MOLECULAR AND CELLULAR BIOLOGY, Feb. 1985, p. 273–280
0270-7306/85/020273-08$02.00/0

Vol. 5, No. 2

# Inheritance of Extrachromosomal Ribosomal DNA During the Asexual Life Cycle of *Dictyostelium discoideum*: Examination by Use of DNA Polymorphisms

DENNIS L. WELKER,† K. PETER HIRTH, AND KEITH L. WILLIAMS‡*

*Max-Planck-Institut für Biochemie, D-8033 Martinsried bei München, Federal Republic of Germany*

Received 12 July 1984/Accepted 6 November 1984

**Wild-type isolates of *Dictyostelium discoideum* exhibited differences in the size of restriction fragments of the extrachromosomal 88-kilobase ribosomal DNA (rDNA) palindrome. Polymorphisms in rDNA also were found among strains derived solely from the NC4 wild-type isolate. These variations involved *Eco*RI fragments II, III, and V; they included loss of the *Eco*RI site separating fragments II and V and deletion and insertion of DNA. More than one rDNA form can coexist in the same diploid or haploid cell. However, one or another parental rDNA tended to predominate in diploids constructed, using the parasexual cycle, between haploid NC4-derived strains and haploid wild-type isolates. In some cases, most if not all of the rDNA of such diploids were of one form after ca. 50 generations of growth. Segregant haploids, derived from diploids that possessed predominantly a single rDNA allele, possessed the same allele as the diploid and did not recover the other form. This evidence implies that replication does not proceed from a single chromosomal or extrachromosomal copy of the rDNA during the asexual life cycle of *D. discoideum*.**

The genes coding for rRNA in eucaryotes are present in multiple copies in each cell, and they are organized in various ways. In general, fungi and metazoans have tandem repeats of chromosomally integrated rDNA (19, 26). In several protozoans, the rDNA genes are predominantly extrachromosomal, existing as linear palindromic dimers with up to several hundred copies per cell. The linear palindromic dimers of rDNA from three such simple eucaryotes are particularly well characterized: *Tetrahymena thermophila*, 22 kilobases (kb) (10); *Physarum polycephalum*, 60 kb (32); and *Dictyostelium discoideum*, 88 kb (2). There is a single chromosomal copy of rDNA in the micronucleus and only extrachromosomal copies in the macronucleus of *T. thermophila* (39). In *Physarum polycephalum* the situation is not clear. If there is a chromosomal copy, assumptions need to be made to explain the mode of inheritance of ribosomal DNA (rDNA) (6). In *D. discoideum* there is no firm evidence supporting the existence of a chromosomal copy of rDNA (13). However, previous work does not exclude there being a chromosomal copy (3). Here we show that the rDNAs of different isolates of *D. discoideum* show considerable restriction fragment length polymorphism. These polymorphisms, plus those found in laboratory strains, are used to examine the inheritance of rDNA in parasexual genetic crosses. These studies imply that replication of the rDNA palindromes does not proceed from a single chromosomal or extrachromosomal copy of rDNA during the asexual life cycle of *D. discoideum*.

## MATERIALS AND METHODS

**Strains and growth of amoebae.** Haploid strains of *D. discoideum* used included 14 wild-type isolates, genetically marked strains derived solely from the NC4 or V12 wild-type isolates, and strains derived by crosses between wild-type isolates. The wild-type isolates were obtained from D. Waddell, Gesamthochschule Wuppertal, Wuppertal, Federal Republic of Germany (strains DD44 and DD61); R. A. Firtel, University of California at San Diego, La Jolla, Calif. (strain OHIO); and K. B. Raper, University of Wisconsin, Madison (strains NC4, V12, WS51, WS380B, WS472, WS576, WS583, WS1956, and WS2054; also strains HU182 and HU188 which are haploids derived from wild-type isolate diploids WS10 and WS585, respectively; for more details see references 5, 18, 28, and 29). Haploid strains, derived solely from the NC4 isolate (25), used in the study of rDNA polymorphisms that arose in the laboratory are M28, TS12 (11), AX2 (34), and AX3 (16), all of which were derived from Sussman's strain DDB which in turn was derived from NC4. Strain HR7 was derived from strain AX3 (30); strain HU878 was derived from strains NC4, M28, TS12, AX3, and HR7 in a complex series of parasexual crosses (35); strain HU1310 is a derivative of strain HU1257 that has lost the D350(III,III) duplication on linkage group III; strain HU1257 can be traced to strains NC4, M28, TS12, AX3, and HR7 via a complex series of parasexual crosses (35); strain HU1628 was derived from strains NC4, M28, TS12, and AX3 in a complex series of parasexual crosses (for genotype, see Table 4); and strain X36 was derived from strains AX3, M28, and TS12 via parasexual crosses (38). Haploid strain HU1231 is a mutant of strain V12 (9). Stock cultures were grown at 21 ± 1°C in association with *Klebsiella aerogenes* on SM agar (37).

**Nomenclature.** To simplify discussion of the different forms of rDNA, allele numbers were allocated according to standard *D. discoideum* genetics nomenclature (21, 37) (Table 1).

**Parasexual genetic techniques.** Diploids were constructed between NC4-derived tester strain HU1628 and haploid wild-type isolates of mating type *matA* or asexual wild-type isolates on the basis of complementation of the recessive *bsgA5 Bacillus subtilis* sensitivity mutation and of dominance of the *cob-354* cobalt resistance mutation which may be plasmid associated (18). Both selector mutations were carried by strain HU1628 which bore easily scorable mark-

* Corresponding author.
† Present address: Department of Biology, Utah State University, Logan, UT 84322.
‡ Present address: School of Biological Sciences, Macquarie University, North Ryde, N.S.W. 2113, Australia.

TABLE 1. Nomenclature of rDNA alleles in haploid *D. discoideum* strains

| Strains | rDNA alleles |
|---|---|
| NC4 and NC4 derivatives: | |
| AX3, AX2 | rDNA350 |
| HR7, HU878, HU1257, HU1310 | rDNA350, rDNA351 |
| NC4, HU1628, M28, TS12, X36 | rDNA350, rDNA352 |
| Other wild-type isolates and their derivatives: | |
| DD44 | rDNA353 |
| WS51 | rDNA354 |
| WS576 | rDNA355 |
| HU188 | rDNA356 |
| WS1956 | rDNA357 |
| WS2054 | rDNA358 |
| WS472 | rDNA359 |
| DD61 | rDNA360 |
| HU182 | rDNA361 |
| OHIO | rDNA362 |
| WS380B | rDNA363 |
| WS583 | rDNA364 |
| V12, HU1231 | rDNA365 |

ers on each of the six established *D. discoideum* linkage groups (D. L. Welker and K. L. Williams, Genetics, in press). This allowed parasexual crosses without the need for inserting a selective marker into the wild-type isolates. In these crosses, diploids were selected at 21 ± 1°C on SM agar plates containing 250 μg of cobaltous chloride per ml which were preinoculated with cobalt-resistant *B. subtilis* BSU2 (Welker and Williams, in press). Haploids were selected from diploids with the antimicrotubule agent thiabendazole (2 μg/ml in SM agar; 36). Scoring of genetic markers was by standard methods (21, 37).

**DNA preparation.** Amoebae for DNA isolation were harvested from 10 to 20 9-cm SM agar plates while in vegetative-growth or early aggregation phase and then incubated overnight at 21 ± 1°C in 300 ml of phosphate-buffered saline (pH 6.8; 36) to allow the amoebae to consume residual bacteria. Amoebae (~$10^7$/ml) were then washed at 4°C by three centrifugations (250 × *g* for 3 min) with resuspension in distilled water at 4°C. The final cell pellet (usually ~5 ml of packed cells) was lysed for the preparation of nuclei with ~100 ml of Nonidet P-40 lysis buffer [10 mM $Mg(CH_3COO)_2$, 10 mM NaCl, 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 10% sucrose, 2% Nonidet P-40 (pH 7.5)]. The nuclei were recovered by differential centrifugation (13,000 × *g* for 10 min), resuspended in 4 ml of Nonidet P-40 lysis buffer, and lysed by the addition of 20 ml of a 0.2 M EDTA–2% sarcosyl solution (pH 8.4) at 65°C. After cesium chloride gradient centrifugation (0.92 g/ml) with ethidium bromide (400 μg/ml), the nuclear DNA bands were isolated and diluted with 1 volume of a solution of 6 mM Tris-hydrochloride, 6 mM NaCl, and 0.2 mM EDTA (pH 7.2). After the addition of 4 volumes of ethanol as a layer over the gradient fraction, the DNA was spooled by gentle mixing of the two layers. The spooled nuclear DNA was removed with a fine glass rod, washed with ethanol, resuspended, and digested with restriction enzymes as described by the manufacturers (Bethesda Research Laboratories or Boehringer Mannheim Biochemicals). Phage λ *Hin*dIII DNA standards were from Bethesda Research Laboratories.

**Electrophoresis and hybridization.** DNA samples were electrophoresed through 0.8% (wt/vol) agarose gels (15) and stained with ethidium bromide. For Southern hybridization (31), DNA was transferred from gels to nitrocellulose filters (BA85; Schleicher & Schuell, Inc.) and hybridized with DNA probes labeled by nick translation with [$\alpha^{32}$P]dATP (27). Either cloned *D. discoideum* rDNA (*Eco*RI fragment V; 20) or *Eco*RI-digested DNA eluted from agarose gels (fragments II and III) was used as a probe. Except where noted, eluted probe DNA was obtained from strain AX2, which is a derivative of NC4, is closely-related to strain AX3, and bears the standard AX3 rDNA350 allele.

## RESULTS

***Eco*RI restriction patterns of rDNA from different isolates of *D. discoideum*.** Fragments of rDNA are readily visualized after restriction enzyme digestion of nuclear DNA, since there are ca. 90 rDNA dimers per haploid genome and the genomic DNA complexity is relatively low. The maps of the rDNA of strain AX3 (rDNA350), a derivative of the type strain NC4, are known for five restriction enzymes (*Hin*dIII, *Eco*RI, *Sal*I, *Pst*I, and *Bam*HI). In particular, *Eco*RI digestion gives seven bands with sizes of 15, 10, 7.2, 5.7, 5.1, 2.8, and 1.5 kb (fragments I, II, III, IV, V, VII, and IXa/IXb, respectively). These fragments have been ordered as shown in Fig. 1 (13).

Examination of nuclear DNA of 14 different wild-type isolates of *D. discoideum* after digestion with *Eco*RI showed that there is considerable variation in the pattern of restriction fragments in different isolates (Fig. 2A). Although fragments I, IV, VII, and IX appeared to be identical in all strains, there were changes observed in the remaining fragments (Table 2).

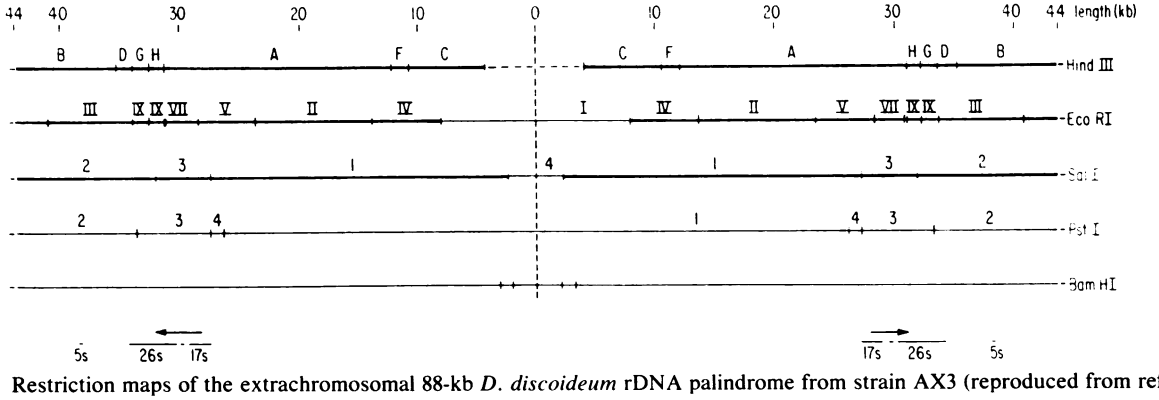


FIG. 1. Restriction maps of the extrachromosomal 88-kb *D. discoideum* rDNA palindrome from strain AX3 (reproduced from reference 13).

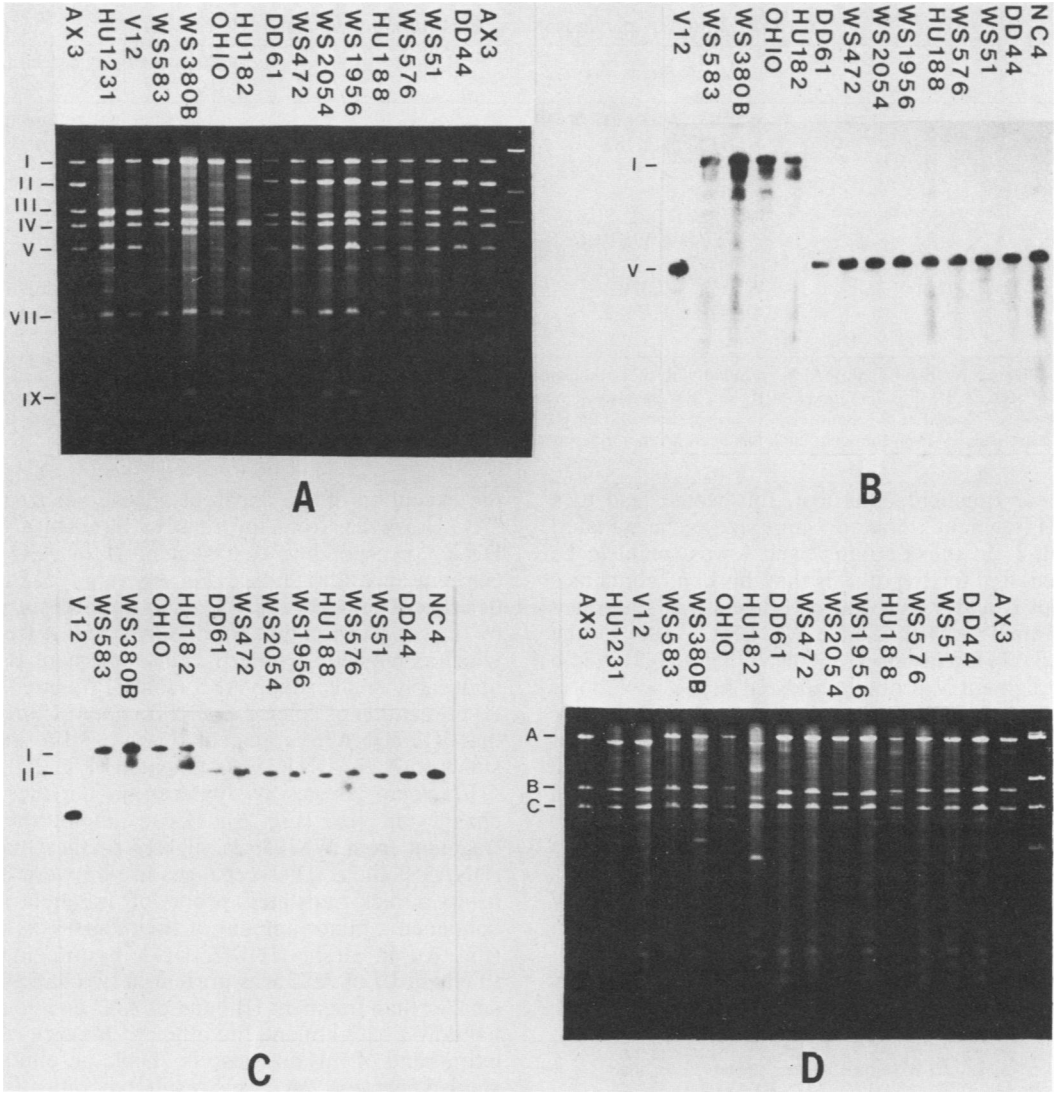


FIG. 2. Polymorphic restriction fragments of rDNA from 14 wild-type isolates digested with *Eco*RI or *Hin*dIII after: *Eco*RI digestion (A), *Eco*RI digestion and Southern hybridization with *Eco*RI fragment V probe (B), *Eco*RI digestion and Southern hybridization with *Eco*RI fragment II probe (C), and *Hin*dIII digestion (D). These rDNA alleles have been designated as follows: NC4 (and its derivatives such as AX3), rDNA350; DD44, rDNA353; WS51, rDNA354; WS576, rDNA355; HU188, rDNA356; WS1956, rDNA357; WS2054, rDNA358; WS472, rDNA359; DD61, rDNA360; HU182, rDNA361; OHIO, rDNA362; WS380B, rDNA363; WS583, rDNA364; V12 (and its derivatives such as HU1231), rDNA365. The bands corresponding to the *Eco*RI and *Hin*dIII fragments of AX3 are indicated on the left of panels A and D, respectively. *Hin*dIII-digested λ DNA standards (23.6, 9.6, 6.6, 4.3, 2.3, and 2.0 kb) are shown on the right in panels A and D.

Prominent bands at ~5.5 kb in *Eco*RI- (Fig. 2A) and at 4 kb in *Hin*dIII- (Fig. 2D) digested DNA of strain WS380B are due to the presence of a newly discovered high-copy-number plasmid in this wild-type isolate (A. Noegel et al., manuscript in preparation). Likewise, the presence of a plasmid in strain OHIO, whose restriction enzyme patterns have not been completely analyzed, explains the additional prominent bands in the *Eco*RI (at ~10, 7, 5.2, 2.6, and 1.9 kb; Fig. 2A) and *Hin*dIII (Fig. 2D) digests of nuclear DNA from this strain.

Fragment V exhibited radical changes; in four cases (strains HU182, OHIO, WS380B, and WS583) it was apparently absent. This was unexpected as fragment V contains part of the region from which the 17S rRNA is transcribed (Fig. 1); absence of fragment V therefore should be lethal. The problem concerning loss of fragment V in strains HU182, OHIO, WS380B, and WS583 was resolved by digestion with *Hin*dIII (Fig. 2D). In three of these strains (OHIO, WS380B, and WS583) *Hin*dIII fragment A which completely overlaps *Eco*RI fragment V (Fig. 1) was apparently unaltered, indicating that the DNA corresponding to *Eco*RI fragment V was still present. In HU182 the largest *Hin*dIII fragment was smaller than the normal fragment A, possibly reflecting a deletion or the presence of an additional *Hin*dIII site in strain HU182 rDNA (Fig. 2D). One explanation for the apparent loss of fragment V is that the *Eco*RI site separating fragments II and V is absent. In three of these strains (OHIO, WS380B, and WS583) rDNA bands similar in size to the AX3 rDNA350 fragment II were not present. In the fourth strain (HU182) a slightly larger band was present. The faintly staining band at ca. 10 kb in strain OHIO is due to an endogenous plasmid and is not homologous to the rDNA350 fragment II (Fig. 2C). The size of *Eco*RI fragment II is 10 kb and of fragment V is 5.1 kb. If the *Eco*RI site

TABLE 2. rDNA polymorphisms in *D. discoideum* isolates[a]

| *Eco*RI band affected | Strain | Nature of polymorphism assuming strain AX3 is wild type |
|---|---|---|
| II | WS583, WS380B, OHIO, HU182 | Increase in size due to loss of *Eco*RI restriction site between fragments II and V |
| II | V12 | Decrease in size |
| III | HU182, DD61, WS2054, WS1956, WS472 | Decrease in size |
| V | WS583, WS380B, OHIO, HU182 | Increase in size due to loss of *Eco*RI restriction site between fragments II and V |

[a] The polymorphisms have been confirmed by probing with nick-translated rDNA fragments (Fig. 2 and 3). Two strains, WS380B, and OHIO, displayed prominent bands in both *Eco*RI (Fig. 2A) and *Hin*dIII (Fig. 2D) digests which were not related to rDNA. Both strains carry high-copy-number plasmids which will be described elsewhere (Noegel et al., manuscript in preparation). Strain HU182 has a complex pattern which has not been fully analyzed. Because all strains given in this table are isolates from the wild, each has been given a different allele number as it is likely that they are all different (Table 1).

separating these fragments was lost, this would lead to a 15.1-kb *Eco*RI fragment, about the same size as the standard AX3 fragment I. In these strains band I was found to be heavily stained, and the hypothesis that this band contains a fused fragment II and V in the *Eco*RI digest was confirmed by Southern analysis with cloned fragment V (20) as the probe (Fig. 2B). With strains WS380B, WS583, OHIO, and HU182, the fragment V probe hybridized in the position of band I; with the other wild-type isolates, *Eco*RI fragment V appeared normal (Fig. 2B). Similarly, with a probe for fragment II (obtained from AX2, a strain closely related to strain AX3 and having the same rDNA restriction pattern [unpublished data]), it was shown that DNA homologous to this fragment also was shifted to the position of band I in these four strains (Fig. 2C). Strain HU182 also carries a second band (~12 kb) homologous to fragment II and V probes; this and other evidence presented below suggest that this strain may in fact carry two rDNA forms.

The *Eco*RI restriction pattern of strain V12 and its derivatives (e.g., HU1231) was similar to that of strain AX3, with the exception of the apparent absence of fragment II (Fig. 2A). However, from the relative intensities of the bands, DNA corresponding to fragment II of AX3 appeared to comigrate with fragment III in digests of V12 DNA due to a deletion of ca. 3 kb (Fig. 2A). This hypothesis was supported by the observation that *Hin*dIII fragment A from V12 DNA, which completely overlaps *Eco*RI fragment II (Fig. 1), was noticeably smaller than AX3 *Hin*dIII fragment A (Fig. 2D). Hybridization of labeled *Eco*RI fragment II probe confirmed that V12 rDNA365 fragment II was ~3 kb smaller than the standard AX3 rDNA350 fragment II (Fig. 2C).

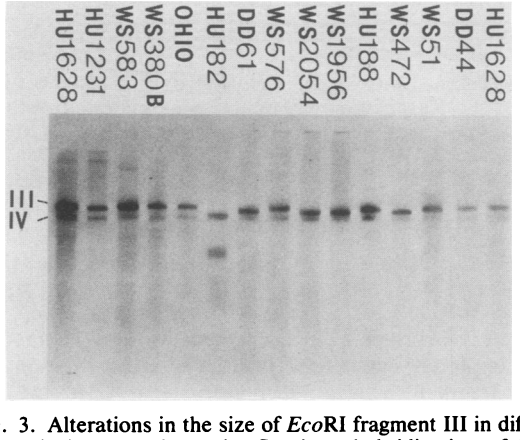


FIG. 3. Alterations in the size of *Eco*RI fragment III in different wild-type isolates as shown by Southern hybridization of *Eco*RI fragment III probe to *Eco*RI restriction fragments of rDNA from the wild-type isolates. For this experiment fragment III DNA from strain AX2 with some cross-contamination with fragment IV DNA was used, and fragment IV DNA here served as an internal size marker. Note the numerous small variations in the size of the fragments homologous to the fragment III probe and the presence of two homologous bands in strain HU182. That DNA from strain HU182 and the other wild-type isolates homologous to the standard rDNA350 *Eco*RI fragment IV was of the same apparent size as that of the AX3 rDNA350 allele was established in separate experiments (unpublished data).

Fragment III was always present, but there were minor changes in size (Fig. 3). For example, the homologous fragment from WS1956 is slightly smaller than that of the rDNA350 allele. These changes in size were highlighted by using a nick-translated probe of fragment III that also contained a minor amount of fragment IV as a size control (Fig. 3). In strain HU182, DNA hybridizing with *Eco*RI fragment III of AX2 was present in two bands: one ~1.5 kb smaller than fragment III band of AX3 and comigrating with band IV (~5.7 kb) and the other with a size of ca. 4 kb. An extra band of this size also is visible on ethidium bromide-stained gels (Fig. 2A). This result may reflect either an extra *Eco*RI site in HU182 DNA homologous to fragment III or the presence of multiple rDNA forms.

To summarize these results, it is clear, even for the two enzymes studied, that restriction enzyme site alterations commonly occur in rDNA palindromes of different *D. discoideum* wild-type isolates. These include a deletion from V12 *Eco*RI fragment II, four cases of the loss of the *Eco*RI site between fragments II and V, several minor changes in the size of *Eco*RI fragment III, and possibly the presence of additional restriction sites. One isolate, HU182, may contain two rDNA forms.

**Changes in *Eco*RI restriction patterns observed in strains derived from the NC4 isolate.** Genetic analysis in *D. discoideum* uses the parasexual cycle, in which two haploid strains are fused to form a diploid, followed by haploidization. Most genetic studies have been done with strains derived from a single isolate, NC4, and we have a large collection of such genetically marked strains (37). Analysis of DNA from 21 NC4-derived strains revealed two rDNA polymorphisms, both of which involve DNA homologous to *Eco*RI fragment II, in our genetic stocks. These have been designated rDNA351 and rDNA352.

The first, rDNA351, which is due to an insertion into *Eco*RI fragment II, was traced to strain HR7 (see above). This leads to an increase of ~3 kb in both *Eco*RI fragment II

(Fig. 4A) and *Hin*dIII fragment A (data not shown) of strain HR7 and its derivatives (e.g., strains HU878 and HU1310; see above). rDNA351 fragment II was not an artifact due to partial digestion of the DNA, as partial digestion could not produce a band of this size (Fig. 1). Strain HR7 and its derivatives also retain a minor population of the unaltered AX3 rDNA350 form. Fragment II DNA of AX2 hybridized both with the mutant rDNA351 fragment II and with a DNA band similar in size to the rDNA350 fragment II of AX3 (Fig. 4B). Under similar conditions, in this region hybridization was not detected to chromosomal DNA from wild-type isolates such as V12, in which the position of fragment II rDNA was altered (Fig. 2C). Hence, the hybridization seen with HR7 DNA in the position of rDNA350 fragment II probably is not due to chromosomal repetitive DNA.

The second rDNA alteration, rDNA352, which was discovered in NC4-derived tester strain HU1628 (see above), consists of an additional band in *Eco*RI digests (Fig. 4A). This extra fragment is present in many of our strains and was traced to the original NC4 wild-type isolate (Fig. 4B). Southern hybridization analysis indicated that this extra band contains DNA homologous with *Eco*RI fragment II of strain AX2. The amount of DNA present in this band clearly varied from strain to strain. Axenic strain AX3, which was used to construct the rDNA map (Fig. 1), contained few if any copies of the rDNA352 allele, whereas strains HU1628 and M28 contained many copies and strain TS12 contained relatively few. In all cases in which the rDNA352 allele was present, the rDNA350 allele also was present. The reasons for this are not clear but if the rDNA352 allele is defective, its presence has no marked effect on either growth or development.

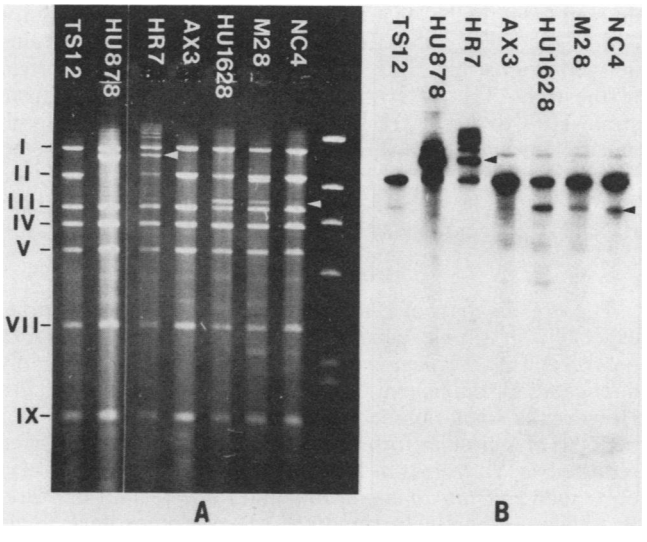


FIG. 4. Polymorphic restriction fragments of rDNA from mutant strains derived from NC4 after: *Eco*RI digestion (A) and *Eco*RI digestion and Southern hybridization with *Eco*RI fragment II probe from strain AX2 (B). The polymorphic bands of the rDNA351 (HR7 and HU878) and rDNA352 (NC4, M28, HU1628, and TS12) alleles are indicated by the arrows. Due to heavier loading, the rDNA352 allele in NC4 was not as cleanly resolved from fragment III; on other gels a distinct ethidium bromide staining band of this size was visible in NC4 preparations. The origin of the additional minor band homologous to the fragment II probe in HU1628 at ~4 kb and the unusually intense staining of bands I and IV in this preparation have not been further investigated. *Hin*dIII-digested λ standards are shown on the right of panel A (see the legend to Fig. 2). The origins of the strains on this figure are given in the text.

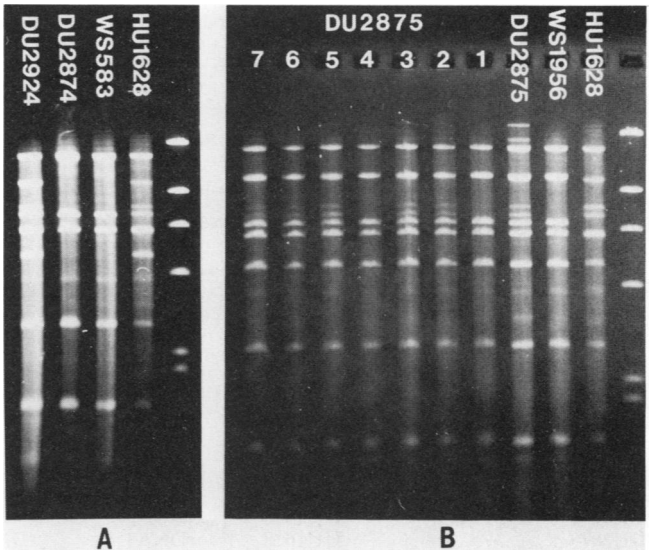


FIG. 5. (A) *Eco*RI restriction fragments of rDNA from strains HU1628 and WS583 and from two independently isolated diploids (strains DU2874 and DU2924) constructed by fusion of strains HU1628 and WS583; (B) *Eco*RI restriction fragments of rDNA from HU1628, WS1956, and diploid DU2875 constructed by fusion of HU1628 and WS1956. Seven clones derived from spores from the initial DU2875 population had considerable variation in the relative amounts of the parental rDNA forms. *Hin*dIII-digested standards are shown on the right of panels A and B.

**Fate of rDNA in crosses between strains with different *Eco*RI restriction patterns.** The differences in *Eco*RI restriction patterns provided genetic markers for rDNA from particular strains and allowed the inheritance of the extrachromosomal rDNA to be studied. In diploids constructed between strain HU1628, a haploid derived from NC4, and a polymorphic wild-type isolate, there was a tendency for the rDNA from one or the other parental haploid to predominate in the diploid within ca. 50 generations of growth. In diploids constructed with some wild-type isolates, in particular WS583, either one or the other parental type predominated (Fig. 5A). With the remaining wild-type isolates, for example WS1956, mixtures of the rDNA forms were seen (Fig. 5B). In the latter cases, equal molarity of the two forms was almost never seen in heterozygous diploids. In some cases the NC4-derived pattern was enriched, and in others that of the wild-type isolate was enriched. In an analysis of one such case involving a diploid constructed from haploids HU1628 and WS1956 (DU2875), subclones derived from the initial diploid cell after ca. 50 generations were shown to have considerable variation in the relative amounts of the two rDNA forms when sampled after another 30 to 40 generations of growth (Fig. 5B).

These data (summarized in Table 3) indicate that if there are chromosomal copies controlling the synthesis of the extrachromosomal rDNA, then replication from one or the other allele is suppressed in most diploids. If suppression occurred, then haploidization of such diploids should lead to the reappearance of either allele in different haploid segregants. This was tested with eight segregant haploids that were isolated from DU2889, a diploid in which essentially only the rDNA from the NC4-derived parent was present. These segregants contained different combinations of NC4-derived and WS380B-derived chromosomes (Table 4). When *Eco*RI fragment V was used as a probe, it was clear that the NC4 pattern of rDNA was found almost exclusively in all

TABLE 3. Inheritance of rDNA in independently isolated diploids constructed between wild-type isolates and the NC4-derived haploid HU1628[a]

| Strain | Haploid parents | | rDNA alleles | | Outcome |
|---|---|---|---|---|---|
| | WI | NC4 | WI | NC4 | |
| DU2885 | DD61 | HU1628 | rDNA360 | rDNA350/352 | NC4-derived forms predominate in both DU2885 and DU2886; rDNA360 possibly absent |
| DU2886 | DD61 | HU1628 | rDNA360 | rDNA350/352 | |
| DU2889 | WS380B | HU1628 | rDNA363 | rDNA350/352 | NC4-derived forms predominate; rDNA363 absent (Fig. 6) |
| DU2924 | WS583 | HU1628 | rDNA364 | rDNA350/352 | NC4-derived forms predominate in DU2924, but WS583-derived rDNA364 form predominates in DU2874 (Fig. 5A) |
| DU2874 | WS583 | HU1628 | rDNA364 | rDNA350/352 | |
| DU2872 | WS1956 | HU1628 | rDNA357 | rDNA350/352 | NC4-derived forms predominate in DU2872, and WS1956-derived rDNA357 form predominates in DU2875 (Fig. 5B); however, all forms clearly present in both diploids |
| DU2875 | WS1956 | HU1628 | rDNA357 | rDNA350/352 | |
| DU2871 | WS2054 | HU1628 | rDNA358 | rDNA350/352 | WS2054-derived rDNA358 form predominates in both diploids, but NC4-derived forms clearly present in DU2876 |
| DU2876 | WS2054 | HU1628 | rDNA358 | rDNA350/352 | |
| DU2887 | HU182 | HU1628 | rDNA361 | rDNA350/352 | HU182-derived rDNA361 form predominates, but NC4-derived forms clearly present |

[a] In this work, diploid strains are designated DU; all other strains are haploids. Because of the nature of the parasexual genetic analysis of *D. discoideum*, diploids can be easily constructed between genetically marked NC4-derived strains (NC4) and wild-type isolates (WI) but not between pairs of wild-type haploids.

haploid segregants (Fig. 6B). For example, with HU2274, in which it was established that at least six of the seven chromosomes were derived from WS380B (Table 4), the WS380B rDNA363 allele was present as a very minor proportion of the rDNA, if at all. Among the segregants of strain DU2889 (Fig. 6A, Table 4), inheritance of the extra band of the rDNA352 pattern also could be followed. As expected from our previous findings, these segregants showed considerable variation in the amounts of this band.

In diploids constructed between polymorphic strains solely derived from the NC4 isolate, there also was a tendency for one or the other rDNA form to predominate. With strains derived from strain HR7, this allowed us to trace the origin of the rDNA351 pattern. In all cases in the lineage from strain HR7 to strain HU1310 the rDNA351 form predominated. However, in a diploid, strain DU1896, constructed from strains HU1257 (the parent of HU1310) and X36 (see above), the X36 rDNA352 pattern predominated. Hence, rDNA351 does not always predominate.

TABLE 4. Genotypes of HU1628 and haploid segregants of diploid DU2889 which was constructed from haploids HU1628 and WS380B[a]

| Strain | Linkage group | | | | | |
|---|---|---|---|---|---|---|
| | I | II | III | IV | VI | VII |
| HU1628 | *cycA1* | *acrA1823* *axeA1* *axeC1* *oaaA1* | *bsgA5* | *whiC351* | *manA2* | *couA351* *frtB353* |
| WS380B | + | + | + | + | + | + |
| HU2274 | + | + | + | + | + | + |
| HU2275 | + | + | + | *whiC351* | + | + |
| HU2281 | + | + | *bsgA5* | + | *manA2* | *couA351* |
| HU2302 | *cycA1* | + | *bsgA5* | + | *manA2* | *couA351* |
| HU2303 | *cycA1* | + | *bsgA5* | *whiC351* | *manA2* | *couA351* |
| HU2305 | + | *acrA1823* | *bsgA5* | + | *manA2* | *couA351* |
| HU2306 | + | *acrA1823* | + | *whiC351* | + | + |
| HU2321 | *cycA1* | + | + | *whiC351* | + | + |

[a] The haploid segregants were derived from strain DU2889 after thiabendazole-induced nondisjunction and chromosome loss. They contain mixtures of the six established linkage groups (chromosomes) from strains HU1628 and WS380B; for historical reasons, the missing seventh linkage group is designated group V. Complete genotypes are given for strains HU1628 and WS380B; in the segregants the *axeA1*, *axeC1*, *oaaA1*, and *frtB353* markers were not scored. Phenotypes of mutations at these loci are: *acrA*, resistance to acriflavin (100 μg/ml), methanol (2 to 3%), and benzimidazole carbamate derivatives; *axe*, ability to grow in axenic media (requires an additional mutation at the *axeB* locus); *bsgA*, inability to use *B. subtilis* as a food source; *couA*, sensitivity to 1.3 mM coumarin with pleiotropic temperature sensitivity (*tsgK21*); *cycA*, resistance to cycloheximide (500 μg/ml); *frtB*, distribution of fruiting bodies in concentric rings; *manA*, α-mannosidase-1 deficient; *oaaA*, ability to develop in the presence of ω-aminocarboxylic acids; *whiC*, absence of the normal yellow spore pigment. Alleles from WS380B are denoted +.

## DISCUSSION

The organization of rDNA genes is surprisingly diverse, especially in simple eucaryotes. In the fungi *Neurospora crassa* and *Saccharomyces cerevisiae* there are tandemly duplicated chromosomal copies of the rDNA genes (8, 24). However, a small number of extrachromosomal copies (~5 per cell) of a circular form of rDNA (3-μm-circle) have been reported in *S. cerevisiae* (1, 14). Some protozoan eucaryotes, such as *Glaucoma chattoni* and *Paramecium tetraurelia*, have, respectively, multiple extrachromosomal single (12) and tandemly repeated (7) rDNA. Other protozoans, such as *T. thermophila*, *Physarum polycephalum*, and *D. discoideum* (the earliest known eucaryote to have diverged from the mainstream of eucaryotic descent [17]), have extrachromosomal palindromic rDNA (2, 4, 10, 32).

The studies reported here show that the linear extrachromosomal rDNA palindrome of *D. discoideum* is highly polymorphic; differences in the basic *Eco*RI restriction pattern were discovered in many wild-type isolates. Moreover, changes have been observed in laboratory strains. This is in contrast to the macronuclear rDNA of *T. thermophila* which is highly conserved between strains (23), although at least one polymorphism is known (22). *Physarum polycephalum*

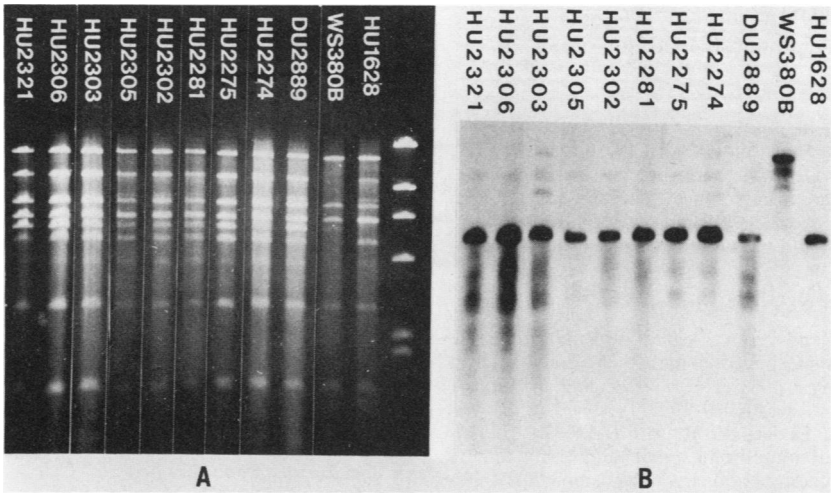


FIG. 6. *Eco*RI restriction fragments of rDNA from strains HU1628 and WS380B, diploid DU2889 (constructed by fusion of strains HU1628 and WS380B), and segregant haploids derived from strain DU2889 (A) and after *Eco*RI digestion and Southern hybridization with cloned *Eco*RI fragment V (20) probe to distinguish minor populations of the WS380B rDNA363 allele (B). *Hin*dIII-digested λ standards are shown on the right of panel A. A WS380B-derived band (~5.5 kb), which is present in all haploid segregants, is unrelated to rDNA and reflects the presence of a high-copy-number plasmid to be described elsewhere.

exhibits levels of polymorphism between those observed in *D. discoideum* and *T. thermophila* (6). This degree of polymorphism correlates with the size of the palindromes: that of *D. discoideum* is ca. 30% bigger than that of *Physarum polycephalum* and four times the size of that of *T. thermophila*. Presumably because most of the *T. thermophila* rDNA is transcribed, most alterations would not be tolerated. The observed alterations in the *D. discoideum* rDNA probably involve nontranscribed regions. Those affecting *Eco*RI fragment II and the *Eco*RI site between fragments II and V certainly do not affect transcribed regions. However, those affecting *Eco*RI fragment III might affect the regions from which the 5S or 26S RNA are transcribed.

Two basic classes of polymorphism were found in *D. discoideum*. The first appears to be loss (or gain) of a restriction enzyme site (e.g., the loss of the *Eco*RI site between fragments II and V in strains WS380B, WS583, OHIO, and HU182). The second class involves more drastic changes such as the insertion or deletion of DNA (e.g., deletion of ~3 kb from *Eco*RI fragment II in strain V12). Two polymorphisms have been observed in laboratory strains derived from the NC4 wild-type isolate. One involves an apparent insertion of ~3 kb in *Eco*RI fragment II of strain HR7, whereas the other involves variation in the amount of a minor rDNA allele (rDNA352) present in the original NC4 wild-type isolate.

These rDNA restriction enzyme fragment length polymorphisms have allowed us to examine the inheritance of rDNA during the asexual life cycle and in parasexual genetic crosses. Such crosses involve fusion of two haploid strains to form a diploid, followed by subsequent haploidization involving random chromosome loss; no meiotic reduction is involved (21). Even in newly isolated diploids, we observed that one or the other parental class of rDNA tended to predominate within 50 generations, but there was no clear evidence for preferential loss of one allele. In particular, in crosses between NC4-derived strain HU1628 and WS583, the different parental rDNAs predominated in different diploids. Most diploids expressing multiple rDNA alleles did not do so in equimolar amounts. Haploid segregants of a diploid, DU2889, that expressed essentially only the rDNA from the NC4-derived parental haploid did not recover the nonexpressed WS380B rDNA363 allele. These studies were conducted with well-marked strains, and in no case did we see any evidence for the association of the rDNA with any particular chromosome. Although chromosome V was not marked in these experiments, sufficient segregants were analyzed to exclude the association of rDNA with this chromosome. We conclude that during the asexual life cycle, rDNA of *D. discoideum* is inherited extrachromosomally. If there is a chromosomal copy, it is not active under these conditions. Nor is the chromosomal copy necessary, as we have constructed strains, for example HU2274, that should have different chromosomal and extrachromosomal rDNAs. At present, it is not possible to search for activation of a putative chromosomal copy of the rDNA during the sexual life cycle of *D. discoideum*.

In *T. thermophila* it was established that there is only a single chromosomal rDNA gene (39) and that a corresponding linear nonpalindromic extrachromosomal rDNA is produced transiently during the formation of the macronucleus (22). The latter molecule has been proposed to be an intermediate in the ultimate formation of palindromic rDNA molecules (22). During vegetative growth the extrachromosomal rDNA copies replicate autonomously. In *Physarum polycephalum* replication of rDNA palindromes occurs throughout the S and G2 cell cycle stages, and some, but not necessarily all, rDNA palindromes serve as replication templates (33). Similar changes to those observed in this work in the relative proportions of polymorphic rDNA palindromes and effects on rDNA inheritance were observed during the sexual cycle of *Physarum polycephalum* (6). In *D. discoideum* NC4-derived haploids, in which multiple rDNA forms exist, more than one rDNA gene must serve as a template. Replication of the rDNA palindromes of *D. discoideum* seems a bulk phenomenon, where several or perhaps all rDNA palindromes can serve as a template. Shifts in the relative proportions of the rDNA forms presumably reflect minor differences in the rates of initiation of replication, transfer to daughter nuclei, or degradation. Although these details of how rDNA replicates in *D. discoideum* remain to be established, we suggest that future studies should concentrate on the extrachromosomal rDNA as we have found no evidence during the asexual life cycle for the

use of a chromosomal rDNA gene as a template for the extrachromosomal rDNA palindromes in this organism.

## ACKNOWLEDGMENTS

We thank G. Gerisch for his encouragement, J. E. Hughes, R. Frankham, and M. Gillings for comments on the manuscript, A. Noegel and B. Metz for advice throughout the course of this work, and H. Dähne and G. Rüger for technical assistance. R. Parish generously provided the cloned *Eco*RI fragment V DNA.

This work was made possible by support from the Max-Planck-Gesellschaft.

## LITERATURE CITED

1. **Clark-Walker, G. D., and A. A. Azad.** 1980. Hybridizable sequences between cytoplasmic ribsomal RNAs and 3 micron circular DNAs of *Saccharomyces cerevisiae* and *Torulopsis glabrata*. Nucleic Acids Res. **8**:1009–1022.
2. **Cockburn, A. F., W. C. Taylor, and R. A. Firtel.** 1978. *Dictyostelium* rDNA consists of non-chromosomal palindromic dimers containing 5S and 36S coding regions. Chromosoma **70**:19–29.
3. **Emery, H. S., and A. M. Weiner.** 1981. An irregular satellite sequence is found at the termini of the linear extrachromosomal rDNA in *Dictyostelium discoideum*. Cell **26**:411–419.
4. **Engberg, J., P. Andersson, V. Leick, and J. Collins.** 1976. Free ribosomal DNA molecules from *Tetrahymena pyriformis* GL are giant palindromes. J. Mol. Biol. **104**:455–470.
5. **Erdos, G. W., K. B. Raper, and L. K. Vogen.** 1973. Mating types and macrocyst formation in *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. U.S.A. **70**:1828–1830.
6. **Ferris, P. J., V. M. Vogt, and C. L. Truitt.** 1983. Inheritance of extrachromosomal rDNA in *Physarum polycephalum*. Mol. Cell. Biol. **3**:635–642.
7. **Findly, R. C., and J. G. Gall.** 1978. Free ribosomal RNA genes in *Paramecium* are tandemly repeated. Proc. Natl. Acad. Sci. U.S.A. **75**:3312–3316.
8. **Free, S. J., P. W. Rice, and R. L. Metzenberg.** 1979. Arrangement of the genes coding for ribosomal ribonucleic acids in *Neurospora crassa*. J. Bacteriol. **137**:1219–1226.
9. **Gregg, J. H., M. Krefft, A. Haas-Kraus, and K. L. Williams.** 1982. Antigenic differences detected between prespore cells of *Dictyostelium discoideum* and *Dictyostelium mucoroides* using monoclonal antibodies Exp. Cell Res. **142**:229–233.
10. **Karrer, K. M., and J. G. Gall.** 1976. The macronuclear ribosomal DNA of *Tetrahymena pyriformis* is a palindrome. J. Mol. Biol. **104**:421–453.
11. **Katz, E. R., and M. Sussman.** 1972. Parasexual recombination in *Dictyostelium discoideum*: selection of stable heterozygotes and stable haploid segregants. Proc. Natl. Acad. Sci. U.S.A. **69**:495–498.
12. **Katzen, A. L., G. M. Cann, and E. H. Blackburn.** 1981. Sequence-specific fragmentation of macronuclear DNA in a holotrichous ciliate. Cell **24**:313–320.
13. **Kimmel, A. R., and R. A. Firtel.** 1982. The organization and expression of the *Dictyostelium* genome, p. 233–324. *In* W. F. Loomis (ed.), The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
14. **Larionov, V. L., A. V. Grishin, and M. N. Smirnov.** 1980. 3 μm DNA—an extrachromosomal ribosomal DNA in the yeast *Saccharomyces cerevisiae*. Gene **12**:41–49.
15. **Loening, U. E.** 1969. The determination of the molecular weight of ribonucleic acid by polyacrylamide-gel electrophoresis. Biochem. J. **113**:131–138.
16. **Loomis, W. F.** 1971. Sensitivity of *Dictyostelium discoideum* to nucleic acid analogues. Exp. Cell Res. **64**:484–486.
17. **McCarroll, R., G. J. Olsen, Y. D. Stahl, C. R. Woese, and M. L. Sogin.** 1983. Nucleotide sequence of the *Dictyostelium discoideum* small-subunit ribosomal ribonucleic acid inferred from the gene sequence: evolutionary implications. Biochemistry **22**:5858–5868.
18. **Metz, B. A., T. E. Ward, D. L. Welker, and K. L. Williams.** 1983. Identification of an endogenous plasmid in *Dictyostelium discoideum*. EMBO J. **2**:515–519.
19. **Miller, O. L.** 1981. The nucleolus, chromosomes, and visualization of genetic activity. J. Cell Biol. **91**:15S–27S.
20. **Ness, P. J., P. Labhart, E. Banz, T. Koller, and R. W. Parish.** 1983. Chromatin structure along the ribosomal DNA of *Dictyostelium*. Regional differences and changes accompanying cell differentiation. J. Mol. Biol. **166**:361–381.
21. **Newell, P. C.** 1982. Genetics, p. 35–70. *In* W. F. Loomis (ed.), The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
22. **Pan, W.-C., and E. H. Blackburn.** 1981. Single extrachromosomal ribosomal RNA gene copies are synthesized during amplification of the rDNA in *Tetrahymena*. Cell **23**:459–466.
23. **Pan, W.-C., E. Orias, M. Flacks, and E. H. Blackburn.** 1982. Allele-specific, selective amplification of a ribosomal RNA gene in *Tetrahymena thermophila*. Cell **28**:595–604.
24. **Petes, T. D.** 1979. Meiotic mapping of yeast ribosomal deoxyribonucleic acid on chromosome XII. J. Bacteriol. **138**:185–192.
25. **Raper, K. B.** 1935. *Dictyostelium discoideum*, a new species of slime mold from decaying forest leaves. J. Agric. Res. (Wash.) **50**:135–147.
26. **Reeder, R. H.** 1974. Ribosomes from eukaryotes: genetics, p. 489–518. *In* M. Nomuro, A. Tissieres, and P. Lengyel (ed.), Ribosomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
28. **Robson, G. E., and K. L. Williams.** 1979. Vegetative incompatibility and the mating-type locus in the cellular slime mould *Dictyostelium discoideum*. Genetics **93**:861–875.
29. **Robson, G. E., and K. L. Williams.** 1980. The mating system of the cellular slime mould *Dictyostelium discoideum*. Curr. Genet. **1**:229–232.
30. **Rothman, F. G., and E. T. Alexander.** 1975. Parasexual genetic analysis of the cellular slime mould *Dictyostelium discoideum* A3. Genetics **80**:715–731.
31. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
32. **Vogt, V. M., and R. Braun.** 1976. Structure of ribosomal DNA in *Physarum polycephalum*. J. Mol. Biol. **106**:567–587.
33. **Vogt, V. M., and R. Braun.** 1977. The replication of ribosomal DNA in *Physarum polycephalum*. Eur. J. Biochem. **80**:557–566.
34. **Watts, D. J., and J. M. Ashworth.** 1970. Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. Biochem. J. **119**:171–174.
35. **Welker, D. L., B. A. Metz, and K. L. Williams.** 1982. Chromosome rearrangements in *Dictyostelium discoideum*. Genetics **102**:711–723.
36. **Welker, D. L., and K. L. Williams.** 1980. Mitotic arrest and chromosome doubling using thiabendazole, cambendazole, nocodazole and ben late in the slime mould *Dictyostelium discoideum*. J. Gen. Microbiol. **116**:397–407.
37. **Welker, D. L., and K. L. Williams.** 1982. A genetic map of *Dictyostelium discoideum* based on mitotic recombination. Genetics **102**:691–710.
38. **Williams, K. L., and P. C. Newell.** 1976. A genetic study of aggregation in the cellular slime mould *Dictyostelium discoideum* using complementation analysis. Genetics **82**:280–307.
39. **Yao, M. C., and J. G. Gall.** 1977. A single integrated gene for ribosomal RNA in a eucaryote, *Tetrahymena pyriformis*. Cell **12**:121–132.