitin or Actinomycin D, indicating that it may not be the result of transcription by any RNA polymerase, but is RNAse sensitive. The most likely mechanism for generating labeled RNAs in the absence of RNA polymerase activity is the template independant CCA addition to some tRNAs (c.f. Mazzara and McClain, 1980). Other potential explanations include RNA ligation events using CTP, or an oligo-C tailing reaction, or perhaps the covalent binding of CTP or oligo-C to proteins via, for example, a phosphoserine linkage or a diphosphate ester bond, or addition of cytidine residues to other structures such as polysaccharides (analogous to the use of thymidine in the synthesis of bacterial cell walls). In most of these cases, the use of a different labeled nucleotide would significantly reduce or eliminate background problems and allow the detection of short run-off products, which would in turn allow the exact positioning of the *in vitro* transcription initiation site. These

experiments are already underway.

Also in progress are experiments designed to delimit the sequences required for RNA polymerase I initiation. The S24d1.5 deletion was subjected to further BAL31 deletion mutagenesis to generate a series of deletions which begin at the Sal I end and run various distances toward the initiation site. Use of these mutants in the in vitro transcription system should allow us to determine which area(s) of the DNA sequence are responsible for RNA polymerase 1 transcription initiation (c.f. Bakken et. al., 1982, Bogenhagen et. al., 1980, Grummt, 1982, Sakonju et. al., 1980 and Tsuda and Suzuki, 1981). A similar series of deletion mutants beginning at the Eco RI end should provide information on the 3' limits of these sequences.

These deletion experiments will only provide a value for the outer limits of the required sequences. If there are several runs of required sequence, as for prokaryotic promoters and polymerase II initiators, the interior sequences which are free to vary would not be determined. Also, upstream enhancer sequences (c.f. Tsuda and Suzuki, 1981) would not be detected.

Summary of S1 Mapping and In Vitro Transcription Results: In the in vitro transcription experiments, only one transcription initiation site was found (or two sites close enough together to be indistinguishable in this type of experiment).

The size determination for the Sal I run-off transcript is not accurate enough to determine whether it matches exactly the size of the large Sal I S1 protection fragment. However, at this time they are assumed to map to the same site, since they are within experimental error, and since the initiation site in vitro has been found to correspond to the in vivo initiation site in other systems (c.f. Grummt, 1981b), and this initiation site should give an S1 protection fragment. It is possible, however, that the S1 map site is downstream from the in vitro initiation site.

The downstream S1 protection start site at 680 nucleotides is never

represented in the in vitro transcription experiments. This argues strongly against the possibility that the downstream protection start site is actually an initiation site. This interpretation is represented schematically in Fig. 11.

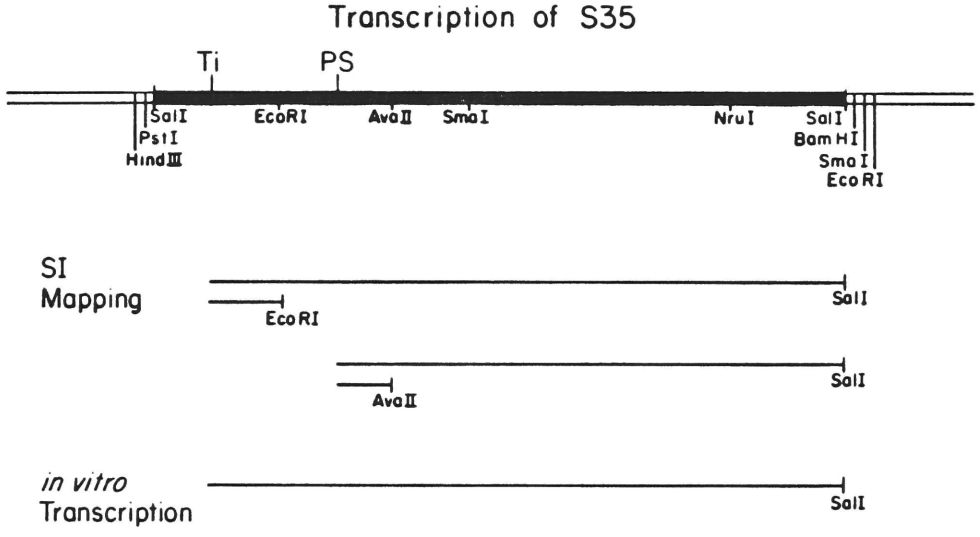


Figure 11: Summary of In Vitro Transcription and S1 Mapping Data. Top line shows the restriction map of PrD100S35 (= PrD100S24). Assigned transcription initiation (Ti) and processing (PS) sites are indicated. Below are shown the two types of results obtained in S1 protection mapping experiments, and their alignment on the S35 map. Bottom line shows the alignment of the in vitro transcription results.

Chromatin Structures on Physarum rDNA

Staphycoccal Nuclease Digestion of Physarum Chromatin: When chromatin from Physarum isolated nuclei or nucleoli is digested for various times with staphycoccal nuclease, and the resulting solublized chromatin fragments sedimented on a sucrose gradient (Johnson et. al., 1978a), the pattern shown in Fig. 12 is generated.

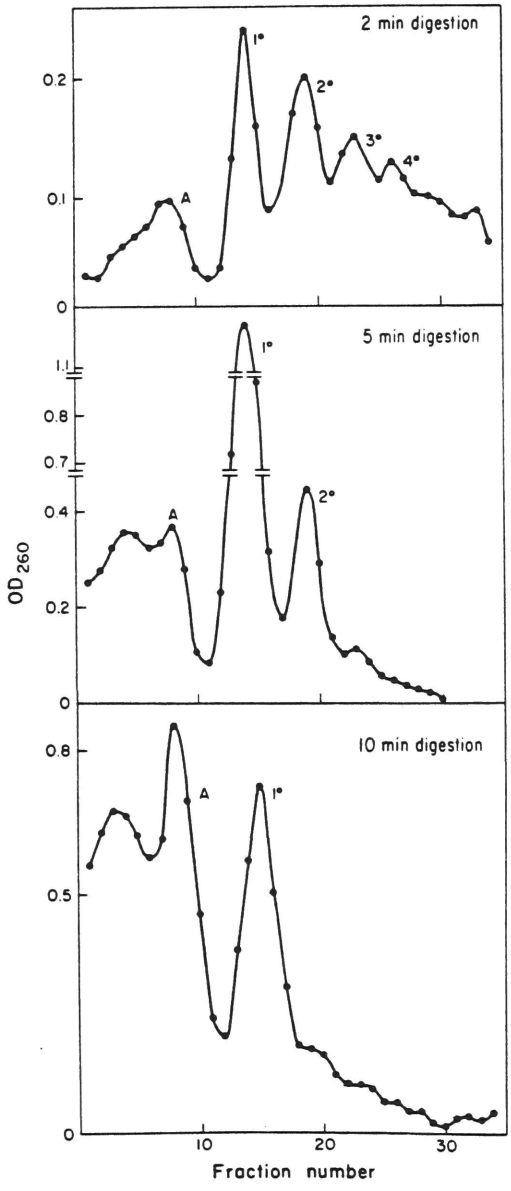


Figure 12: Chromatin Subunits of Physarum polycephalum. Sucrose gradients of soluble chromatin fragments from Physarum nuclei following staphylococcal nuclease digestion for the indicated times. Top of the gradient is to the left. Peak A is approximately 5.7 S, monomers 11 S. Chromatin concentration was determined by reading UV absorbance at 260 nm.

As can be seen, the lower portion of the gradient represents a normal series of nucleosomes beginning with monomers. However, in contrast to other findings (c.f. Lacy and Axel, 1975), a sharp peak in the region of 5.8 S is generated. That this peak, called "peak A", is not merely a stable subnucleosomal fragment is indicated by the fact that it appears in the same relative yeild in all preparations, and by the fact that is is stable upon extended digestion with nuclease while nucleosome fragments become increasingly smaller. In addition, DNA extracted from peak A is 144 nucleotides long, the same length as monomer nucleosome DNA.

Hybridization of DNA Derived from Chromatin Fractions to rRNA and rDNA Restriction Fragments: In order to determine whether the various types of nucleosomal particles arise from different areas of the rDNA, an excess of DNA isolated from peak A, monomer nucleosomes, or higher oligomers (4-mers and larger) was hybridized in solution with various labeled rRNA species, or with rDNA restriction fragments, and the extent of hybridization was determined by measuring the amount of label remaining after RNAse or S1 nuclease digestion, respectively.

Results of the hybridizations with rRNAs have been presented previously (Johnson et. al., 1978a), and show that the 19 S and 26 S rRNA genes are largely packaged into peak A type nucleosomes.

To determine more exactly the boundaries of the region packaged into peak A nucleosomes, various rDNA fragments were used as hybridization probes. Hybridization was carried out as described (Gallimore et. al., 1974 and Sharp et. al., 1974) except that S1 resistance was used as the criterion for hybrid formation rather than binding to hydroxylapatite. S1 digestion was carried out as for S1 mapping experiments (Methods - S1 Mapping). Data were analyzed by linearization of the rate of hybridization (Gallimore et. al., 1974). As shown in Fig. 13, the Hin c fragment (8.0-13.0 kb on the rDNA map, containing portions of the 19 S and 26 S genes and the entire internal transcribed spacer region and 5.8 S gene), and the Eco c fragment (5.3-7.6 kb, containing portions of both intervening sequences and the 26 S gene β region) both hybridize extensively to DNA isolated from peak A nucleosomes 65% and 55% of the

total amount of hybridization, respectively), and to monomer nucleosomes (35% and 42%, respectively), but only very slightly to DNA isolated from higher oligomers (undetectably and 2%, respectively).

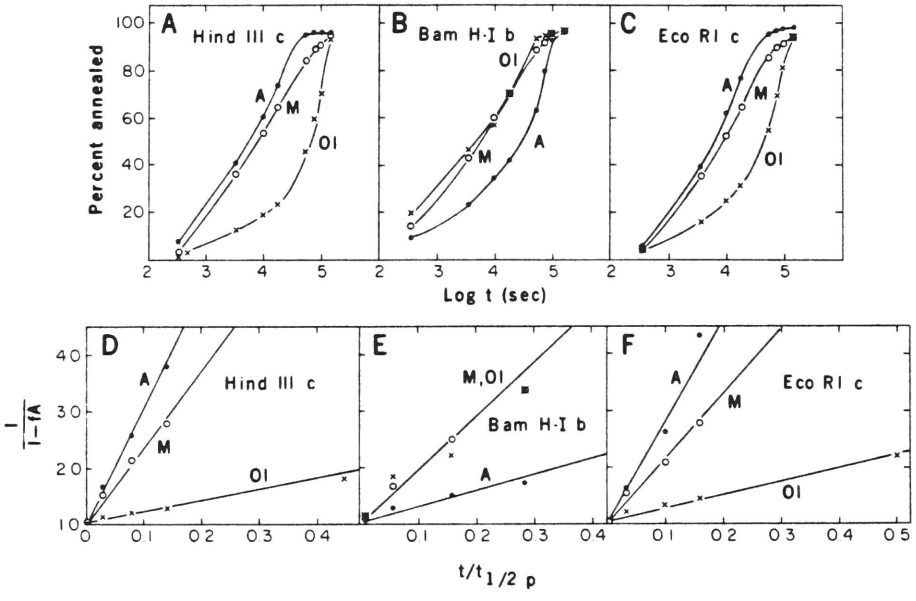


Figure 13: Hybridization of Isolated Chromatin Subunit DNA to rDNA Restriction Fragments. ^{32}P-labeled rDNA restriction fragments Hin c (19 S, ITS and 26 S α coding regions), Bam b (CSP untranscribed region), and Eco c (26S β and IS-1 and 2) were hybridized with DNA derived from isolated Peak A (A), monomer (M) and higher oligomer (Ol) nucleosome fractions. After various times the extent of hybridization was determined by assaying for S1-resistant label.
Upper: Percent hybridization is plotted against the log of the time (in seconds) of hybridization.
Lower: Linearization of the data (see Methods). 1 / (1-fA) is plotted against $t / (t_{1/2p})$, where fA is the fraction of initial restriction fragment annealed, t is time (sec) and $t_{1/2p}$ is the time required for one-half of the probe to anneal in the absence of added chromatin DNA. A steeper slope indicates more extensive hybridization.

In contrast, the Bam b fragment (23.9-37.3 kb, spanning the central spacer region) hybridizes extensively to DNA from higher oligomers and monomer nucleosomes (47% to each), but only slightly with peak A nucleosomal DNA (5.5% of total hybridization). This indicates that peak A nucleosomes are not randomly arrayed throughout the rDNA molecule, but

occur only in regions which are associated with the transcribed region, whereas the slowly digested series of compacted nucleosomes are primarily present in the central, untranscribed spacer region.

Attempts to perform similar hybridizations with rDNA terminal fragments, such as Bam HI c (0-2.7 kb, containing only terminal untranscribed regions) led to highly anomolous hybridization kinetics in all cases. This is probably due to the presence of tandem inverted repeat sequences in this region (see below), which would lead to snap-back formation and hence protection of some sequences from S1 nuclease even in the absence of nucleosomal DNA. We therefore cannot use this method to determine whether the terminal untranscribed region is packaged in compacted nucleosomes or in the extended peak A configuration.

rDNA Termini

Cloning of rDNA Terminal Fragments: The cloning strategy is shown schematically in Fig. 2. Total purified Physarum rDNA was digested to completion with Eco RI, and the terminal fragments isolated. In order to remove any potential blocking hairpins or single-stranded regions from the extreme termini, these fragments were treated briefly with S1 nuclease under conditions which should result in the removal of single-stranded regions without causing breakdown of the double-stranded DNA (Vogt, 1973). Following precipitation, the DNA was redissolved in ligase buffer with a ten-fold molar excess of polynucleotide kinase-labeled Eco RI linkers, and ligated overnight at 20°C. The resultant DNA was then Eco RI treated, and ligated to Eco RI cut-Charon 13 DNA. Phage were packaged, and used to infect lawns of K802 cells. The recombinant plaques were picked by making filter replicas of the plates, and screening with nick-translated ^{32}P-labeled rDNA Bam c fragment (Benton and Davis, 1977). Positive plaques were replated and rescreened as before. Positives were again picked, and saved for further analysis.

Several of the recombinant plaques were restriction mapped. In most cases, it was found that the Charon 13 dispensible fragment was

retained, but an insert fragment was also present. The sizes of these insert fragments ranged from 1.7 to 5.3 kb. The largest, designated PrD229, was selected for further study, since it was the size expected for a complete Eco RI rDNA terminal fragment.

The efficiency of such a cloning experiment is difficult to estimate, due to the uncertain recoveries of clonable fragments during the preparative steps (such as the S1 treatment, and the Sepharose column employed after the linker ligation), but seems to be higher than that of the transcription initiation region. This may be due to the further purification removing some inhibitory material (such as polysaccharide). Alternatively, there may not be a strong selective pressure against cloning this region, as there seems to be against the initiator region.

Hybridization of PrD 229 DNA with Authentic rDNA Termini: PrD 229 DNA was digested with Eco RI, or Eco RI plus Bam HI, and the fragments separated by gel electrophoresis. The fragments were transferred to nitrocellulose, and the filter replica was then probed using nick-translated ^{32}P labeled rDNA Bam c or Pst d fragments. The results are shown in Fig. 14. Only the insert hybridizes with the rDNA termini. This hybridization is specific, as it cannot be competed out by additional carrier DNA. This indicated that the clone PrD 229 insert is in fact derived from a region of the rDNA termini. However, this hybridization does not indicate how far the clone extend toward the actual terminus.

Partial Denaturation Mapping of the Clone PrD 229: During the previously described R-loop mapping of the rRNA genes it was noted that a large proportion of the rDNA termini possessed secondary structure after incubation under partially denaturing conditions. It was reasoned that PrD 229 DNA, being derived from this region of the rDNA, should also have secondary structure after partial denaturation. Isolated insert DNA was therefore incubated under the same conditions as had been used for R-loop mapping, and the preparation spread for electron microscopic visualization.

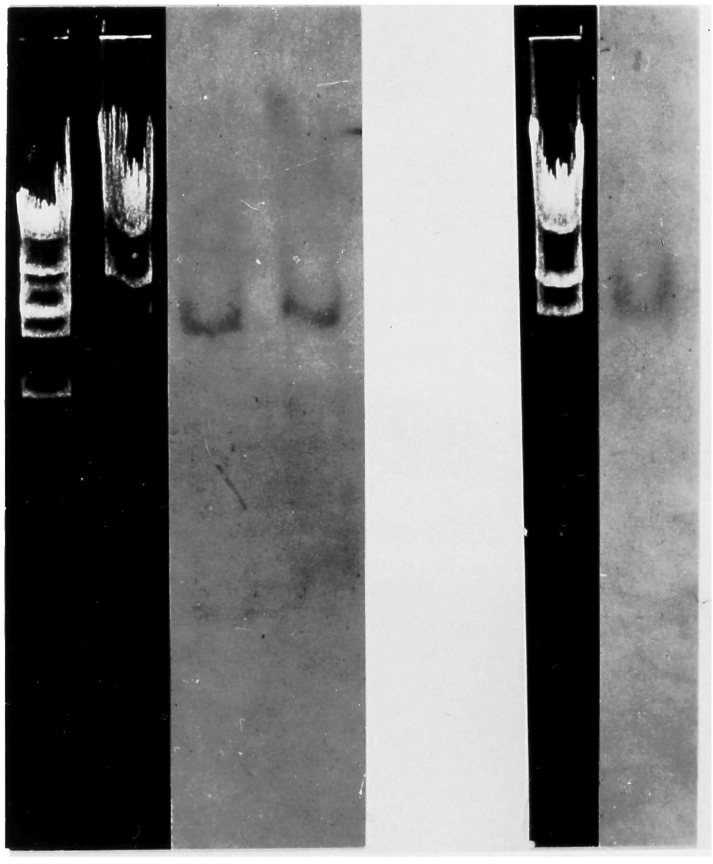


Figure 14: Hybridization of PrD229 Restriction Fragments with Labeled rDNA Terminal Restriction Fragments.

a. Ethidium stained gel pattern of PrD229 DNA digested with Eco R1 and Bam HI.

b. Gel pattern of PrD229 DNA digested with Eco R1.

c. Southern transfer of a. hybridized to isolated, nick translated rDNA Bam c fragment.

d. Southern transfer of b. hybridized as in c.

e. Gel Pattern of PrD229 DNA digested with Eco R1.

f. Southern transfer of e. hybridized with isolated, nick translated rDNA Pst c fragment.

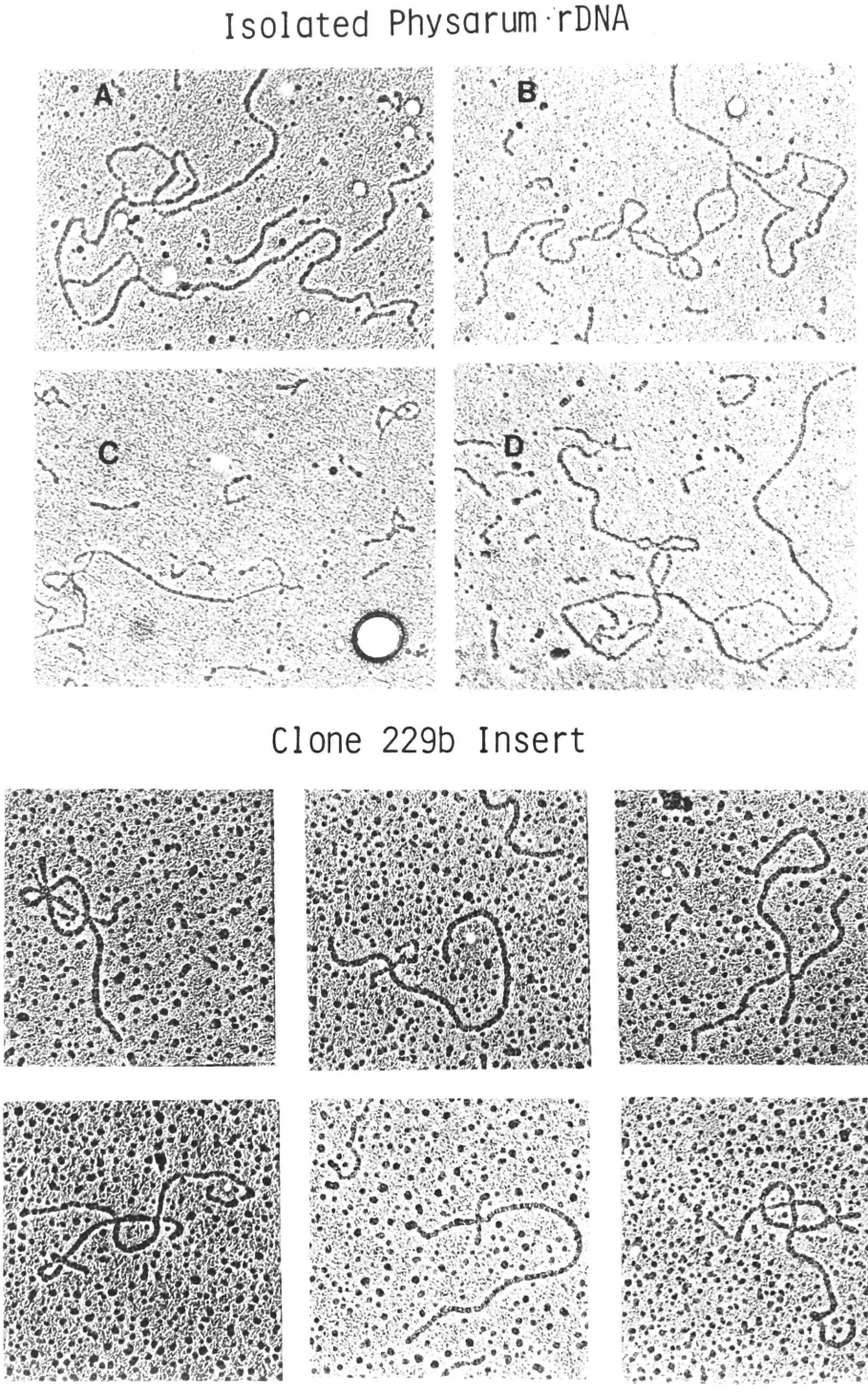


Figure 15: Secondary Structures at rDNA Termini and in PrD229 Insert. Upper: Termini of R-looped Physarum rDNA, demonstrating typical secondary structures. A. and D. show cruciform/rabbit ear structures, which can interconvert by rotation. B. and C. show Y shapes, with some single-stranded regions in C.
Lower: Isolated PrD229 insert DNA incubated under R-looping conditions (with no RNA). Cruciform/rabbit ear structures and possible loops are seen.
Scale bar represents 1 μm.

Secondary structure was indeed found, as shown in Fig. 15. Moreover, the secondary structures were of approximately the same size and type as those seen in rDNA termini, and were the same distance from the end of the insert as the rDNA secondary structures were from the end of the rDNA molecule. This result indicates that the PrD 229 insert contains most (if not all) of the rDNA terminal sequences.

Restriction Mapping of the PrD229 Clone: A restriction map of this clone was created using standard mapping techniques with infrequently cutting restriction enzymes. Mapping of this clone revealed that the Bam HI site expected to appear near the center of the insert was in fact not present in the majority of the recombinant DNA isolated. It was, however, present in a subpopulation of these molecules, as revealed by end labeling the insert molecule with γ-^{32}P-ATP and polynucleotide kinase followed by digestion with Bam HI and gel electrophoresis (data not shown). This indicated that the Bam HI site was probably originally present in the recombinant, but that early during the amplification of this clone a mutation, resulting in the loss of this site, occurred in one or more molecules. The mutant molecules were then preferentially amplified, either through random selection of an inoculum, or through a competitive advantage over the unmutated recombinants. In addition to the loss of the Bam HI site, two or three Hind III sites were found, one or two very near one end of the insert and the other in the center. This is shown schematically in Fig. 16. Not shown in this figure is the possibility that a Bam HI site may exist approximately 400 nucleotides from the Eco RI end which is not near a Hind III site. This is the end of the rDNA that originated from the extreme terminus of the rDNA molecule. These sites may be present in a small subpopulation of the rDNA molecules isolated from Physarum and have been cloned and picked randomly, or they may have arisen by a mutational event similar to that assumed to be responsible for the loss of the Bam HI site. Even if these changes resulted from mutations, the bulk of the sequence is still representative of the rDNA termini since they hybridize with terminal probes under stringent conditions.

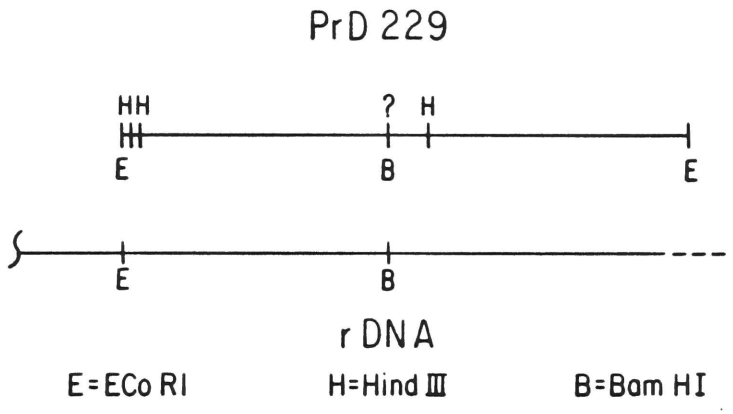


Figure 16: Simplified Restriction Map of PrD229 DNA.
Below: Partial restriction map of rDNA, showing Eco R1 and Bam HI sites near the terminus. No Hind III sites are present in this area.
Upper: PrD229 insert restriction map, showing expected (below the line) Eco R1 and Bam HI sites, and the new (above the line) Hind III sites. The ? above the line indicates that the Bam HI site is apparently not present in all PrD229 DNA molecules (see text).

Since the terminus apparently consists mostly of inverted repeat sequences (Hardman et. al., 1979), restriction enzymes that cleave frequently may give a recognizable repeating ladder. To determine whether this is the case, PrD229b insert DNA was end labeled at the Eco RI sites with γ-^{32}P-ATP and polynucleotide kinase, recut with Hind III, and the products separated on an agarose gel. The large band, representing the terminal end of the rDNA molecule and labeled at the outer end, was electroeluted. This end-labeled DNA was then subjected to partial digestion with several frequently-cutting restriction enzymes, including Hae III, Hinf I, Alu I, Mnl I and Sau 3a. The Sau 3a and Hae III digests gave a relatively straight-forward repeating ladder, indicating evenly spaced repeating sites. These sites are mapped onto the cloned DNA in Fig. 17.

The repeat unit is approximately 140 base pairs, but varies from repeat to repeat, indicating that the repeats are not exact but have some length heterogeneity. Also some repeat units contain interior Hae

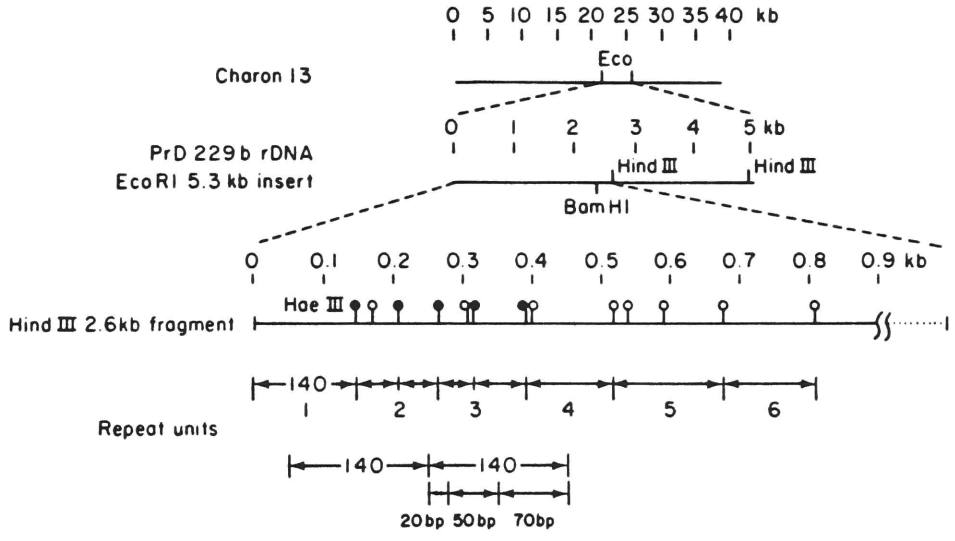


Figure 17: PrD229 DNA - Hae III Partial Restriction Map.
Top: Schematic of PrD229 showing the relationship of the rDNA insert to the phage arms.
Line 2: The insert fragment was isolated and end labeled with ^{32}P-γ-ATP and polynucleotide kinase. This isolated insert was then restricted with Hind III, and the singly end-labeled (rDNA terminal) fragment was isolated and partially digested with other restriction fragments.
Line 3: Restriction map determined for Hae III sites, showing approximately 140 bp repeating units, some of which contain additional Hae III sites. Closed circles represent favored cleavage sites which are relatively more intense in the autoradiograph of the partial digestion gel.
Line 4: Repeat pattern of the Hae III sites, showing 6 repeating units.
Line 5: Schematic diagram of two repeating units, one with and one without interior Hae III sites. Lengths of each unit are averages, and vary from repeat to repeat.

III sites, usually at the center of the repeat, or about 20 nucleotides from one end. This again shows heterogeneity in the repeats, and also indicates that the rotational symmetry within each repeat is not exact.

Subcloning of PrD 229: In order to determine the sequence of the terminal end of PrD 229, purified PrD 229 DNA was digested with Eco RI, and the total digestion product was ligated with Eco RI-cut M13mp7.

Recombinant (clear) plaques were picked, and designated the 229E series. Several of these recombinants were sequenced. One such sequencing result is shown in Fig. 18. Two sequences were found, as expected. However, when RF minipreps were made of these 229E clones, none were found to be 5.3 kb in length. This indicates that the M13mp7 clones have undergone some deletion. This deletion is apparently not near either end, however, since the sequences for all 229E clones were identical to one of the two prototype sequences for as far as could be determined. The sequence which corresponds to the rDNA terminus is presented in Fig. 19 (e4).

In an effort to obtain an M13 clone which contained an undeleted terminal region, PrD 229 DNA was digested with Eco RI, and electrophoresed. The insert fragment was then recovered by electroelution, and digested with Hind III. This digestion was again electrophoresed, and the Eco RI-Hind III 2.7 kb fragment was recovered by electroelution. This fragment was then ligated to Eco RI, Hind III-cut M13mp9, and the mixture transformed into JM103. Recombinants were picked and plaque purified, and designated the 229EH series. Sequencing of these recombinants gave the same sequence as the 229E series clones of the 229E4 type. However, RF miniprep restriction digestions again revealed that the clones had undergone some deletion in each case examined.

To determine the sequence of the entire region, the shotgun sequencing strategy of Messing (Messing et.al., 1981) was used. PrD 229 insert DNA was purified by gel electrophoresis and electroelution. The purified insert was then partially digested with either Hae III or Sau 3A, and ligated to Sma I-cut or Bam HI-cut M13mp9 DNA, respectively. Recombinants were designated the 229H or 229S series, respectively, and picked for sequencing at random. Sequences from some of these clones are also presented in Fig. 19. Following a similar strategy, Alu I partial digests were cloned into the Sma I site of M13mp9. Clones from this library are being sequenced currently. These clones seem to derive from the Hind III end of the PrD229 insert, and so are not necessarily connected to the inverted repeats near the terminus.

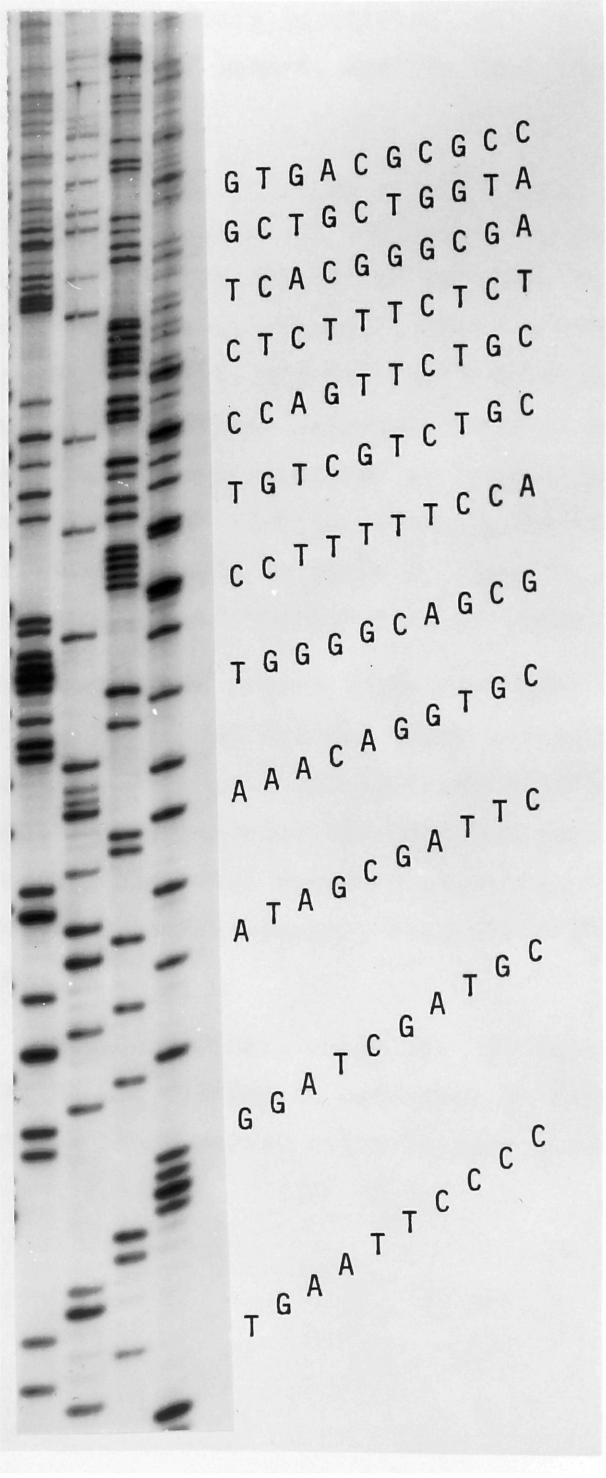


Figure 18: Sequencing Gel Autoradiograph for an E4 Sequencing Reaction. Sequencing gel, and the sequence determined from it, are presented for a PrD22 Eco R1 subclone into M13mp7. The sequence shown represents the rDNA terminal end of the PrD229 clone.

So far, several complete repeating units have been sequenced. Partial sequences are available from others. However, few overlaps have been unambiguously identified, due to the strong preference for one orientation of insert, and the fact that the repeats being sequenced are very similar.

Structural Features of the Repeat Unit from the rDNA Terminal Clone PrD229. Analysis of the repeating unit sequences obtained above indicates that these repeats do not have any simple repeating sequence in the normal sense. However, there are several sequence features that are almost invariant, and many more which are common, throughout the repeating units. These sequence features are present in a fixed order along the DNA, and are separated by regions of extremely variable sequences that range in length from 0 to 40 nucleotides. The order of these structural motifs is shown in Fig. 19, along with the sequence of 6 subclones which demonstrate many of these features.

Note that, when present, inverted gaps sites define a central symmetry axis. A second axis of local 2-fold symmetry is usually found between the C-T rich region (nucleotides 115-130) and the A-G rich region (137-154). The degenerate Sau 3A sites near the beginning of each repeat may also contain local symmetry elements. Potential secondary structures involving these symmetry elements are considered in the Discussion section.

Also note that, while the different sequences contain different lengths of variable sequences in different sites, the total length of most of these repeat units is very close to 140 bp.

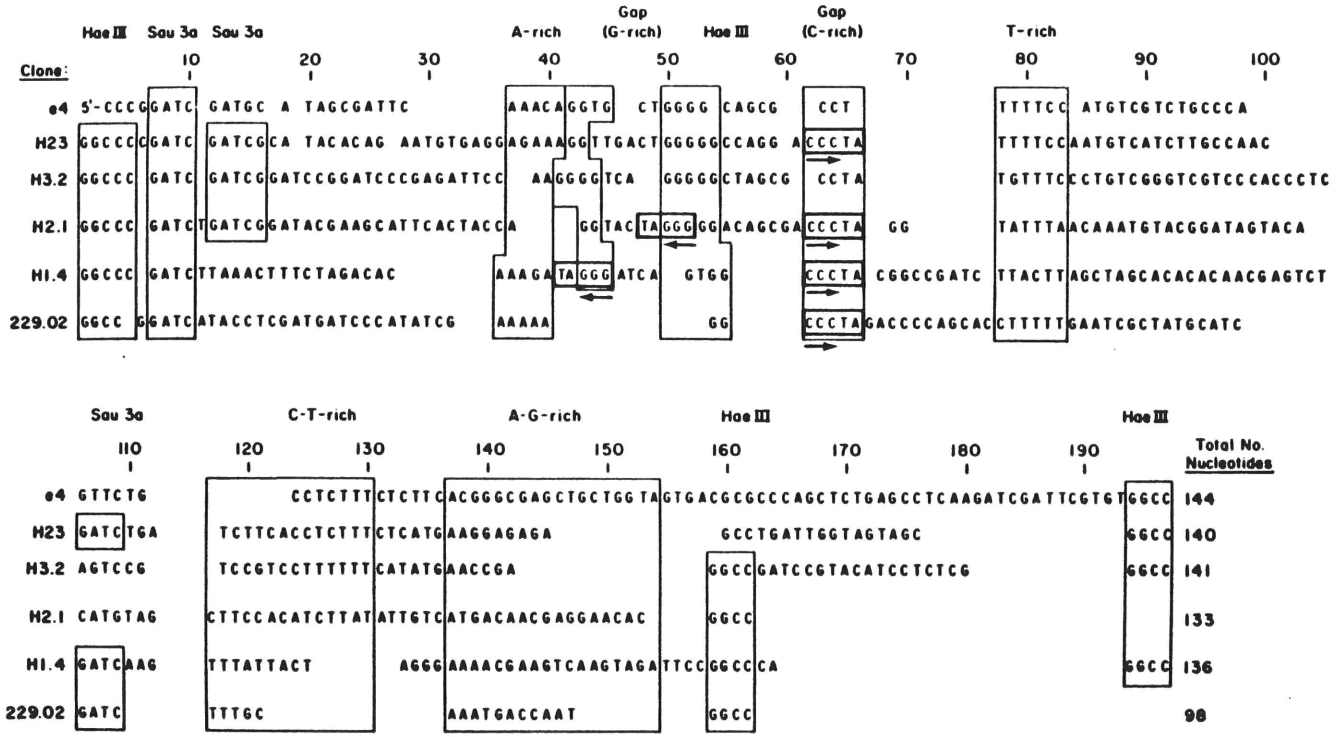


Figure 19: Features of the Inverted Repeat Sequences from the rDNA Terminal Clone PrD229. Alignment of the sequences from various subclones allows identification of regions of conserved structure, shown boxed. Areas between are of variable length and apparently random sequence. See text for discussion.

DISCUSSION

The research described in this thesis has focused on the ribosomal RNA genes and flanking sequences of Physarum polycephalum. The numerous advantages of this system were already described in the Introduction. Use of this system has allowed several questions to be addressed, notably 1) What is the structure of the transcription unit, and how do its gene order and the presence of intervening sequences compare with other ribosomal RNA genes in other organisms? 2) What sequences are present at the transcription initiation region, and what common features do these sequences share with other transcription initiators? 3) What chromatin structure is present on the transcribing genes, and how does it differ from that on untranscribed regions? 4) What structure(s) are prresent at the ends of this linear molecule that allow the completion of replication without the continual loss of sequences from the end? These questions are considered separately below.

Transcription Unit

A Map of the rRNA Transcription Unit of Physarum polycephalum: A map of the coding regions has been presented previously (Figs. 5 and 6). The transcription initiation site has been localized to 18.1 kb (Sun et. al., 1979, and the S1 and in vitro transcription results shown here). Transcription proceeds from the initiation site through the 19 S, 5.8 S, and 26 S α, β and γ regions, respectively, and including all sequences located between these various coding regions, notably including the intervening sequences (Gubler et. al., 1980). Termination occurs at 4.7 kb, probably just a few nucleotides downstream from the end of the 26 S γ coding region (Kukita et. al., 1981), in a region that includes tandem inverted repeats.

Other workers (Gubler et. al., 1980) have extended the R-loop mapping to the 5.8 S gene. Their results indicate that the 5.8 S gene is located approximately 450 nucleotides from the 26 S gene. This is similar to the distances found in other systems, including Drosophila (Pellegrini et. al., 1977) and yeast (Philippsen et. al., 1978). (See also Mazzara et. al., 1980 and Long and Dawid, 1980, for reviews). In many other systems, the 5.8 S rRNA gene has been localized to the transcribed spacer region, but not within it (c.f. Frankel et. al., 1977, Cory et. al., 1977, and Boseley et. al., 1978).

Presence of Two Intervening Sequences in the 26 S rRNA R-loops hybrids such as shown in Fig. 4, and the large number of similar types of hybrids, clearly indicate that the 26 S rRNA gene contains two intervening sequences or introns.

Other species have been found which have one intron interrupting their large rRNA gene. The Tetrahymena rRNA intervening sequence (Niles et. al., 1981a and Niles and Jain, 1981)) is in the position of the second intervening sequence of Physarum, while that of Chlamydomonas mitochondrial rRNA (Allet and Rochaix, 1979) occurs at the position of the first Physarum intervening sequence. Still other species, such as Drosophila melanogaster and Leishmania donovani, have large rRNAs which contain a physical break, resulting in the presence of two distinct R-loops (Wellauer and Dawid, 1977 and Leon et. al., 1978). In both of these cases, the interruption in the gene occurs approximately at the midpoint of the gene, in a position similar to that of the Physarum first intervening sequence.

Some of the rDNA genes in Drosophila also contain an intervening sequence (Glover and Hogness, 1977, Pellegrini et. al., 1977, Wellauer and Dawid, 1977 and White and Hogness, 1977). This intervening sequence is in the position which corresponds to the second Physarum intervening sequence. However, apparently all of the genes in Drosophila which are interrupted are not transcribed (Long and Dawid, 1979). The reason for this lack of transcription does not appear to be changes in the transcription initiation region (Long et. al., 1981). Long et. al.

hypothesize that the presence of the intervening sequence itself inactivates the transcription unit. This would be in stark contrast to Physarum, where the genes with two intervening sequences are transcribed (Gubler et. al., 1980).

The two intervening sequences have been shown to be spliced out in random order very quickly, often before transcription is completed at the 3' end of the 26 S gene (Gubler et. al., 1980). This suggests that the processing mechanism may be autocatalysis, as occurs in the Tetrahymena rRNA precursor (Kruger et. al., 1982, Zaug and Cech, 1982 and Cech et. al., 1981).

The sequence for both intervening sequences and the surrounding rRNA coding regions has been determined (Nomiyama et. al., 1981 and Nomiyama et. al., 1980). The junction of the coding and noncoding sequences has been unambiguously determined by these workers, and does not conform to the consensus mRNA intron-exon junction sequence. The junctions of other rRNA intervening sequences also do not conform to this consensus sequence. It therefore seems that the processing of these introns does not involve the same mechanism as that for protein-coding RNAs. No general rule has been formulated for rRNA intron-exon junctions, other than that the nucleotides involved in the splicing event are a T at the end of the exon, and a G as the last nucleotide of the intron (Nomiyama et.al., 1981). Greater homology has been noted between the intron/exon junction sequences of the two Physarum intervening sequences and that of the Tetrahymena rRNA intervening sequence (Thomas R. Cech, U.C.L.A. Symposium on DNA Replication and Recombination, Keystone, 1983) further strengthening the case that the removal of these sequences in Physarum may be autocatalytic.

Transcription Initiation Site

Localization of the Transcription Initiation Point: The transcription initiation region was located to within a few nucleotides in a small Sal I fragment which has been cloned in the M13 system. Investigation of this region with S1 protection mapping experiments and in vitro transcription have demonstrated that there is a single (or possibly two very closely spaced) initiation point(s) in this region, and that all of the sequence information required to promote accurate transcription initiation is located within a region from 70 nucleotides upstream from the initiation nucleotide, to a point potentially 800 nucleotides downstream.

The results from other systems (c.f. Grummt, 1982) have shown that the RNA polymerase I promoter lies considerably closer to the initiation site, within a region from 50 nucleotides upstream to 10-20 nucleotides downstream. It is likely that this will be found to be the case in this system also, when the appropriate experiments are carried out.

In Vitro Transcription of S24 DNA: Previous work has demonstrated the possibility of using cell-free transcription systems to determine the site of initiation and the sequence requirements for polymerase recognition and initiation (c.f. Grummt, 1982, Tsuda and Suzuki, 1981 and Weil et. al., 1979a and b). Initial work centered on the use of isolated nuclei (Davies and Walker, 1977, Sarma et. al., 1976, Sun et. al., 1979 and Udvarty and Seifart, 1976) or on cell-derived chromatin (Davies and Walker, 1977 and Marzluff and Huang, 1975). More recent work has shown that naked DNA templates can be recognized as templates for transcription (c.f. Grummt, 1981 a and b, Manley et. al., 1980, and Weil et. al., 1979a and b). although isolated chromatin would be the preferred template (c.f. Sawada et. al., 1982 and Seebeck et. al., 1979 for Physarum). An alternative system is the microinjection of cloned DNA into Xenopus oocytes, and either biochemical or electron microscopic assay of the new transcription products (c.f. Moss, 1982).

As described earlier, the S100 of Weil et. al., using naked DNA, was used for these experiments because of the ease with which it may be set up, and the possibilities it presents for determining the exact position and extent of the promotor sequences by varying the template.

The Presence of a Processing Site Very Near the Transcription Initiation Site: The S1 protection mapping experiments indicated that there is more than one possible 5' end for the pre-rRNA transcript. Since there are not two initiation sites in the in vitro transcription experiments, the most likely explanation for this finding is that the pre-rRNA transcript undergoes a 5' terminal cleavage soon after transcription begins. Such 5' terminal processing events are common in eukaryotic systems (c.f. Lewin, 1980).

This interpretation could also explain a previous result. Sun et. al. (1979) noted a large amount of small RNAs in their nuclear transcription experiments. These small RNAs, on the order of 100 to 200 nucleotides and containing the 5' tri(γ-thio)phosphate, were originally assumed to result from premature termination near the initiation site (like an attenuator in some bacterial genes). These fragments may instead represent the 5' terminus of a long precursor RNA, which has been cleaved off in an early processing step and is not degraded immediately in isolated nuclei. The size of 100 to 200 nucleotides agrees well with the expected 125 nucleotides between the upstream transcription initiation site and the downstream protection start site.

Extremely fast processing such as postulated here is known to occur in other systems. Dictyostelium discoidum rRNA transcription matrices show the effects of a processing cleavage taking place during transcription (Grainger and Maizels, 1980), and RNAs in several systems seem to be rapidly processed since the 5' triphosphate is often missing from isolated precursor RNAs.

Additionally, Physarum is known to have an extremely unstable pre-rRNA (Jacobson and Holt, 1973, Kathy V. Scotto and Peter W. Melera, personal communication and unpublished observations). As mentioned previously, the intervening sequences are usually spliced out prior to the

termination of transcription (Gubler et. al., 1980). In addition, electron microscopic observations of transcribing rDNA consistently shows a region of rDNA upstream from the transcription matrix which is not packaged into nucleosomes but appears to be extended like the transcription unit, and which appears to be associated with proteins (Grainger and Ogle, 1978). This region may represent the actual start of the transcription unit and associated RNA polymerases, with the RNA transcript being cleaved off before it becomes visible to electron microscopy.

Sequence of the Transcription Initiation Region: A partial sequence of the S24 clone, which includes the transcription initiation site, is shown in Fig. 9. Since the area of the initiation site and its polarity are now known, comparison of the sequence of this area with other RNA polymerase initiation sites is now informative.

Published initiation site sequences examined included 5 S RNA (polymerase III) promoters from Xenopus (Bogenhagen et. al., 1980 and Sakonju et. al., 1980) and yeast (Valenzuela et. al., 1977), a "typical" RNA polymerase II promoter (Breathnach and Chambon, 1981, Corden et. al., 1980, and Gannon et. al., 1979), a "prototype" prokaryotic Pribnow box sequence (Scherrer et. al., 1978), and rRNA initiation sites from yeast (Bayev et. al., 1980 and Klemenz and Geiduschek, 1980), human (Miesfeld and Arnheim, 1982 and Financek et. al., 1982), Tetrahymena (Niles et. al., 1981 and Saiga et. al., 1982), mouse (Bach et. al., 1981 Grummt, 1982 and Urano et. al., 1980), rat (Rothblum et. al., 1982), Drosophila (Long et. al., 1981), and E. coli rRNA genes D and X (Young and Steitz, 1979).

The published sequences were compared to the entire Physarum S24d1.5 sequence (177 nucleotides, including the region between the Eco RI and far Sal I sites of S24, which includes the S1 protection mapping site and the in vitro transcription start site) in both orientations, using a 70% homology limit over small windows. Positive homology was reported if a region of more than 20 nucleotides in length had a greater than 60% homology. These regions are shown in Fig. 20.

In addition to those shown, scattered apparently random local homologies were found throughout the sequences. These homologies do not fit any pattern and are not associated with regions near a transcription initiation site. Since all of the information necessary for accurate RNA polymerase I initiation in the mouse resides within the region from 50 nucleotides upstream from the promoter to less than 10 nucleotides downstream (Grummt, 1982), homologies outside of this immediate region have not been presented, or further analyzed. Random homologies would be expected to arise approximately once every 1400 nucleotides examined, approximately the frequency observed here.

As can be seen, there is not any single area of the Physarum sequence which corresponds to the rRNA transcription initiation point of most, or even of several, other species. (Those positions at which two initiation sequences align represent either two rRNA genes from the same species [human] or very closely related species [rat and mouse].) One homology is in the "wrong" orientation, and some rRNA initiation sequences do not share any detectable homology with Physarum. Not surprisingly, no homology was found with RNA polymerase II or III initiation sites, and only weak homologies with a "typical" E. coli promoter.

This indicates that the exact sequence of the rRNA initiation point, and the transcription initiation signals in this area, are not highly conserved across species.

The lack of homology in rRNA initiators is in contrast to polymerase II initiation sites, which are extremely highly conserved. Probably the less stringent conservation of sequence reflects the fact that the polymerase involved is highly specialized, transcribing only this gene. The polymerase and the DNA sequence specifying initiation could evolve in a coordinate fashion in this specialized system much more easily than would be the case for polymerse II or III, which must recognize several different initiation sites, and must differentiate between "strong" and "weak" initiation signals. In these cases changes in the polymerase would have myriad effects, most of which would likely be

In view of the inverted homology with the Drosophila rRNA transcription initiation site, it is possible that the functional sequences involved in the initiation process are not required to be in a specific orientation. This would be possible if their primary function were not polarized. One example of this would be the local disruption of chromatin structure, leading to either a local phasing of nuclesomes, or a localized bare spot on the DNA (which may correspond to a nuclease hypersensitive site). Another example of nonpolarized function would be localized melting at the oligo-T tracts frequently upstream from the initiation site. A third possibility is a conformation change in the DNA itself (possibly to Z-DNA), such as has been proposed by Larsen and Weintraub (1982) to explain their S1-sensitive sites in initiator regions.

It is interesting to note that sequence homologies do exist in the rDNA spacer sequences of widely divergent species (c.f. Triezenberg et. al., 1982). It is possible that these conserved sequences, outside the initiation region, are important for some unknown function. Such functions could include regulation of the extent of transcription in the rRNA gene repeat, upstream enhancer effects, upstream binding and promotion of the RNA polymerase, or structural functions related to the assembly of the nucleolar organizer.

A Conserved Feature of rRNA Transcription Initiation Sites: While there are no conserved sequences among the rRNA transcription initiation sites, there is a conserved structural feature of these sequences. In all cases, within 60 nucleotides upstream from the initiation site there is a region with local two-fold symmetry, which could form a more or less extensive hairpin loop.

This feature may represent an important feature of the promoter. Regions of dyad symmetry, which could potentially form hairpin loops, have been found associated with many types of protein recognition sites, including other transcription initiation sites (Ziff and Evans, 1978, although in this case it includes the initiation nucleotide). Such regions could be used to control nonspecific protein interactions such

as nucleosome binding, or may function directly as a recognition site for a dimeric or cooperative protein.

Chromatin Structure on the Physarum rDNA

Chromatin Structures in Transcribed and Untranscribed Region of the rDNA: Physarum chromatin is digested by staphylococcal nuclease into nucleosome-sized fragments, much as any other chromatin, although a note of caution must be sounded since staphylococcal nuclease does show some sequence specificity (Pauli et. al., 1982). Unlike other systems, however, an altered, slowly sedimenting nucleosome is also formed. This altered nucleosome was initially dubbed "peak A". It contains 144 bp of DNA, a full-length nucleosomal amount, but sediments at 5.7 S rather than the 11 S which is usual for compacted nucleosomes. As isolated initially, it contained histones H2A and H2B, but was depleted in H3 and H4. It also contained non-histone proteins, but did not contain histone H1 (Johnson et. al., 1978a).

As indicated in the Results section, peak A nucleosomes are found only in regions of the rDNA which are transcribed. The exact extent of the region occupied by these altered, extended nucleosomes has not been determined by hybridization studies since probes were not available at the time. However, electron microscopic observations (Grainger and Ogle, 1978) have shown that the untranscribed spacer region, spanning some 22-24 kb, is in a uniformly compacted, nucleosomal arrangement, whereas the transcribed region, beginning at or immediately before the transcription initiation site, is almost fully extended. The terminal, untranscribed spacer region is also in a compacted nucleosomal arrangement. Neither electron microscopy nor our hybridization results indicate any difference between the chromatin structure on coding regions, transcribed spacers, or intervening sequences. A representation of this is shown in Fig. 21.

The chromatin structure of the extreme terminus of the rDNA palindrome is not yet known. Probes from this region show highly unusual

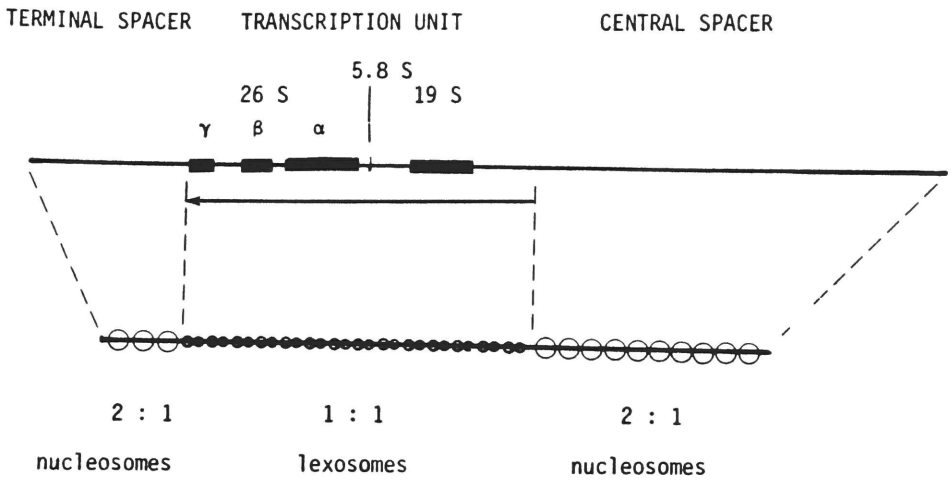


Figure 21: Chromatin Subunit Arrangement on Physarum rDNA. Top line shows the coding regions on the rDNA. Arrow indicates the direction and extent of transcription. Lower line shows that the terminal and central untranscribed spacers are compacted into nucleosomal structures, while the transcribed region is extended in a "lexosomal" packaging arrangement. Ratios indicated were determined by electron microscopic observation (Grainger and Ogle, 1979).

hybridization kinetics, even in the absence of chromatin-derived DNA sequences to hybridize with, making the determination of the chromatin structure in this region impossible by this method. Grainger and Ogle (1978) find most of the length of the terminal, untranscribed spacer region packaged into nucleosomes, but cannot determine the chromatin structure at the extreme termini. Tetrahymena rDNA palindromes, which may be packaged in a similar manner, do not have nucleosomes at their termini (Blackburn and Chiou, 1981 and Cech and Karrer, 1980). The presence of a covalently bound protein (Cheung et. al., 1981) near the termini also argues against nucleosomal packaging.

These findings have been confirmed and extended by others (Pierron et. al., 1982). Peak A has been found to be highly enriched in RNA polymerase II, indicating that it is associated not only with rRNA transcribing genes, but with transcribing genes in general. Areas of the

chromosome which are packaged in this manner are replicated early in the S phase, and this replication is necessary after mitosis to get transcription.

This also data has been extended in other ways in our laboratory (Prior, 1982 and Prior et. al., 1983), which indicate that the peak A nucleosomes are in fact extended, appearing rod-like in electron microscopic visualization, and that specific non-histone proteins are associated with a nucleosomal core which contains a full octamer of histones, but in which histones H3 and H4 are highly modified.

This model of chromatin structure on the actively transcribing rRNA genes, dubbed the "lexosome" model, (Prior et. al., 1983, Prior, 1982, Prior et. al., 1980) is consistent with the observations that actively transcribing rRNA genes are in a DNAse I sensitive configuration (Stalder et. al., 1978), but are still cleaved into nucleosome-sized fragments by staphylococcal nuclease (Johnson et. al., 1976, Johnson et. al., 1978a, Stalder and Braun, 1978, and Stalder et. al., 1979). It is expected that this model will apply also to other transcribing genes in Physarum. Support for this generalization has emerged from other electron microscopic observations which indicate that 10-12% of Physarum total chromatin is in a smooth configuration, and that nascent RNA chains are associated with this smooth configuration rather than with the beaded, nucleosomal configuration (Scheer et. al., 1981).

The lexosome model is more attractive in light of the recent discovery (Baer and Rhodes, 1983) that nucleosome cores from transcribed regions in mouse cells will form stable complexes with mouse RNA polymerase II in vitro, implying that these nucleosomes have an altered conformation which is recognized by the polymerase.

Physarum isolated nucleoli and purified rDNA chromatin fragments will be extremely useful as a model chromatin system, because of the ease with which biochemical amounts of the altered nucleosomes can be isolated.

A Model for the Activation of an rRNA Transcription Unit: In the

discussion dealing with the scattering of transcription initiation site homologies, it was suggested that the transcription initiation region may function by altering its conformation, or that of the immediately surrounding chromatin, rather than by a simple sequence-dependant protein recognition event. This suggestion can be combined with the chromatin results in the immediately preceeding section to give a model for the flow of events during the activation of an rRNA transcription unit.

In the inactive stages of the Physarum life cycle, the rDNA is completely packaged into compacted nucleosomal subunits. The nucleosome cores are at a relatively constant distance from each other, but each individual nucleosome can occupy any one of several positions along the DNA (see Lewin, 1980 for discussion and references), and may even be able to move from one position or phase to another by "rolling", "sliding", or "hopping" (Beard, 1978). This is represented by the arrows above the first line of Fig. 22. If nucleosomes are placed randomly into any of these possible phases, because nucleosomes occupy 144 bp of DNA while an average linker region is only 19-40 bp, any given sequence will be tightly associated with a nucleosome core in the majority of the rDNA molecules (or the majority of the time). Such tight association is expected to reduce the accessibility of this sequence to other proteins, such as those involved in transcription and transcriptional control. Restriction enzymes have been found to have access to only 15% of their recognition sites in randomly phased chromatin (c.f. Pfeiffer and Zachau, 1980 and references therein).

During the first stages of activation of the rRNA genes, secondary structures may form in the DNA at critical points such as immediately upstream from the initiation region (see above), and at the termination point (Kukita et. al., 1981, see also Niles et. al., 1981a). Additional secondary structures may form within the gene itself, since the mature rRNAs have a high degree of secondary structure. These secondary structures would have the effect of forcing the local chromatin structure into only one of the several potential phases (c.f. Wasylyk et. al., 1979), and may result in the complete clearing away of nucleosomes from

A Possible Mechanism for Activation of the Transcription Unit

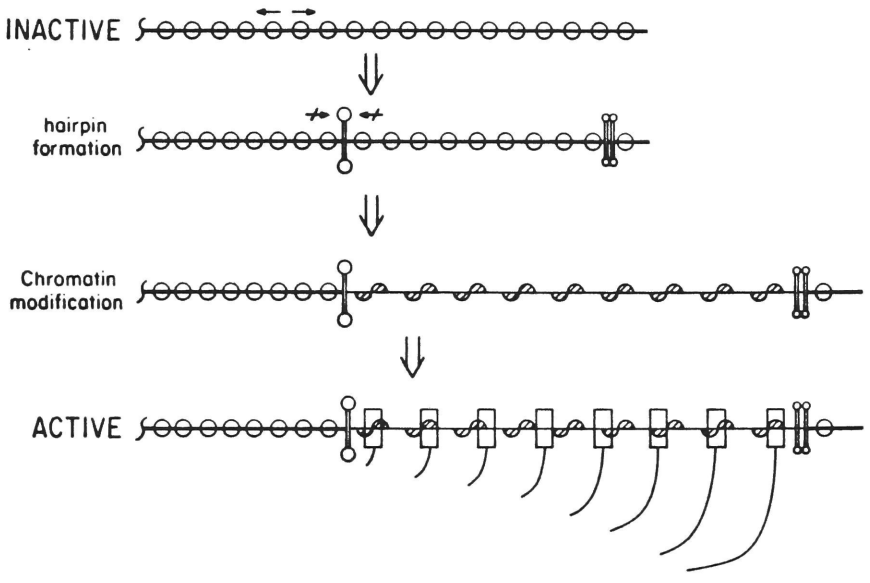


Figure 22: A Possible Mechanism for Activation of the Transcription Unit. Inactive genes are compacted into nucleosomes, which are free to occupy any one of several possible phases, and may be able to move from one phase to another (arrows). Secondary structure formation blocks any movement, and constrains the nucleosome to occupy only one phase. Following an unspecified recognition event, chromatin modification results in the formation of lexosomes on the transcription unit, extending it to full length. The extended gene may now be transcribed by RNA polymerase, even through it is still associated with chromatin subunits. See text for additional discussion.

a small region of the DNA (second line, Fig. 22). These regions would become the DNAse I hypersensitive sites found immediately upstream from active genes (Elgin, 1981 and Wu and Gilbert, 1981), and in some cases downstream (Kuo et. al., 1979 and Wu and Gilbert, 1981). The secondary structure formation would also result in the creation of an S1 nuclease sensitive site, also seen immediately upstream from the transcription initiation point in active genes (Larsen and Weintraub, 1982 and unpublished observations), and regions which are accessible to other nucleases (Groudine and Weintraub, 1981 and McGhee et. al., 1981).

However, no S1 sensitive or DNAse 1 hypersensitive sites have been reported in the rDNA for Physarum (Richard Braun, personal communication, and this laboratory, unpublished observations).

The formation of these secondary structures may require the the mediation of a specific non-histone protein to stabilize the structure, or the prior modification of the inactive chromatin (such as modification of core histones). In fact two classes of inactive chromatin have been observed in chicken erythrocytes (Fulmer and Bloomfield, 1981) which differ in their solubilities. These solubility differences are not the result of large protein differences, since a full and equal histone complement was found in each class, and no non-histone protein differences were detectable. The differences are postulated to be due to phosphorylation and/or ADP-ribosylation of core histones. These two classes could represent the activatable and non-activatable portions of the genome, and be morphologically represented by heterochromatin and euchromatin.

Once specific areas of the DNA have been exposed, sequence-specific DNA binding proteins can interact with these regions in a reproducible manner with all of the rDNA molecules, further stabilizing the secondary structures and possibly further altering the local chromatin structure. Such sequence-specific binding proteins are widely known from prokaryotes, but their presence in eukaryotes has until recently only been conjecture. A recent report of a topoisomerase bound to DNA near the DNAse I hypersensitive sites in Tetrahymena rDNA is one example of such an interaction (Bonven and Westergaard, 1982). This topoisomerase could also function to allow further secondary structure formation at these regions.

After secondary structure formation, exposure of specific DNA sequences and a specific recognition event, further chromatin modifications occur in the regions bounded by the secondary structures (third line, Fig. 22). This step probably requires a DNA replication cycle (Groudine and Weintraub, 1981). These modifications include the loss of H1, covalent modification (probably specific acetylation, see Vidali et.

al., 1978) of core nucleosomes, the binding of specific HMG proteins (Weisbrod and Weintraub, 1979) and the binding of specific non-histone chromatin proteins to each nucleosome core within the activating gene. The modified chromatin would now be in the DNAse I sensitive configuration associated with active or potentially active genes, as first reported by Weintraub and Groudine (1976).

The chromatin modifications proposed are not necessarily limited to proteins. DNA methylation is known to vary with the activity of a given sequence, and changes in methylation have been correlated to changes in DNAse I sensitivity (Kuo et. al., 1979). Other DNA base or sugar modifications, while not presently known to occur to a large extend in eukaryotes, may also be involved in this shift to an "open" configuration.

Such an "open" gene will not necessarily be transcribed. Further sequence-specific interactions seem to be required before transcription can occur in polymerase II-transcribed genes (see Miller et. al., 1978). It appears that, in the specific case of rRNA genes, once such an interaction has taken place, the gene is immediately transcribed at a maximal rate. Up- and down-regulation do not appear to occur in individual transcription units (c.f. Lewin, 1980 for discussion and references). Transcription proceeds through the gene even though the DNA in this region is still associated with nucleosomal core proteins (last line, Fig. 22). These nucleosomes, and their specific associated proteins (AP1 and 3, see Prior, 1982 and Prior et. al., 1983) are not in a beaded configuration, but are extended to virtually the full length of the DNA sequence (Johnson et. al., 1978b Johnson et. al., 1979 and Scheer et. al., 1981). This chromatin configuration is based on the "lexosome" model proposed by Prior and coworkers (1982 and 1983).

Telomeric Regions

Structure of the rDNA Termini in Vivo: The terminal restriction fragments of rDNA isolated from Physarum plasmodia are heterogeneous in size, varying by about ±400 base pairs. The sequence throughout this region is made up of inverted repeats, which readily form secondary structures visible in the electron microscope (Hardman et. al., 1979, Campbell et. al., 1979).

In addition to the size heterogeneity, they show some limited sequence heterogeneity, as demonstrated by the presence of subpopulations which contain extra restriction sites near the termini. Whether the same sequence variant is present on both ends of any given molecule is unknown. However, the findings of Ferris and Vogt (1982) demonstrate that the central spacer is not a perfect palindrome, so it seems likely that the ends need not be, either.

Another interesting feature of the telomeric regions in vivo is the presence of non-ligatable, single-stranded gaps at any one or more of several potential gap sites within 3 kb of the end (Johnson, 1980). These gaps are associated with the inverted repeat sequences, and always seem to be one nucleotide in length. The nucleotide that is missing is a C, and it is followed (5' to 3') by the sequence CCTA. The average distance between gaps is approximately 400 nucleotides, but varies over a range of several hundred nucleotides in the most extreme cases. In all probability at any given time there is a population of rDNA molecules which are ungapped.

Due to the presence of the inverted repeats and gaps, the phrase "end of the molecule" may prove difficult to define. Any gapped hairpin loop will act as a functional "end" of the molecule, and it is possible that the termini exist as a mixture of many different secondary structure conformations, each of which would have different gapped hairpin loops. Also, there may not be any requirement for a continuously equal

number of nucleotides associated with each strand, so the "end" may actually move from one potential hairpin gap to another.

Also associated with the inverted repeat sequences, near but not at the end of the rDNA, is a covalently bound protein molecule (Cheung et. al., 1981). The exact nature of this protein, and its attachment to the DNA, remain unknown. It is attractive to hypothesize that they may be either bound to the gaps, or responsible for their creation or both.

In comparison, the termini of Dictyostelium extrachromosomal rDNA also has terminal repeats, as indicated by Eco RI cleavage at many closely-spaced sites in this region (Cockburn et. al., 1978). The extreme terminus consists of an irregular, repeated C_nT satellite-like sequence (Emery and Weiner, 1981). The extrachromosomal rDNAs of the hypotrichs Euplotes, Oxytricha, Stylonichia and Tetrahymena consist of a variable number of repeats of a simple C_4-A_2 sequence (Blackburn and Gall, 1978 and Klobutcher et. al., 1981). All of these termini show the same kind of length heterogeneity on gels as the Physarum rDNA termini. Tetrahymena rDNA termini also contain single-nucleotide gaps (Blackburn and Gall, 1978).

A covalently bound protein molecule has not been reported near or at the termini of the rDNAs of any of the hypotrichs, or of Dictyostelium. Such a protein has been found in adenovirus (Rekosh et. al., 1977 and Robinson and Bellett, 1977), bacteriophage ø29 and several families of RNA viruses (see Wimmer, 1982 for review). In adenovirus and ø29 this protein serves to prime DNA synthesis at the extreme 5' end of the viral genome. Since DNA replication is known to begin near the center of the rDNA in Physarum, and the protein is not located at the extreme 5' end of the molecule, it cannot be closely analogous to the adenovirus terminal protein.

Sequence of Terminal Clones from Physarum rDNA: To determine the sequence of an rDNA terminus, the terminal restriction fragment was cloned, and shotgun subcloned using various procedures for sequencing. Apparently none of these various procedures gave rise to random inserts, as one orientation is highly favored, especially in the case of the Hae

III subclones. In addition, at least some of these clones are unstable in M13 (notably h3.1 and h2.1), giving rise to sequence changes as they are propagated. This instability is a function of the sequences in the insert, as other inserts from other regions of the rDNA do not change in culture (data not shown).

From these sequences, and further restriction enzyme mapping data, a tenative continuous sequence of 800 nucleotides from the (rDNA terminal) Eco RI end of the PrD229 insert has been assigned. While the problems of obtaining convincing overlaps between sequences in a series of tandem, degenerate repeats are obvious, and compounded by the favored orientation and start sites of the clones, the continuous sequence presented in Fig. 23 is, in all likelyhood, accurate to within a few percent.

These sequences are arranged into a series of degenerate 144 bp repeats (the figure 144 comes also from restriction mapping experiments). The degeneracy is evident both from the variation in the lengths of the various repeats, and in the presence in some but not all of the repeats of interior Hae III sites. These regions have been identified as repeats on the basis of their shared structural features. These features, aligned with the sequences from several clones, were presented in Fig. 19.

One of the most notable of these features is the central axis of symmetry. While the palindrome is far from exact in any case, there is sufficient inverted repeat nature in many of these repeats to allow the formation of stable secondary structures. Three examples of such structures are shown in Fig. 24.

Another notable feature is the presence in some of the repeats of the sequence CCCTA, which has been identified as a potential gap site. Whenever this sequence appears, it is near the center of symmetry. In several cases, two such sites appear as elements of the inverted repeat, being present in a head-to-head configuration approximately 10 to 30 nucleotides apart. The potential gap sites are within the stable secondary structures that can be formed, and are quite often at one or both

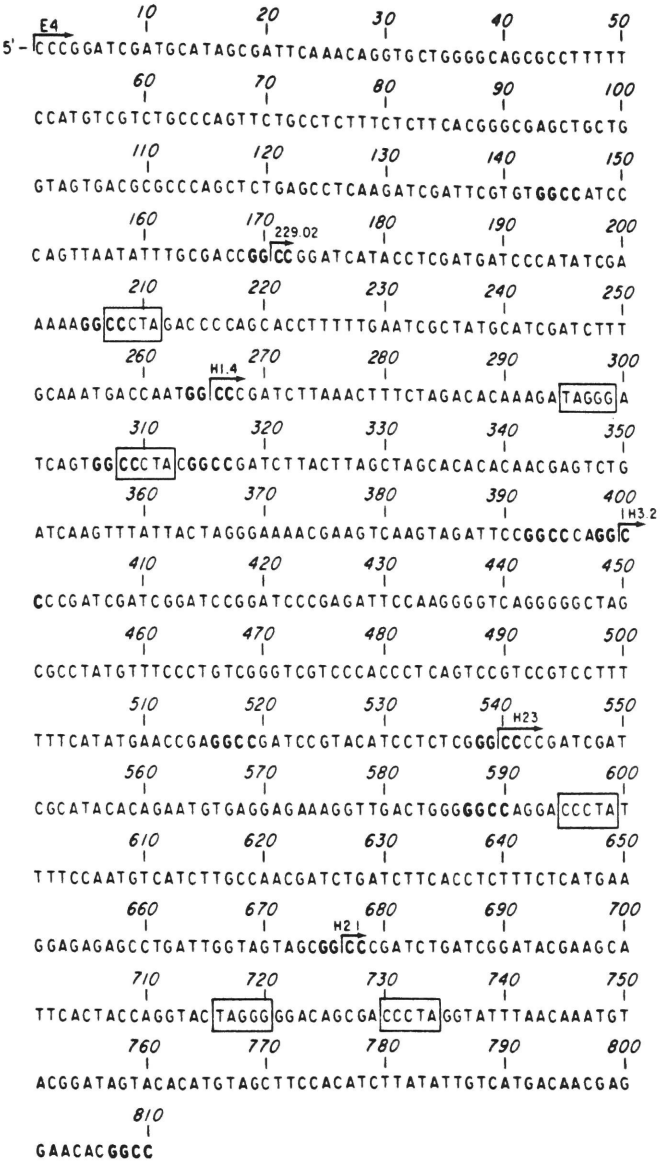


Figure 23: Continuous Sequence from the rDNA Terminal End of the Clone PrD229. 800 nucleotides of continuous sequence from the end of the rDNA terminal clone PrD229 was determined by restriction mapping and sequencing. This end was identified as the rDNA terminal-proximal end.

ends of a hairpin loop, as for example in Fig. 24c. If such a structure were gapped, a hairpin stem with a single-stranded tail would result. Such a structure would provide an excellent substrate for a recombination process, beginning with the invasion of another repeat unit by the single-stranded tail.

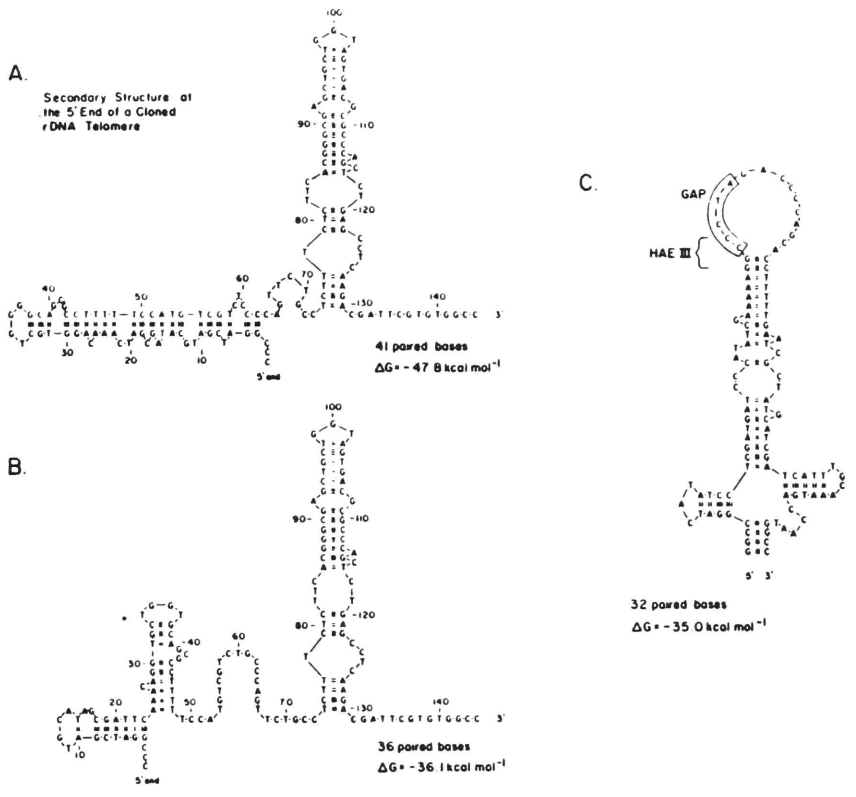


Figure 24: Potential Secondary Structure in Repeat Units from PrD229. Stable secondary structures from repeating units of the clone PrD229 insert. ΔG is calculated as described by Tinoco et. al., 1973 for RNA. Potential gap site sequences are usually found at the loop of these hairpins, as shown in C.

Other common features of the repeat units include the early presence of tandem Mbo I (Sau 3A) sites, and regions of unusual nucleotide composition as noted in Fig. 19. The possible functions of these features, if any, remains to be determined. It is tempting to speculate that they may provide a specific DNA conformation at discrete intervals to provide a series of protein recognition sites, or to provide a substrate for some enzyme involved in the terminal replication completion process.

Also still to be determined are the position(s) of the covalently bound protein associated with these sequences, and whether the terminal

sequence presented here represents the extreme terminus of an intact rDNA molecule (if such a statement can be made) or is merely very close to it.

This complex inverted repeating structure is in great contrast with other types of terminal sequences studied so far. Dictyostelium, Tetrahymena and other hypotrichs have a simple repeated sequence at their termini (C_nT in the case of Dictyostelium, and C_4A_2 for the hypotrichs, including Tetrahymena) (Emery and Weiner, 1981, Blackburn and Gall, 1977 and Klobutcher et. al., 1982). Yeast telomeres, on the other hand, are more complicated and may be more similar to Physarum rDNA termini.

Some or all of the features of the termini are probably required for the function of this region in allowing the completion of replication of the linear end. To determine which of these features are important, experiments are planned in which the isolated rDNA termini will be ligated to a linear plasmid vector and transfected into various types of host cells. Previous experiments have demonstrated that the simple hexanucleotide repeat terminal sequence of Tetrahymena is capable of supporting the replication of such a linear yeast vector (Szostak and Blackburn, 1982), showing that the telomere function is conserved enough to cross this evolutionary distance. Therefore it is conceivable that the Physarum rDNA telomere will prove capable of supporting the replication of either the linear yeast plasmid, or of a linearized hybrid vector (containing both a pBR322 and an SV40 origin of replication) in cultured HeLa cells. Alternately, cloning experiments could be done using the rDNA origin of replication and termini with some marker sequence or gene between them. Once a successful linear vector has been established using the Physarum rDNA termini, experiments can begin to establish which sequences are responsible for their function. The conservation of other features in the linear vector would be evidence for their function in terminus replication.

A Model for the Replication of the Extreme Termini of the Physarum Linear rDNA Molecule: The presence of the various features discussed

above at the termini of Physarum rDNA has suggested a model for the completion of replication of this terminus. This model is presented in Fig. 25. A nearly completed replicating rDNA molecule will have a small single-stranded region at each end, due to the removal of the 5' RNA primer. Such a molecule can form into various stable or quasi-stable foldback structures (I. in Fig. 25). These foldback structures may be stabilized by the covalently bound protein, and may involve more than one repeat unit.

The formation of such hairpins causes the creation of single-stranded regions in the loops. In addition, the loops may be gapped at this time, leaving a single-stranded tail. In either case, the single-stranded region can invade the terminal repeat region of another DNA molecule, forming a D-loop (II.). The invading strand does not necessarily have to invade at its exactly complementary position in a donor rDNA, but may invade at another repeat further towards the center, or may invade a homologous chromosomal sequence. Whether there is a preferred invasion site is unknown.

If gapping has not already occurred, it will occur now, beginning a strand transfer event similar to that originally proposed by Holliday (1964)(III.). The incompletely synthesized strand from the replicating molecule will be transferred to the donor DNA molecule beginning at the specific gap and running to the 5' end of the strand. The donor strand will transfer to the replicating rDNA, beginning at the position of the gap and running to the extreme 5' terminus.

The cross-over structure is resolved by a normal strand scission event, such as has been postulated for rec A-mediated homologous recombination (IV.). The scission events will leave the terminus of the replicating molecule complete, but containing a nick or gap at the original gap site. The donor molecule will be incomplete, having not only a nick or gap at the 3' end of the newly replicated DNA fragment, but a substantial single-stranded region at its 5' end. In addition, due to the degenerate nature of the repeats, each molecule is likely to contain regions of mismatch.

The exact position of the strand scission event may or may not be determined by the end of the recipient rDNA molecule. Any inexactness in matching the position of this strand scission and the end of the rDNA will be reflected in the loss or gain of additional repeat sequences (or other chromosomal sequences) at the new 5' end. If extra sequences are added, they would provide a template for extension of the 3' end of the complementary strand, and be more or less permanently added to the replicating rDNA. If the replicating terminus still has a 5' undershot end, it may involve itself in additional recombinations, or may eventually lose the extreme end. The ends thus generated will in all probability be of random lengths (as observed), depending on the specificity of the strand scission event. Sequence specificity of strand scission would lead to preferred but not unique endpoints of the rDNA, and possibly to loss or gain of sequence only in repeat unit blocks.

The final step of this model is the gap-filling DNA replication required to complete the donor molecule DNA strand, and to fill any gaps which are unwanted (V.). This DNA synthesis is completely internal in the DNA strand, and each synthesis site is provided with a 3'-OH DNA strand primer. Repair synthesis would also be required to repair the mismatched bases contained within the exchanged repeats, and to fill in the complement of any new 5' sequences obtained from the recombination.

This model requires two unusual features of the rDNA terminus, namely the presence of an array of inverted repeats which have sufficient homology between themselves or with chromosomal sequences to be able to exchange complementary DNA strands, and the presence of a specific gap. Both of these requirements are met by the rDNA (Johnson, 1980 and Bergold et. al., 1983). Notice that the array of repeats could be as small as two, although a larger array would probably allow a more rapid D-loop formation due to the higher immediate concentration of homologous sequences. Also, the closer to exact homology the repeats are, the easier the strand exchange event will be. However, this must be balanced against the propensity for repeated inverted repeats to undergo deletions (as for instance in our M13 clones), which would destroy the ability of the repeat array to continue functioning. The

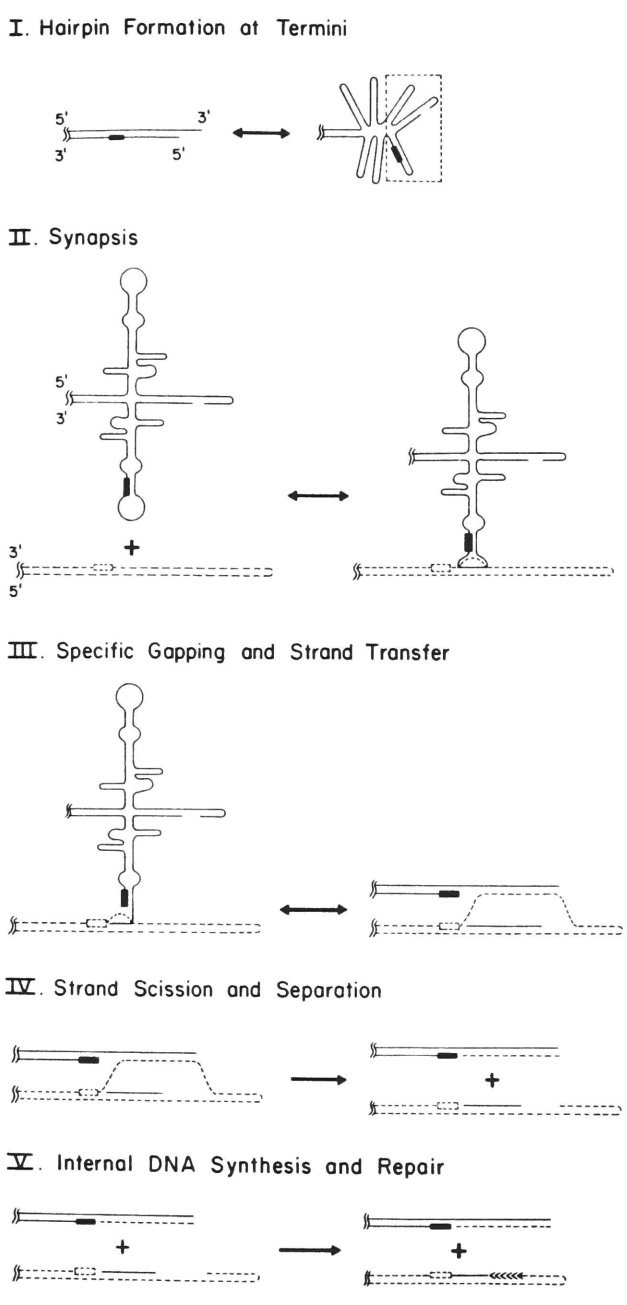


Figure 25: A Model for the Mechanism of Completion of Replication at a Linear rDNA End.
I. An incompletely replicated DNA strand may undergo secondary structure formation at the terminus.
II. The single-stranded regions at the end of the hairpin loops invade a homologous sequence on another DNA molecule (or further up the same molecule).
III. Specific gapping at CCCTA sequences near the hairpin loop initiates a strand transfer event, giving rise to recombination intermediates.
IV. Recombination intermediates are resolved by strand scission, allowing the DNA molecules to separate.
V. Internal DNA synthesis and repair of any mismatches completes the new rDNA terminus and regenerates the homologous sequence.

degeneracy of the repeats may be a partial defense against such deletions, as the inexactness would reduce the likelihood of the initial recombination event, while still allowing a large potential target area for such events.

This model suggests that the extreme terminus of the rDNA molecule will be heterogeneous in length, due to the random loss and gain of sequences at the end during the recombination scission step. Such heterogeneity is seen, not only in our termini (Campbell et. al., 1979) but in other extrachromosomal rDNAs in Tetrahymena (Blackburn and Gall, 1978) and Dictyostelium (Emery and Weiner, 1981).

The proposed mechanism also results in the generation of a large number of potential recombination "hot spots" during each replication cycle. It would therefore be surprising if some recombination intermediates, such as that proposed in Fig. 25 (II. and III.) were not seen when a total DNA or rDNA preparation is spread directly for electron microscopy. In fact such recombination intermediates are observed, at a frequency of about 2.5% of the full-length rDNA molecules found (Bergold et. al., 1983). One such intermediate is shown in Fig. 26.

Since the length of time required for completion of the recombination event is not known, no statement can be made concerning the expected frequency of observation of recombinant intermediates. It should be noted, however, that in order to replicate sufficient rDNA each generation some 300 such replications need to occur per generation, with two recombination events per complete replication. The generation time for Physarum is approximately 8.5 hours. This allows us to compute that one rDNA replication should be completed approximately every 1.7 minutes. In order for the ratio of recombining ends to reach 2.5%, at any given time 7.5 ends should be recombinant. This means that if terminal completion is the only event which requires recombination, the resolution time of the recombination would be approximately 6.3 minutes (7.5 ends x 1.7 min./2 ends), an extremely long time for such an event. (Phage T4 undergoes many rounds of recombination during the six minutes between DNA replication onset and first appearance of mature infectious

phage (c.f. Lewin, 1977).) One may therefore assume that not all recombinations that occur lead to terminal completion. The rDNA terminus may thus be considered a recombination-prone structure.

It is interesting to note that the same mechanism could work intramolecularly. A hairpin loop on, for example, the 5' strand could invade another repeat unit further out on the 3' strand (or a hairpin loop on the 3' strand could invade a more central repeat unit on the 5' strand), giving the first D loop. This would result in the formation of a closed loop at the rDNA terminus. Such loops are also observed at high frequency in isolated rDNA molecules (Bergold et. al., 1983). Gapping and strand transfer would then result in the displacement of the 5' (incomplete) strand terminal fragment outward along the complementary strand, and create a large single-stranded region in the interior of the 5' strand. This region could then be filled and ligated, resulting in the completion of the terminus. For this mechanism to replicate the extreme terminus of the rDNA, it is necessary for the inverted repeat units to extend all of the way to the terminus.

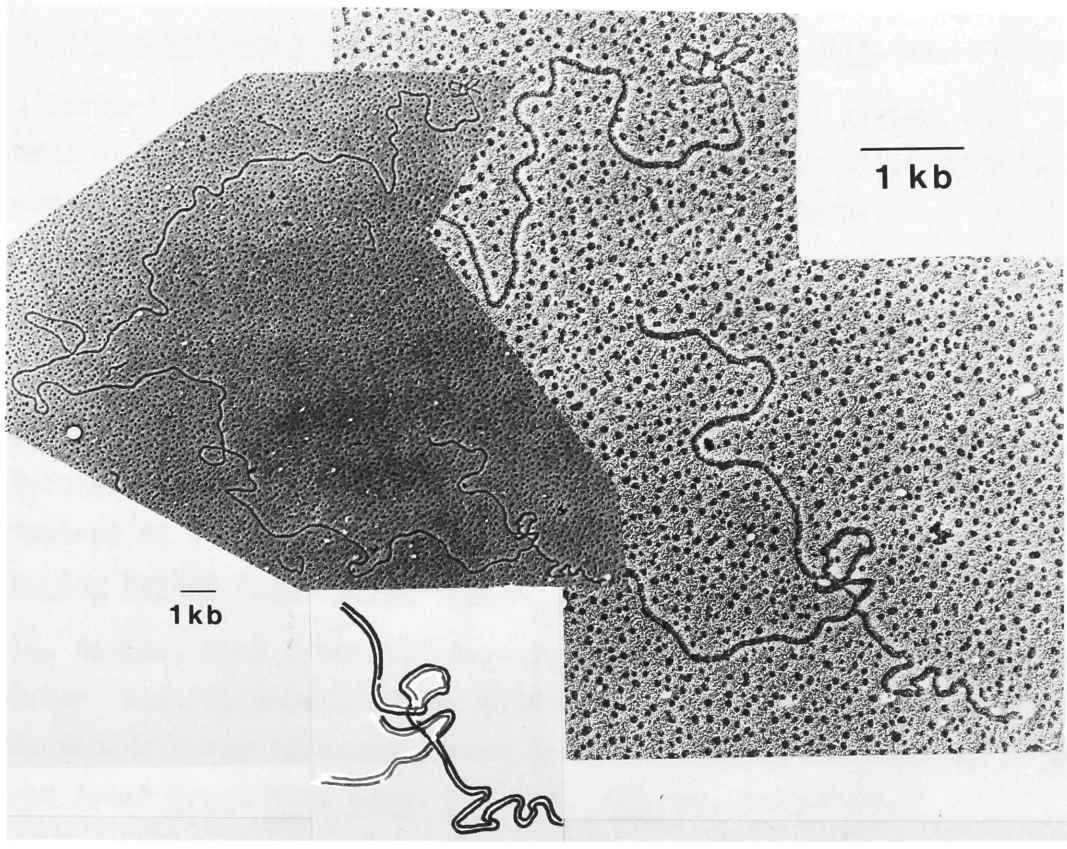


Figure 26: Electron Micrograph of Full-Length rDNA Molecule with Recombinant Structures at Its Ends.
Left: The full length of the recombinant rDNA molecule. Scale bar at left represents 1 kb of fully extended DNA.
Right: Enlargement of the ends of the rDNA molecule, showing details of the recombinant structures. Scale bar above right represents 1 kb.
Inset: Interpretation of the lower recombination intermediate, representing the structure as the of step IV. in Fig. 25, with one strand scission event having already taken place.

REFERENCES

Affolter, Hans-Urs and Richard Braun (1978) "Ribosomal DNA in Spores of Physarum polycephalum" Biochim. Biophys. Acta V. 519, pp. 118-124.

Allet, Bernard and Jean-Davis Rochaix (1979) "Structure Analysis at the Ends of the Intervening DNA Sequences in the Chloroplast 23 S Ribosomal Genes of C. reinhardii" Cell V. 18, pp. 55-60.

Allet, B., P. G. N. Jeppsen, K. J. Katagiri and H. Delius (1973) "Mapping the DNA Fragments Produced by Cleavage of λ DNA with Endonuclease RI" Nature V. 241, pp. 120-122.

Allfrey, V. G., E. M. Johnson, I. Y.-C. Sun, V. C. Littau, H. R. Matthews and E. M. Bradbury (1978) "Structural Organization and Control of the Ribosomal Genes in Physarum polycephalum" Cold Spring Harbor Symp. Quant. Biol. V. 42, pp. 505-514.

Arnheim, Norman, Mark Krystal, Roy Schmickel, Golder Wilson, Oliver Ryder and Elizabeth Zimmer (1980) "Molecular Evidence for Genetic Exchanges Among Ribosomal Genes on Nonhomologous Chromosomes in Man and Apes" Proc. Nat. Acad. Sci. V. 77, pp. 7323-7327.

Arnheim, Norman, Peter Seperack, Julian Banerji, Rhonda B. Lang, Roger Miesfeld and Kenneth B. Marcu (1980) "Mouse rDNA Nontranscribed Spacer Sequences are Found Flanking Immunoglobulin C_H Genes and Elsewhere Throughout the Genome" Cell V. 22, pp. 179-185.

Bach, Rene, Ingrid Grummt and Bernard Allet (1981) "The Nucleotide Sequence of the Initiation Region of the Ribosomal Transcription Unit from Mouse" Nuc. Acids Res. V. 9, pp. 1559-1569.

Baer, Bradford W. and Daniela Rhodes (1983) "Eukaryotic RNA Polymerase II Binds to Nucleosome Cores from Transcribed Genes" Nature V. 301, pp. 482-488.

Baker, C. C., J. Herise, G. Courtois, F. Galibert and E. Ziff (1979) "Messenger RNA for the Ad2 DNA Binding Protein: DNA Sequences Encoding the First Leader and Heterogeneity at the mRNA 5' End" Cell V. 18, pp. 569-580.

Bakken, Aimee, Garry Morgan, Barbara Sollner-Webb, Judith Roan, Sharon Busby and Ronald H. Roeder (1982) "Mapping of Transcriptional Initiation and Termination Signals of Xenopus laevis Ribosomal DNA" Proc. Nat. Acad. Sci. V. 79, pp. 56-60.

Bateman, A. J. (1975) "Simplification of Palindromic Telomere Theory" Nature V. 253, p. 379.

Bayev, A. A., O. I. Georgiev, A. A. Hadjiolov, M. B. Kermekchiev, N. Nikolaev, K. G. Skryabis and U. M. Zakharyev (1980) "The Structure of the Yeast Ribosomal RNA Genes: 2. The Nucleotide Sequence of the Initiation Site for Ribosomal RNA Transcription" Nuc. Acids Res. V. 8, pp. 4919-4926.

Beard, Peter (1978) "Mobility of Histones on the Chromosome of Simian Virus 40" Cell V. 15, pp. 955-967.

Benoist, Christophe and Pierre Chambon (1981) "In Vivo Sequence Requirements of the SV40 Early Promoter Region" Nature V. 290, pp. 304-310.

Benton, W. David and Ronald W. Davis (1977) "Screening λgt Recombinant Clones by Hybridization to Single Plaques in Situ" Science V. 196, pp. 180-182.

Bergold, Peter, Gerald R. Campbell, Virginia C. Littau and Edward M. Johnson (1983) "Sequence and Hairpin Structure of an Inverted Repeat Series at Termini of the Physarum Extrachromosomal rDNA Molecule" Cell in press.

Bird, Adrian P. (1980) "Gene Reiteration and Gene Amplification" in Cell Biology: A Comprehensive Treatise (ed. Lester Goldstein and David M. Prescott) V. 3, pp. 61-111. Academic Press, New York.

Bird, Adrian P. and Mary H. Taggert (1980) "Variable Patterns of Total DNA and rDNA Methylation in Animals" Nuc. Acids Res. V. 8, pp. 1499-1504.

Birnboim, H. C. and J. Doly (1979) "A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA" Nuc. Acids Res. V. 7, pp. 1513-1523.

Birnstiel, M. L., M. Chipchase and J. Spiers (1971) "The Ribosomal RNA Cistrons" Prog. Nuc. Acids Res. Mol. Biol. V. 11, pp. 351-389.

Blackburn, Elizabeth and San-San Chiou (1981) "Non-Nucleosomal Packaging of a Tandemly Repeated DNA Sequence at Termini of Extrachromosomal DNA Coding for rRNA in Tetrahymena" Proc. Nat. Acad. Sci. V. 78, pp. 2263-2267.

Blackburn, Elizabeth H. and Joseph G. Gall (1978) "A Tandemly Repeated Sequence at the Termini of the Extrachromosomal Ribosomal RNA Genes in Tetrahymena" J. Mol. Biol. V. 120, pp. 33-53.

Blattner, Fred (1978) "Procedure for Cloning with Charon Phages" personal communication.

Blattner, Frederick R., Bill, G. Williams, Ann E. Blechl, Katherine Denniston-Thompson, Harvey F. Faber, Lesley-Anne Furlong, David J. Grunwald, Delight O. Kiefer, David D. Moore, James W. Schumm, Edward L. Sheldon and Oliver Smithies (1977) "Charon Phages: Safer Derivatives of Bacteriophage Lambda for DNA Cloning" Science V. 196, pp. 161-169.

Bogenhagen, Daniel F. and Donald D. Brown (1981) "Nucleotide Sequences in Xenopus 5 S DNA Required for Transcription Termination" Cell V. 24, pp. 261-270.

Bogenhagen, Daniel F., Shigeru Sakonju and Donald D. Brown (1980) "A Control Region in the Center if the 5 S RNA Gene Directs Specific Initiation of Transcription: II. The 3' Border of the Region" Cell V. 19, pp. 27-35.

Bohnert, Hans-Jurgen, Barbara Schiller, Roswita Bohme and Helmut W. Sauer (1975) "Circular DNA and Rolling Circles in Nucleolar rDNA from Mitotic Nuclei of Physarum polycephalum" Eur. J. Biochem. V. 57, pp. 361-369.

Bonven, Bjourne and Ole Westergaard (1982) "DNAse I Hypersensitive Regions Correlate with a Site-Specific Endogenous Nuclease Activity on the r-Chromatin of Tetrahymena" Nuc. Acids Res. V. 10, pp. 7593-7608.

Boseley, Paul G., Anne Tuyns and Max L. Birnstiel (1978) "Mapping of the Xenopus laevis 5.8 S rDNA by Restriction and DNA Sequencing" Nuc. Acids Res. V. 5, pp. 1121-1138.

Bradbury, E. M., H. R. Matthews, J. McNaughton and H. V. Molgaard (1973) "Sub-Nuclear Components of Physarum polycephalum" Biochim. Biophys. Acta V. 335, pp. 19-29.

Bradbury, E. Morton, Harry R. Matthews and Harald V. Molgaard (1975) "Restriction Enzyme Digests of the Heavy Nucleolar Satellite Deoxyribonucleic Acid of Physarum polycephalum" Biochemical Society Transactions 558th Meeting, Edinburgh, V. 3, pp. 1015-1017.

Braun, Richard and Thomas E. Evans (1969) "Replication of Nuclear Satellite and Mitochondrial DNA in the Mitotic Cycle of Physarum" Biochim. Biophys. Acta V. 182, pp. 511-522.

Braun, R., L. Hall, M. Schwarzler and S. S. Smith (1977) "The Mitotic Cycle of Physarum polycephalum" in Proc. Intnatl. Symp. Cell Diff. in Microorgs., Plants and Animals (ed. L. Nover and K. Mathes) pp. 149-166, Gustav Fischer, Stuttgart, W. Ger.

Braun, Richard and Helen Wili (1969) "Time Sequence of DNA Replication in Physarum" Biochim. Biophys. Acta V. 174, pp. 246-252.

Brown, Donald D. and Igor B. Dawid (1968) "Specific Gene Amplification in Oocytes" Science V. 160, pp. 272-280.

Breathnach, Richard and Pierre Chambon (1981) "Organization and Expression of Eucaryotic Split Genes Coding for Proteins" Ann. Rev. Biochem. V. 50, pp. 349-383.

Burgess, Ann Baker and Richard R. Burgess (1974) "Purification and Properties of Two RNA Polymerases from Physarum polycephalum" Proc. Nat. Acad. Sci. V. 71, pp. 1174-1177.

Campbell, Gerald R., Virginia C. Littau, Peter W. Melera, Vincent G. Allfrey and Edward M. Johnson (1979) "Unique Sequence Arrangement of Ribosomal Genes in the palindromic rDNA Molecule of Physarum polycephalum" Nuc. Acids Res. V. 6, pp. 1433-1447.

Cavalier-Smith, T. (1974) "Palindromic Base Sequences and Replication of Eukaryotic Chromosome Ends" Nature V. 250, pp. 467-470.

Cech, Thomas R. and Susan L. Brehm (1981) "Replication of the Extrachromosomal Ribosomal RNA Genes of Tetrahymena thermophila" Nuc. Acids Res. V. 9, pp. 3531-3543.

Cech, Thomas R. and Kathleen M. Karrer (1980) "Chromatin Structure of the Ribosomal RNA Genes of Tetrahymena thermophila as Analyzed by Trimethylpsoralen Crosslinking in Vivo" J. Mol. Biol. V. 136, pp. 395-416.

Cech, Thomas R., Arthur J. Zaug and Paula J. Grabowski (1981) "In Vitro Splicing of the Ribosomal RNA Precursor of Tetrahymena: Involvement of a Guanosine Nucleotide in the Excision of the Intervening Sequence" Cell V. 27, pp. 487-496.

Cheung, Margaret K., Dimitrios Drivas, Virginia C. Littau and Edward M. Johnson (1981) "Protein Tightly Bound Near the Termini of the Physarum Extrachromosomal rDNA Palindrome" J. Cell Biol. V. 91, pp. 309-314.

Cockburn, Andrew F., William C. Taylor and Richard A. Firtel (1978) "Dictyostelium rDNA Consists of Non-chromosomal Palindromic Dimers Containing 5 S and 36 S Coding Regions" Chromosoma V. 70, pp. 19-29.

Corden, J., B. Wasylyk, A. Buchwalder, P. Sassone-Corsi, C. Kedinger, and Pierre Chambon (1980) "Promoter Sequences of Eukaryotic Protein-Coding Genes" Science V. 209, pp. 1406-1413.

Cory, Suzanne and Jerry M. Adams (1977) "A Very Large Repeating Unit of Mouse DNA Containing the 18 S, 28 S and 5.8 S rRNA Genes" Cell, V. 11, pp. 795-805.

Dancis, Barry M. and Gerald P. Holmquist (1979) "Telomere Replication and Fusion in Eukaryotes" J. Theor. Biol. V. 78, pp. 211-224.

Daniel, John W. and Helen H. Baldwin (1964) "Methods of Culture for Plasmodial Myxomycetes" in Meth. Cell Physiol. (ed. D. M. Prescott) V. 1, pp. 9-41, Academic Press, New York.

Davies, K. E. and I. O. Walker (1977) "In Vitro Transcription of RNA in Nuclei, Nucleoli and Chromatin from Physarum polycephalum" J. Cell Sci. V. 26, pp. 267-279.

Davis, Ronald W., Martha Simon and Norman Davidson (1971) "Electron Microscope Heteroduplex Methods for Mapping Regions of Base Sequence Homology in Nucleic Acids" Meth. Enzymol. V. 21, (ed. Lawrence Grossman and Kivie Moldave) pp. 413-428.

Dawid, Igor B., Eric O. Long, Pier Paolo DiNocera and Mary Lou Pardue (1981) "Ribosomal Insertion-Like Elements in Drosophila melanogaster Are Interspersed with Mobile Sequences" Cell V. 25, pp. 399-408.

Denhardt, David T. (1966) "A Membrane-Filter Technique for the Detection of Complementary DNA" Biochem. Biophys. Res. Comm. V. 23, pp. 641-646.

Dover, Gabriel (1982) "Molecular Drive: a Cohesive Mode of Species Evolution" Nature V. 299, pp. 111-117.

Elgin, Sarah C. R. (1981) "DNAse I Hypersensitive Sites in Chromatin" Cell V. 27, pp. 413-415.

Emery, Herschell S. and Alan M. Weiner (1981) "An Irregular Satellite Sequence is Found at the Termini of the Linear Extrachromosomal rDNA in Dictyostelium discoidum" Cell V. 26, pp. 411-419.

Engberg, Jan, Paul Andersson, Vagn Leick and John Collins (1976) "Free Ribosomal DNA Molecules from Tetrahymena pyriformis GL are Giant Palindromes" J. Mol. Biol. V. 104, pp. 455-470.

Farr, David R. and Marc Horisberger (1978) "Structure of a β-Galactan from the Nuclei of Physarum polycephalum" Biochim. Biophys. Acta V. 539, pp. 37-40.

Federoff, Nina (1979) "On Spacers" Cell V. 16, pp. 697-710.

Ferris, Patrick J. and Volker M. Vogt (1982) "Structure of the Central Spacer Region of the Extrachromosomal Ribosomal DNA in Physarum polycephalum" J. Mol. Biol. V. 159, pp. 359-381.

Financsek, Istvan, Kiyohisa Mizumoto, Yukio Mishima and Masami Muramatsu (1982) "Human Ribosomal RNA Gene: Nucleotide Sequence of the Transcription Initiation Region and Comparison of Three Mammalian Genes" Proc. Nat. Acad. Sci. V. 79, pp. 3092-3096.

Findley, R. Craig and Joseph G. Gall (1978) "Free Ribosomal RNA Genes in Paramecium are Tandemly Repeated" Proc. Nat. Acad. Sci. V. 75, pp. 3312-3316.

Frankel, Gary, Andrew F. Cockburn, Karen L. Kindle and Richard A. Firtel (1977) "Organization of the Ribosomal RNA Genes of Dictyostelium discoidum. Mapping of the Transcribed Region" J. Mol. Biol. V. 109, pp. 539-558.

Fulmer, Andrew W. and Victor A. Bloomfield (1981) "Chicken Erythrocyte Nucleus Contains Two Classes of Chromatin that Differ in Micrococcal Nuclease Sensitivity and Solubility at Physiological Ionic Strength" Proc. Natl. Acad. Sci. V. 78, pp. 5968-5972.

Gall, Joseph G. (1969) "The Genes for Ribosomal RNA During Oogenesis" Genetics V. 61 supp. 1 part 2, pp. 121-132.

Gall, Joseph G. (1974) "Free Ribosomal RNA Genes in the Macronucleus of Tetrahymena" Proc. Nat. Acad. Sci. V. 71, pp. 3078-3081.

Gallimore, Philip H., Phillip A. Sharp and Joe Sambrook (1974) "Viral DNA in Transformed Cells" J. Mol. Biol. V. 89, pp. 49-72.

Gannon, F., K. O'Hare, F. Perrin, J. P. LePennec, C. Benoist, M. Cochet, R. Breathnach, A. Royal, A. Garapin, B. Cami and P. Chambon (1979) "Organisation and Sequences at the 5' End of a Cloned Complete Ovalbumin Gene" Nature V. 278, pp. 428-434.

Glover, David M. and David S. Hogness (1977) "A Novel Arrangement of the 18 S and 28 S Sequences in a Repeating Unit of Drosophila melanogaster rDNA Cell V. 10, pp. 167-176.

Goodman, Eugene M. (1980) "Physarum polycephalum: A Review of a Model System Using a Structure-Function Approach" Intnatl. Rev. Cytol. V. 63, pp. 1-58.

Gornicki, Stella Z., Susan B. Vuturo, Timothy V. West and Robert F. Weaver (1974) "Purification and Properties of Deoxyribonucleic Acid-Dependant Ribonucleic Acid Polymerases from the Slime Mold Physarum polycephalum" J. Biol. Chem. V. 249, pp. 1792-1798.

Grainger, Robert C. and Nancy Maizels (1980) "Dictyostelium Ribosomal RNA is Processed During Transcription" Cell V. 20, pp. 619-623.

Grainger, Robert M. and Roy C. Ogle (1978) "Chromatin Structure of the Ribosomal RNA Genes in Physarum polycephalum" Chromosoma V. 65, pp. 115-126.

Grant, William D. (1972) "The Effect of α-Amanitin and $(NH_4)_2SO_4$ on RNA Synthesis in Nuclei and Nucleoli Isolated from Physarum polycephalum at Different Times during the Cell Cycle" Eur. J. Biochem. V. 29, pp. 94-98.

Grazziani, Franco, Ruggero Caizzi and Silvana Gorgano (1977) "Circular Ribosomal DNA During Ribosomal Magnification in Drosophila melanogaster" J. Mol. Biol. V. 112, pp. 49-64.

Greenberg, Michael (1980) "Cleared Lysate Procedure for Plasmid Purification" personal communication.

Gross-Bellard, Maria, Pierre Oudet and Pierre Chambon (1973) "Isolation of High-Molecular-Weight DNA from Mammalian Cells" Eur. J. Biochem. V. 36, pp. 32-38.

Grosschedl, R. and M. L. Birnstiel (1980) "Identification of Regulatory Sequences in the Prelude Sequences of an H2A Histone Gene by the Study of Specific Deletion Mutants in Vivo" Proc. Natl. Acad. Sci. V. 77, pp. 1433-1436.

Groudine, Mark and Harold Weintraub (1981) "Activation of Globin Genes During Chicken Development" Cell V. 24, pp. 393-401.

Grummt, Ingrid (1981a) "Mapping of a Mouse Ribosomal DNA Promoter by In Vitro Transcription" Nuc. Acids Res. V. 9, pp. 6093-6102.

Grummt, Ingrid (1981b) "Specific Transcription of Mouse Ribosomal DNA in a Cell-Free System that Mimics Control in Vivo" Proc. Nat. Acad. Sci. V. 78, pp. 727-731.

Grummt, Ingrid (1982) "Nucleotide Sequence Requirements for Specific Initiation of Transcription by RNA Polymerase I" Proc. Nat. Acad. Sci. V. 79, pp. 6908-6911.

Gubler, Ueli, Toni Wyler and Richard Braun (1979) "The Gene for the 26 S rRNA in Physarum Contains Two Insertions" FEBS Lett. V. 100, pp. 347-350.

Gubler, Ueli, Toni Wyler, Thomas Seebeck and Richard Braun (1980) "Processing of Ribosomal Precursor RNAs in Physarum polycephalum" Nuc. Acids Res. V. 8, pp. 2647-2664.

Hall, Len and Richard Braun (1977) "The Organisation of Genes for Transfer RNA and Ribosomal RNA in Amoebae and Plasmodia of Physarum polycephalum" Eur. J. Biochem. V. 76, pp. 165-174.

Hall, Len, Ueli Gubler and Richard Braun (1978) "Metabolic Stability of the Extrachromosomal Ribosomal RNA Genes in the Slime Mould Physarum polycephalum" Eur. J. Biochem.

Hall, Leonard and Geoffrey Turnock (1976) "Synthesis of Ribosomal RNA During the Mitotic Cycle in the Slime Mould Physarum polycephalum" Eur. J. Biochem. V. 62, pp. 471-477.

Hall, Leonard, Geoffrey Turnock and Brian J. Cox (1975) "Ribosomal RNA Genes in the Amoebal and Plasmodial Forms of the Slime Mould Physarum polycephalum" Eur. J. Biochem. V. 51, pp. 459-465. V. 86, pp. 45-50.

Hardman, Norman, Peter L. Jack, Alistair J. P. Brown and Alan McLachlan (1979) "Characterization of Ribosomal Satellite in Total Nuclear DNA from Physarum polycephalum" Biochim. Biophys. Acta V. 562, pp. 365-376.

Hildebrandt, Armin and Helmut W. Sauer (1977) "Transcription of Ribosomal RNA in the Life Cycle of Physarum May Be Regulated by a Specific Nucleolar Initiation Inhibitor" Biochem. Biophys. Res. Comm. V. 74, pp. 466-472.

Hohn, Barbara (1982) "In Vitro Packaging of λ and Cosmid DNA" Meth. Enzymol. V. 68, pp. 299-309.

Holliday, Robin (1964) "A Mechanism for Gene Conversion in Fungi" Genet. Res. Camb. V. 5, pp. 282-304.

Holmes, David S. and Michael Quigley (1981) "A Rapid Boiling Method for the Preparation of Bacterial Plasmids" Anal. Biochem. V. 114, pp. 193-197.

Holt,Charles E. (1980) "The Nuclear Replication Cycle in Physarum polycephalum" in Growth and Differentiation in Physarum polycephalum (ed. William F. Dove and Harold P. Rusch) Princeton Univ. Press, Princeton, N. J. pp. 9-63.

Hoopes, Barbara C. and William R. McClure (1981) "Studies on the Selectivity of DNA Precipitation by Spermine" Nuc. Acids Res. V. 9, pp. 5493-5504.

Hu, Shiu-Lok and James L. Manley (1981) "DNA Sequences Required for Initiation of Transcription in Vitro From the Major Late Promoter of Adenovirus 2" Proc. Natl. Acad. Sci. V. 78, pp. 820-824.

Jacobson, David N. and Charles E. Holt (1973) "Isolation of Ribosomal RNA Precursors from Physarum polycephalum" Arch. Biochem. Biophys. V. 159, pp. 342-352.

Jockusch, Brigitte M. and Ian O. Walker (1974) "The Preparation and Preliminary Characterisation of Chromatin from the Slime Mould Physarum polycephalum" Eur. J. Biochem. V. 48, pp. 417-425.

Johnson, Edward M. (1980) "A Family of Inverted Repeat Sequences and Specific Single-Strand Gaps at the Termini of the Physarum rDNA Palindrome" Cell V. 22, pp. 875-886.

Johnson, Edward M., Vincent G. Allfrey, E. Morton Bradbury and Harry R. Matthews (1978a) "Altered Nucleosome Structure Containing DNA Sequences Complementary to 19 S and 26 S Ribosomal RNA in Physarum polycephalum" Proc. Nat. Acad. Sci. V. 75, pp. 1116-1120.

Johnson, Edward M, Gerald R. Campbell and Vincent G. Allfrey (1979) "Different Nucleosome Structures on Transcribing and Non-Transcribing Ribosomal Gene Sequences" Science V. 206, pp. 1192-1194.

Johnson, Edward M., Virginia C. Littau, Vincent G. Allfrey, E. Morton Bradbury and Harry R. Matthews (1976) "The Subunit Structure of Chromatin from Physarum polycephalum" Nuc. Acids Res. V. 3, pp. 3313-3329.

Johnson, Edward M., Harry R. Matthews, Virginia C. Littau, Leonard Lothstein, E. Morton Bradbury and Vincent G. Allfrey (1978b) "The Structure of Chromatin Containing DNA Complementary to 19 S and 26 S Ribosomal RNA in Active and Inactive Stages of Physarum polycephalum" Arch. Biochem. Biophys. V. 191, pp. 537-550.

Kahn, Michael, Roberto Kolter, Christopher Thomas, David Figurski, Richard Meyer, Eric Remaut and Donald R. Helinski (1979) "Plasmid Cloning Vehicles Derived from Plasmids ColE1, F, R6K, and RK2" Meth. Enzymol. (ed. Ray Wu) V. 68, pp. 268-280, Academic Press, New York.

Karn, Jonathan, Sydney Brenner, Leslie Barnett and Gianni Cesareni (1980) "Novel Bacteriophage λ Cloning Vector" Proc. Nat. Acad. Sci. V. 77, pp. 5172-5176.

Karrer, Kathleen M. and Joseph G. Gall (1976) "The Macronuclear Ribosomal DNA of Tetrahymena pyriformis is a Palindrome" J. Mol. Biol. V. 104, pp. 421-453.

Keuhn, Glenn D., Hans-Urs Affolter, Valerie J. Atmar, Thomas Seebeck, Ueli Gubler and Richard Braun (1979) "Polyamine-Mediated Phosphorylation of a Nucleolar Protein from Physarum polycephalum that Stimulates rRNA Synthesis" Proc. Nat. Acad. Sci. V. 76, pp. 2541-2545.

King, Balas O. and Meng-Chao Yao (1982) "Tandemly Repeated Hexanucleotide at Tetrahymena rDNA Free End is Generated from a Single Copy During Development" Cell V. 31, pp. 177-182.

Klemenz, Roman and E. Peter Geiduschek (1980) "The 5' Terminus of the Precursor Ribosomal RNA of Saccharomyces cerevisiae" Nuc. Acids Res. V. 8, pp. 2679-2689.

Klenow, Hans, Kay Overgaard-Hansen and Shamkant A. Patkar (1971) "Proteolytic Cleavage of Native DNA Polymerase into Two Different Catalytic Fragments" Eur. J. Biochem. V. 22, pp. 371-381.

Klobutcher, Lawrence A., Marshal T. Swanton, Pierluigi Donini and David M. Prescott (1981) "All Gene-Sized DNA Molecules in Four Species of Hypotrichs Have the Same Terminal Sequence and an Unusual 3' Terminus" Proc. Nat. Acad. Sci. V. 78, pp. 3015-3019.

Kruger, Kelly, Paula J. Grabowski, Arthur J. Zaug, Julie Sands, Daniel E. Gottschling and Thomas R. Cech (1982) "Self-Splicing RNA: Autoexcision and Autocyclization of the Ribosomal RNA Intervening Sequence of Tetrahymena" Cell V. 31, pp. 147-157.

Kukita, Toshio, Yoshiyuki Sakaki, Hisayuki Nomiyama, Takeshi Otsuka, Satoru Kuhara and Yasuyuki Takagi (1981) "Structure Around the 3' Terminus of the 26 S Ribosomal RNA Gene of Physarum polycephalum" Gene V. 16, pp. 309-315.

Kuo, M. T., J. L. Mandel and P. Chambon (1979) "DNA Methylation: Correlation with DNAse I Sensitivity of Chicken Ovalbumin and Conalbumin Chromatin" Nuc. Acids Res. V. 7, pp. 2105-2114.

Lacy, Elizabeth and Richard Axel (1975) "Analysis of DNA of Isolated Chromatin Subunits" Proc. Nat. Acad. Sci. V. 72, pp. 3978-3982.

Larsen, Alf and Harold Weintraub (1982) "An Altered DNA Conformation Detected by S1 Nuclease Occurs at Specific Regions in Active Chick Globin Chromatin" Cell V. 29, pp. 609-629.

Leon, Wilson, David L. Fouts and Jerry Manning (1978) "Sequence Arrangement of the 16 S and 26 S rRNA Genes in the Pathogenic Haemoflagellate Leishmania donovani" Nuc. Acids Res. V. 5, pp. 491-504.

LeSturgeon, Wallace M. and Harold P. Rusch (1973) "Localization of Nucleolar and Chromatin Residual Acidic Protein Changes During Differentiation in Physarum polycephalum" Arch. Biochem. Biophys. V. 155, pp. 144-158.

Lewin, Benjamin (1977) Gene Expression 3 John Wiley & Sons, New York.

Lewin, Benjamin (1980) "Gene Expression 2" 2nd ed., John Wiley & Sons, New York.

Lima De Faria, A., M. Birnstiel and Halina Jaworska (1969) "Amplification of Ribosomal Cistrons in the Heterochromatin of Acheta" Genetics V. 61, supp. 1 part 2, pp. 145-159.

Long, Eric O. and Igor B. Dawid (1979) "Expression of Ribosomal DNA Insertions in Drosophila melanogaster" Cell V. 18, pp. 1185-1196.

Long, Eric O. and Igor B. Dawid (1980a) "Repeated Genes in Eukaryotes" in Ann. Rev. Biochem. V. 49, pp. 727-764.

Long, Eric O. and Igor B. Dawid (1980b) "Repeated Genes in Eukaryotes" in Cell Biology: A Comprehensive Treatise (ed. Lester Goldstein and David M. Prescott) V. 3, pp. 727-764. Academic Press, New York.

Long, Eric O., Martha L. Rebbert and Igor B. Dawid (1981) "Nucleotide Sequence of the Initiation Site for Ribosomal RNA Transcription in Drosophila melanogaster: Comparison of Genes With and Without Insertions" Proc. Nat. Acad. Sci. V. 78, pp. 1513-1517.

MacGregor, Herbert C. (1972) "The Nucleolus and Its Genes in Amphibian Oogenesis" Biol. Reviews V. 47, pp. 177-210.

McDonald, Michael W., Martha N. Simon and F. William Studier (1977) "Analysis of Restriction Fragments of T7 DNA and Determination of Molecular Weights by Electrophoresis in Neutral and Alkaline Gels" J. Mol. Biol. V. 110, pp. 119-146.

Mandel, M. and A. Higa (1970) "Calcium Dependant Bacteriophage DNA Infection" J. Mol. Biol. V. 53, pp. 159-162.

Maniatis, T., E. F. Fritsch and J. Sambrook (1982) "Molecular Cloning: A Laboratory Manual" Cold Spring Harbor Laboratories, New York.

Maniatis, Tom, Ross C. Hardison, Elizabeth Lacy, Joyce Lauer, Catherine O'Connell, Diana Quon, Gek Kee Sim and Argiris Efstratiadis (1978) "The Isolation of Structural Genes from Libraries of Eucaryotic DNA" Cell V. 15, pp. 687-701.

Manley, James L., Andrew Fire, Amparo Cano, Phillip A. Sharp and Malcolm L. Gefter (1980) "DNA-Dependant Transcription of Adenovirus Genes in a Soluble Whole-Cell Extract" Proc. Nat. Acad. Sci. V. 77, pp. 3855-3859.

Martin, Diane J. and Pierre Chambon (1981) "The SV40 Early Region TATA Box is Required for Accurate in Vitro Initiation of Transcription" Nature V. 290, pp. 310-315.

Marzluff, William F. Jr. and Ru Chih C. Huang (1975) "Chromatin Directed Transcription of 5 S and tRNA Genes" Proc. Nat. Acad. Sci. V. 72, pp. 1082-1086.

Matthews, Harry R., Edward M. Johnson, Wendy M. Steer, E. Morton Bradbury and Vincent G. Allfrey (1978) "The Use of Netropsin with CsCl Gradients for the Analysis of DNA and Its Application to Restriction Nuclease Fragments of Ribosomal DNA from Physarum polycephalum" Eur. J. Biochem. V. 82, pp. 569-576.

Mazzara, Gail P., Guy Plunkett III and William H. McClain (1980) "Maturation Events Leading to Transfer RNA and Ribosomal RNA" in Cell Biology: A Comprehensive Treatise (ed. Lester Goldstein and David M. Prescott) V. 3, pp. 439-545. Academic Press, New York.

Mazzara, Gail P. and William H. McClain (1980) "tRNA Synthesis" in Transfer RNA: Biological Aspects (ed. Dieter Soll, John. N. Abelson and Paul R. Schimmel), Cold Spring Harbor Monograph Series 9B, pp. 3-28, Cold Spring Harbor Laboratories, New York.

Melera, P. W. and H. P. Rusch (1973) "A Characterization of Ribonucleic Acid in the Myxomycete Physarum polycephalum" Exptl. Cell Res. V. 82, pp. 197-209.

Melera, Peter W. (1980) "Transcription in the Myxomycete Physarum polycephalum" in Growth and Differentiation in Physarum polycephalum (ed. William F. Dove and Harold P. Rusch) Princeton Univ. Press, Princeton, N. J. pp. 64-97.

Messing, Joachim (1980) "A Manual for the Use of M13mp2 and Derivatives" personal communication.

Messing, Joachim, Roberto Crea and Peter H. Seeburg (1981) "A System for Shotgun DNA Sequencing" Physarum polycephalum" Nuc. Acids Res. V. 8, pp. 2647-2664.

Miesfeld, Roger and Norman Arnheim (1982) "Identification of the In Vivo and In Vitro Origin of Transcription in Human rDNA" Nuc. Acids Res. V. 10, pp. 3933-3949.

Miller, D. M., P. Turner, A. W. Nienhaus, D. E. Axelrod and T. V. Gopalakrishnan (1978) "Active Conformation of the Globin Genes in Uninduced and Induced Mouse Erythroleukemia Cells" Cell V. 14, pp. 511-521.

Miller, Kathryn G. and Barbara Sollner-Webb (1981) "Transcription of Mouse rRNA Genes by RNA Polymerase I: In Vitro and In Vivo Initiation and Processing Sites" Cell V. 27, pp. 165-174.

Miller, Oscar L. Jr. and Barbara R. Beatty (1969) "Extrachromosomal Nucleolar Genes in Amphibian Oocytes" Genetics V. 61 supp. 1 part 2, pp. 133-143.

Mohberg, Joyce and H. P. Rusch (1971) "Isolation and DNA Content of Nuclei of Physarum polycephalum" Exptl. Cell Res. V. 66, pp. 305-316.

Molgaard, Harald V., Harry R. Matthews and E. Morton Bradbury (1976) "Organisation of Genes for Ribosomal RNA in Physarum polycephalum" Eur. J. Biochem. V. 68, pp. 541-549.

Moss, Tom (1982) "Transcription of Cloned Xenopus laevis Ribosomal DNA Microinjected into Xenopus Oocytes, and the Identification of an RNA Polymerase I Promoter" Cell V. 30, pp. 835-842.

Moss, Tom, Paul G. Bosely and Max L. Birnstiel (1980) "More Ribosomal Spacer Sequences from Xenopus laevis" Nuc. Acids Res. V. 8, pp. 467-485.

Newlon, Carol Shaw, Gail E. Sonenshein and Charles E. Holt (1973) "Time of Synthesis of Genes for Ribosomal Ribonucleic Acid in Physarum" Biochem. V. 12, pp. 2338-2345.

Niles, Edward G., Kathryn Cunningham and Rakesh Jain (1981a) "Structure of the Tetrahymena pyriformis rRNA Gene" J. Biol. Chem. V. 256, pp. 12857-12860.

Niles, Edward G. and Rakesh Jain (1981) "Physical Map of the Ribosomal Ribonucleic Acid Gene from Tetrahymena pyriformis" Biochem. V. 20, pp. 193-197.

Niles, Edward G., Joyce Sutiphong and Shabid Haque (1981b) "Structure of the Tetrahymena pyriformis rRNA Gene" J. Biol. Chem. V. 256, pp. 12849-12856.

Nomiyama, Hisayuki, Satoru Kuhara, Toshio Kukita, Takeshi Otsuko and Yoshiyuki Sakaki (1981) "Nucleotide Sequence of the Ribosomal RNA Gene of Physarum polycephalum: Intron 2 and Its Flanking Regions of the 26 S rRNA Gene" Nuc. Acids Res. V. 9, pp. 5507-5520.

Nomiyama, Hisayuki, Yoshiyuki Sakaki and Yasuyuki Takagi (1982) "Nucleotide Sequence of a Ribosomal RNA Gene Intron from Slime Mold Physarum polycephalum" Proc. Nat. Acad. Sci. V. 78, pp. 1376-1380.

Otsuka, Takeshi, Hisayuki Nomiyama, Yoshiyuki Sakaki and Yasuyuki Takagi (1982) "Nucleotide Sequence of Physarum polycephalum 5.8 S rRNA Gene and its Flanking Regions" Nuc. Acids Res. V. 10, pp. 2379-2385.

Pan, Wei-Chun and Elizabeth H. Blackburn (1981) "Single Extrachromosomal Ribosomal RNA Genes are Synthesized During Amplification of the rDNA in Tetrahymena" Cell V. 23, pp. 459-466.

Pauli, Urs H., Thomas Seebeck and Richard Braun (1982) "Sequence-Specific Cleavage of Chromatin by Staphyococcal Nuclease can Generate an Atypical Nucleosome Pattern" Nuc. Acids Res. V. 10, pp. 4121-4133.

Pech, Michael, Rolf E. Streeck and Hans G. Zachau (1979) "Patchwork Structure of a Bovine Satellite DNA" Cell V. 18, pp. 883-893.

Pellegrini, Maria, Jerry Manning and Norman Davidson (1977) "Sequence Arrangement of the rDNA of Drosophila melanogaster" Cell V. 10, pp. 213-224.

Pfeiffer, Wolfgang and Hans G. Zachau (1980) "Accessibility of Expressed and Non-Expressed Genes to a Restriction Nuclease" Nuc. Acids Res. V. 8, pp. 4621-4638.

Philippsen, Peter, Marjorie Thomas, Richard A. Kramer and Ronald W. Davis (1978) "Unique Arrangement of Coding Sequences for 5 S, 5.8 S, 18 S and 26 S Ribosomal RNA in Saccharomyces cerevisiae as Determined by R-Loop and Hybridization Analysis" J. Mol. Biol. V. 123, pp. 387-404.

Pierron, Gerard, Helmut W. Sauer, Barbara Toublan and Raymond Jalouzot (1982) "Physical Relationship Between Replicons and Transcription Units in Physarum polycephalum" Eur. J. Cell Biol. V. 29, pp. 104-113.

Poncz, M., D. Solowiejczyk, M. Ballantine, E. Schwartz and S. Surrey (1982) "'Nonrandom' DNA Sequence Analysis in Bacteriophage M13 by the Dideoxy Chain-Termination Method" Proc. Nat. Acad. Sci. V. 79, pp. 4298-4302.

Prior, Christopher P. (1982) "Fluorescence Studies on Chromatin Subunits During Replication and Transcription" thesis submitted to Columbia University.

Prior, Christopher P., Charles R. Cantor, Edward M. Johnson and Vincent G. Allfrey (1980) "Incorporation of Exogenous Pyrene-Labeled Histone into Physarum Chromatin: A System for Studying Changes in Nucleosomes Assembled in Vivo" Cell V. 20, pp. 597-608.

Prior, Christopher P., Charles R. Cantor, Edward M. Johnson and Vincent G. Allfrey (1983) "Molecular Structure of the Lexosome, A Subunit of Transcriptionally Active Chromatin on Ribosomal Genes of Physarum polycephalum" in preparation.

Rae, Peter M. M. and Robert E. Steele (1979) "Absence of Cytosine Methylation at C-C-G-G and G-C-G-C Sites in the rDNA Coding Regions and Intervening Sequences of Drosophila and the rDNA of Other Higher Insects" Nuc. Acids Res. V. 6, pp. 2987-2995.

Rekosh, D. M. K., W. C. Russell, A. J. D. Bellett and A. J. Robinson (1977) "Identification of a Protein Linked to the Ends of Adenovirus DNA" Cell V. 11, pp. 283-295.

Ritossa, F. (1972) "Procedure for Magnification of Lethal Deletions of Genes for Ribosomal RNA" Nature New Biology V. 240, pp. 109-111.

Ritossa, F. (1973) "Crossing Over Between X and Y Chromosomes During Ribosomal DNA Magnification in Drosophila melanogaster" Proc. Nat. Acad. Sci. V. 70, pp. 1950-1954.

Robinson, A. J. and A. J. D. Bellett (1974) "A Circular DNA-Protein Complex from Adenoviruses and Its Possible Role in DNA Replication" Cold Spring Harbor Symp. Quant. Biol. V. 39, pp. 523-531.

Rothblum, Lawrence, Ramachandra Reddy and Brandt Cassidy (1982) "Transcription Initiation Site of Rat Ribosomal RNA" Nuc. Acids Res. V. 10, pp. 7345-7362.

Rusch, Harold (1969) "Some Biochemical Events in the Growth Cycles of Physarum polycephalum" Fed. Proc. V. 28, pp. 1761-1770.

Ryser, U., S. Fakan and R. Braun (1973) "Localization of Ribosomal RNA Genes by High Resolution Autoradiography" Exptl. Cell Res. V. 78, pp. 89-97.

Saiga, Hidetoshi, Kiyohisa Mizumoto, Takashi Matsui and Toru Higashinakagawa (1982) "Determination of the Transcription Initiation Site of Tetrahymena pyriformis rDNA Using In Vitro Capping of 35 S Pre-rRNA" Nuc. Acids Res. V. 10, pp. 4223-4236.

Sakonju, Shigeru, Daniel F. Bogenhagen and Donald D. Brown (1980) "Control Region in the Center of the 5 S RNA Gene Directs Specific Initiation of Transcription: I. The 5' Border" Cell V. 19, pp. 13-25.

Sanger, F., S. Nicklen and A. R. Coulson (1977) "DNA Sequenceing with Chain Terminating Inhibitors" Proc. Nat. Acad. Sci. V. 74, pp. 5463-5467.

Sarma, M. H., E. R. Feman and C. Baglioni (1976) "RNA Synthesis in Isolated HeLa Cell Nuclei" Biochim. Biophys. Acta V. 418, pp. 29-38.

Sauer, H. W. (1978) "Regulation of Gene Expression in the Cell Cycle of Physarum polycephalum" in Cell Cycle Regulation (ed. J. R. Jeter, I. L. Cameron, G. M. Padilla, and A. M. Zimmerman) pp. 149-166, Cell Biology Series, Academic Press, New York.

Sawada, Fumio, Yasuhiko Miyauchi, Hiroshi Tanaka and Shinji Matsumoto (1982) "Preparation of Metaphase Chromatin of Physarum polycephalum Without the Loss of Repressed RNA Synthesis" Biochem. Biophys. Res. Comm. V. 104, pp. 657-663.

Scheer, Ulrich, Hanswalter Zentgraf and Helmut W. Sauer (1981) "Different Chromatin Structures in Physarum polycephalum" Chromosoma V. 84, pp. 279-290.

Scherrer, Gunther E. F., Malcolm D. Walkinshaw and Struther Arnott (1978) "A Computer-Aided Oligonucleotide Analysis Provides a Model Sequence for RNA Polymerase Recognition in E. coli" Nuc. Acids Res. V. 5, pp. 3759-3773.

Seebeck, Thomas, Jurg Stalder and Richard Braun (1979) "Isolation of a Minichromosome Containing the Ribosomal Genes from Physarum polycephalum" Biochem. V. 18, pp. 484-490.

Sharp, Philip A., Ulf Pettersson and Joe Sambrook (1974) "Viral DNA in Transformed Cells" J. Mol. Biol. V. 86, pp. 709-726.

Sollner-Webb, Barbara and Ronald H. Roeder (1979) "The Nucleotide Sequence of the Initiation and Termination Sites for Ribosomal RNA Transcription in X. laevis" Cell V. 18, pp. 485-499.

Southern, E. M. (1975) "Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis" J. Mol. Biol. V. 98, pp. 503-517.

Staden, R. (1980) "A New Computer Method for the Storage and Manipulation of DNA Gel Reading Data" Nuc. Acids Res. V. 8, pp. 3673-3694.

Stalder, Jurg and Richard Braun (1978) "Chromatin Structure of Physarum polycephalum Plasmodia and Amoebae" FEBS Lett. V. 90, pp. 223-227.

Stalder, Jurg, Thomas Seebeck and Richard Braun (1978) "Degradation of the Ribosomal Genes by DNAse I in Physarum polycephalum" Eur. J. Biochem. V. 90, pp. 391-395.

Stalder, Jurg, Thomas Seebeck and Richard Braun (1979) "Accessibility of the Ribosomal Genes to Micrococcal Nuclease in Physarum polycephalum" Biochim. Biophys. Acta V. 561, pp. 452-463.

Steer, Wendy M., Harald V. Molgaard, E. Morton Bradbury and Harry R. Matthews (1978) "Ribosomal Genes in Physarum polycephalum: Transcribed and Non-Transcribed Sequences Have Similar Base Compositions" Eur. J. Biochem. V. 88, pp. 599-605.

Subramanian, K. N., J. Pan, S. Zaiw and S. M. Weissman (1974) "The Mapping and Ordering of Fragments of SV40 DNA Produced by Restriction Endonucleases" Anal. Biochem. V. 1, pp. 727-752.

Sun, Irene Yi-Chi, Edward M. Johnson and Vincent G. Allfrey (1979) "Initiation of Transcription of Ribosomal Deoxynucleic Acid Sequences in Isolated Nuclei of Physarum polycephalum: Studies Using Nucleoside 5'-[γ-S]Triphosphates and Labeled Precursors: Biochem. V. 18, pp. 4572-4580.

Szostak, Jack W. and Elizabeth H. Blackburn (1982) "Cloning Yeast Telomeres on Linear Plasmid Vectors" Cell V. 29, pp. 245-255.

Temin, Howard M. (1981) "Structure, Variation and Synthesis of Retrovirus Long Terminal Repeat" Cell V. 27, pp. 1-3.

Temin, Howard M. (1982) "Function of the Retrovirus Long Terminal Repeat" Cell V. 28, pp. 3-5.

Thomas, Marjorie, Raymond L. White and Ronald W. Davis (1976) "Hybridization of RNA to Double-Stranded DNA: Formation of R-loops" Proc. Nat. Acad. Sci. V. 73, pp. 2294-2298.

Thuring, A. W. J., J. P. M. Sanders and P. Borst (1975) "A Freeze-Squeeze Method for Recovering Long DNA from Gels" Anal. Biochem. V. 66, pp. 213-220.

Tinoco, Ignacio, Jr., Olke C. Uhlenbeck and Mark Levine (1971) "Estimation of Secondary Structure in Ribonucleic Acids" Nature V. 230, pp. 362-367.

Tinoco, Ignacio, Jr., Philip N. Borer, Barbara Dengler, Mark Levine, Olke C. Uhlenbeck, Donald M. Crothers and Jay Gralla (1973) "Improved Estimation of Secondary Structure in Ribonucleic Acids" Nature New Biology V. 246, pp. 40-41.

Triezenberg, Steven J., Caroline Rushford, Ronald P. Hart, Kathleen L. Berkner and William R. Folk (1982) "Structure of the Syrian Hamster Riboosomal DNA Repeat and Identification of Homologous and Nonhomologous Regions Shared by Human and Hamster DNAs" J. Biol. Chem. V.

257, pp. 7826-7833.

Truett, Martha A. and Joseph G. Gall (1977) "The Replication of rDNA in the Macronucleus of Tetrahymena" Chromosoma V. 64, pp. 295-304.

Tsuda, Masaki and Yoshiaki Suzuki (1981) "Faithful Transcription Initiation of Fibroin Gene in a Homologous Cell-Free System Reveals an Enhancing Effect of 5' Flanking Sequences Far Upstream" Cell V. 27, pp. 175-182.

Udvarty, Andor and Klaus H. Seifart (1976) "Transcription of Specific Genes in Isolated Nuclei from HeLa Cells in Vitro" Eur. J. Biochem. V. 62, pp. 353-363.

Urano, Yoshio, Ryo Kominami, Yukio Mishima and Masami Muramatsu (1980) "The Nucleotide Sequence of the Putative Transcription Initiation Site of a Cloned Ribosomal RNA Gene of the Mouse" Nuc. Acids Res. V. 8, pp. 6043-6058.

Valenzuela, Pablo, Graeme I. Bell, Alejandro Venegas, Elaine T. Sewell, Frank R. Masiarz, Louis J. DeGennaro, Fanyela Weinberg and William J. Rutter (1977) "Riboosomal RNA Genes of Saccharomyces cerevisiae" J. Biol. Chem. V. 252, pp. 8126-8135.

Varmus, Harold and Ronald Swanstrom (1982) "Replication of Retroviruses" in Mol. Biol. of Tumor Viruses 2nd ed. RNA Tumor Viruses, pp. 369-512, (ed. Robin Weiss, Natalie Teich, Harold Varmus and John Coffin) Cold Spring Harbor Laboratories, New York.

Vidali, Giorgio, Lidia C. Boffa, E. Morton Bradbury and Vincent G. Allfrey (1978) "Butyrate Suppression of Histone Deacetylation Leads to Accumulation of Multi-acetylated Forms of Histones H3 and H4 and Increased DNAse I Sensitivity of the Associated DNA Sequences" Proc. Natl. Acad. Sci. V. 75, pp. 2239-2243.

Vogt, Volker M. (1973) "Purification and Further Properties of Single-Strand-Specific Nuclease from Aspergillus oryzae" Eur. J. Biochem. V. 33, pp. 192-200.

Vogt, Volker M. and Richard Braun (1976a) "Repeated Structure of Chromatin in Metaphase Nuclei of Physarum" FEBS Lett. V. 64, pp. 190-192.

Vogt, Volker M. and Richard Braun (1976b) "Structure of Ribosomal DNA in Physarum polycephalum" J. Mol. Biol. V. 106, pp. 567-587.

Vogt, Volker M. and Richard Braun (1977) "The Replication of Ribosomal DNA in Physarum polycephalum" Eur. J. Biochem. V. 80, pp. 557-566.

Walker, B. W., M. E. Christensen, A. L. Beyer and W. M. LeSturgeon (1980) "The Nuclear Proteins of Physarum polycephalum: A Comparative View" in Growth and Differentiation in Physarum polycephalum (ed. William F. Dove and Harold P. Rusch) Princeton Univ. Press, Princeton, N. J. pp. 98-128.

Wasylyk, B., P. Oudet and P. Chambon (1979) "Preferential in Vitro Assembly of Nucleosome Cores on Some A-T Rich Regions of SV40 DNA" Nuc. Acids Res. V. 7, pp. 705-713.

Weil, P. Anthony, Donald S. Luse, Jacqueline Segall and Robert G. Roeder (1979a) "Selective and Accurate Initiation of Transcription at the Ad2 Major Late Promoter in a Soluble System Dependant of Purified RNA Polymerase II and DNA" Cell V. 18, pp. 469-484.

Weil, P. Anthony, Jacqueline Segall, Barry Harris, Sun-Yu Ng and Robert G. Roeder (1979b) "Faithful Transcription of Eukaryotic Genes by RNA Polymerase III in Systems Reconstituted with Purified DNA Templates" J. Biol. Chem. V. 254, pp. 6163-6173.

Weintraub, Harold and Mark Groudine (1976) "Chromosomal Subunits in Active Genes Have an Altered Conformation" Science V. 193, pp. 848-856.

Weisbrod, Stuart and Harold Weintraub (1979) "Isolation of a Subclass of Nuclear Proteins Responsible for Conferring a DNAse-I Sensitive Structure on Globin Chromatin" Proc. Natl. Acad. Sci. V. 76, pp. 630-634.

Wellauer, Peter K. and Igor B. Dawid (1977) "The Structural Organization of Ribosomal DNA in Drosophila melanogaster" Cell V. 10, pp. 193-212.

Wellauer, Peter K., Igor B. Dawid and Kenneth D. Tartof (1978) "X and Y Chromosomal Ribosomal DNA of Drosophila: Comparison of Spacers and Insertions" Cell V. 14, pp. 269-278.

White, Raymond L. and David S. Hogness (1977) "R Loop Mapping of the 18 S and 28 S Sequences in the Long and Short Repeating Units of Drosophila melanogaster rDNA" Cell V. 10, pp. 177-192.

Wille, John J. and W. L. Steffens (1979) "Cycle Specific Association of Nascent Chromatin with Nuclear Envelope Components in Physarum polycephalum" Nuc. Acids Res. V. 6, pp. 3323-3339.

Wimmer, Eckard (1982) "Genome-Linked Proteins of Viruses" Cell V. 28, pp. 199-201.

Wu, Carl and Walter Gilbert (1981) "Tissue-Specific Exposure of Chromatin Structure at the 5' Terminus of the Rat Preproinsulin Gene" Proc. Natl. Acad. Sci. V. 78, pp. 1577-1580.

Yao, Meng-Chao (1981) "Ribosomal RNA Amplification in Tetrahymena May Be Associated with Chromosome Breakage and DNA Elimination" Cell V. 24, pp. 765-774.

Yao, Meng-Chao, Elizabeth Blackburn and Joseph Gall (1981) "Tandemly Repeated C-C-C-C-A-A Hexanucleotide of Tetrahymena rDNA is Present Elsewhere in the Genome and May Be Related to the Alteration of the Somatic Genome" J. Cell Biol. V. 90, pp. 515-520.

Yao, Meng-Chao, Alan R. Kimmel and Martin A. Gorovsky (1974) "A Small Number of Cistrons for Ribosomal RNA in the Germinal Nucleus of a Eukaryote, Tetrahymena pyriformis" Proc. Nat. Acad. Sci. V. 71, pp. 3082-3086.

Yao, Meng-Chao and Joseph G. Gall (1977) "A Single, Integrated Gene for Ribosomal RNA in a Eucaryote, Tetrahymena pyriformis" Cell, V. 12, pp. 121-132.

Young, Richard A. and Joan A. Steitz (1979) "Tandem Promoters Direct E. coli Ribosomal RNA Synthesis" Cell V. 17, pp. 225-234.

Zachau, Hans G. and Tibor Igo-Kemenes (1981) "Face to Phase with Nucleosomes" Cell V. 24, pp. 597-598.

Zaug, Arthur J. and Thomas R. Cech (1982) "The Intervening Sequence Excised from the Ribosomal RNA Precursor of Tetrahymena Contains a 5'-Terminal Guanosine Resides Not Encoded by the DNA" Nuc. Acids. Res. V. 10, pp. 2823-2835.

Zellweiger, Ariene, Ueli Ryser and Richard Braun (1972) "Ribosomal Genes of Physarum: Their Isolation and Replication in the Mitotic Cycle" J. Mol. Biol. V. 64, pp. 681-691.

Ziff, Edward M. and Ronald M. Evans (1978) "Coincidence of the Promoter and Capped 5' Terminus of RNA from the Adenovirus 2 Major Late Transcription Unit" Cell V. 15, pp. 1463-1475.

End