

I: Sequence Organization of Drosophila melanogaster
5S rRNA and 4S RNA Genes

II: In vitro Studies on Replication
of Plasmid DNAs

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Susan E. Conrad

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Abstract

I. The sequence organizations of Drosophila melanogaster 4S RNA (tRNA) and 5S rRNA genes have been investigated. Segments of Drosophila DNA containing these genes have been propagated in recombinant plasmids using Escherichia coli as a host and ColE1 as a vector.

Electron microscope partial denaturation mapping, mapping by ferritin labeling and restriction enzyme-gel electrophoresis analysis all indicate that the Drosophila DNA inserts of the 5S rRNA gene containing plasmids consist of tandem repeats of 5S genes and spacer regions. The repeat length is approximately 380 nucleotide pairs (ntp), corresponding to a gene of length 120 ntp and a spacer of length 260 ntp. Little heterogeneity in the lengths of the repeats has been detected.

A tRNA gene containing clone has been characterized by electron microscopic methods and by restriction endonuclease mapping. Four tRNA genes were detected on a 9.34 kb fragment of DNA. Three of these genes appear to be identical and different from the fourth. No evidence was found for extensive sequence homology in the sequences surrounding the genes. In situ hybridization with cRNA transcribed from the plasmid showed localization at region 42A of chromosome 2R.

II. An improved system for in vitro replication of Escherichia coli plasmid DNAs has been developed and characterized. Endogenous DNA is removed from the extracts, making replication dependent upon exogenous DNA even when plasmid containing cells are used as a source of extracts. Replication in this system requires E. coli DNA polymerases I and III, RNA polymerase, DNA gyrase, and the products of at least five genes required for E. coli replication (dnaB, dnaC, dnaG, dnaP, dnaZ).

This system has been used to study the replication properties of the ampicillin resistant plasmid RSF1030. We have found that, like ColE1, RSF1030 replicates unidirectionally from a unique origin. We have also investigated the replication of pFH118, a high copy number mutant derived from ColE1. The mutation in pFH118 is due to the insertion of 20-30 base pairs into the coding sequence for a 100 nucleotide RNA that is transcribed during DNA replication. Results of reversion studies suggest that this RNA plays a role in determining plasmid copy number. Furthermore, the RNAs transcribed from ColE1 and RSF1030 have significant sequence homology, although the plasmids were isolated independently and have been thought to have no sequence homology.

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Part I

Sequence Organization of
Drosophila melanogaster 4S RNA and 5S RNA Genes

Introduction

Part I of this thesis reports work that was done in Dr. Norman Davidson's laboratory. Along with Pauline Yen, Dave Hershey, and Ann Sodja, I investigated the sequence organization of the 4S RNA (tRNA) and 5S ribosomal RNA genes of Drosophila melanogaster using recombinant DNA techniques. Many of these techniques were new to Caltech at the time this work was done, so we arranged a collaboration with Christine Ilgen and John Carbon at UCSB for the cloning part of the project. We prepared Drosophila DNA and sent it to Santa Barbara where it was joined to the vector ColE1 and used to transform E. coli. The facts that pure 4S and 5S RNAs are relatively easy to obtain in large quantities and that the genes coding for them are repeated many times in the genome made these genes amenable to both isolation and characterization. In our original screening of the Drosophila melanogaster library, we found several clones containing 5S RNA genes and several containing tRNA genes.

Characterization of the 5S gene containing clones showed that in Drosophila, as in other organisms, the genes are arranged in tandemly repeated arrays of G/C rich genes and A/T rich spacers. We found, however, that unlike other organisms, Drosophila exhibits little heterogeneity in the lengths of the repeats.

Characterization of a tRNA-gene containing clone designated pCIT12 suggested that the tRNA genes in Drosophila melanogaster are arranged in a complex manner. We identified four tRNA genes coding for at least two different tRNA species on this cloned fragment of DNA. There is not a constant distance between the genes, and we found no evidence for extensive sequence homology in the regions adjacent to the tRNA genes. Subsequent work done primarily by

Pauline Yen has shown that the initial characterization of pCIT12 only began to explore this tRNA cluster. pCIT12 itself now seems to contain the genes for at least three different tRNA species (asn, lys, arg). The total tRNA cluster in this region covers 46 kb of DNA, and contains at least 18 tRNA genes. It is not known how many isoaccepting species are represented in these 18 genes, and there is still no evidence for extensive sequence homology in the regions adjacent to the genes. Current work is aimed towards identifying the different genes and sequencing these regions in order to understand the regulation of tRNA gene expression.

Chapter 1

The Sequence Arrangement of
Drosophila melanogaster 5S DNA
Cloned in Recombinant Plasmids

N. Davis Hershey, Susan E. Conrad,* Ann Sodja,
Pauline H. Yen, Maurice Cohen, Jr. and
Norman Davidson

Department of Chemistry
and *Division of Biology
California Institute of Technology
Pasadena, California 91125

Christine Ilgen† and John Carbon
Section of Biochemistry and Molecular Biology
Department of Biological Sciences
University of California
Santa Barbara, California 93106

Summary

Segments of *Drosophila melanogaster* DNA containing 5S rRNA genes have been propagated in recombinant plasmids using *E. coli* as a host and Col E1 as a vector. Electron microscope partial denaturation mapping, mapping by ferritin labeling and restriction enzyme-gel electrophoresis analysis all indicate that the *Drosophila* DNA inserts of these plasmids consist of tandem repeats of 5S genes and spacer regions. The repeat length is approximately 380 nucleotide pairs (ntp), corresponding to a gene of length 120 ntp and a spacer of length 260 ntp. The insert in one plasmid (pCIT9) consists of 32 contiguous repeats. Restriction enzyme-gel electrophoresis analysis shows that all these repeats have the same length within ± 5 nucleotides. This repeat length is estimated as 370 ± 20 ntp by gel electrophoresis and 390 ± 40 ntp by partial denaturation mapping. A second plasmid (pCIT19) contains three complete genes, two complete spacers and incomplete flanking spacer sequences. The two complete repeat units released by suitable restriction endonuclease digestions differ in length by 20 ± 5 ntp, with estimated lengths of 370 and 390 ntp. The positions and spacings of the genes on this plasmid have been observed directly by ferritin labeling and by partial denaturation mapping. The A+T content of the 5S DNA spacer region is calculated to be 68%. By in situ hybridization, cRNA transcribed from one plasmid hybridizes to polytene chromosomes only at band 56F, the known locus of the 5S rRNA genes. Spontaneous excision of some of the tandem repeat units from the recombinant plasmids occurs during growth in *E. coli*; the frequency of excision does not depend upon the *recA* character of the host, but is greatly increased by chloramphenicol treatment.

† Present address: Department of Biochemical Sciences, Princeton University, Princeton, New Jersey 08540.

Introduction

The approximately 165 copies of the 5S rRNA genes of *Drosophila melanogaster* (Procunier and Tartoff, 1975) are localized at band 56F on chromosome 2R (Prensky, Steffensen and Hughes, 1973; Grigliatti et al., 1973). Procunier and Tartoff (1976) have reported that these genes occur in a tandemly repetitious arrangement with 80 genes in each of two clusters separated by a sequence of unknown length. The repeat length observed by these investigators is 290 nucleotide pairs (ntp). In a similar experiment, C. A. Hutchison (personal communication) has determined a repeat length of 400 ntp.

We report on a further study of the sequence organization of *Drosophila* 5S rRNA genes by electron microscopic and restriction endonuclease-gel electrophoresis investigations of two *Drosophila* DNA segments propagated in a procaryotic vector. Detailed studies on the 5S rRNA genes of *Xenopus laevis* and *Xenopus mulleri* have shown that most of these genes are organized as tandem repeats of lengths 720 and 1400 ntp, respectively (Brown, Wensink and Jordan, 1971; Brown and Sugimoto, 1973). Since the G + C-rich gene has a length of 120 ntp, the A + T-rich spacers have lengths of 600 and 1280 ntp in these two species. The spacers of *Xenopus laevis* display some length heterogeneity arising from the presence of variable numbers of 15 ntp subrepeats (Carroll and Brown, 1976a). The existence of these subrepeats is supported by sequencing data (Brownlee, Cartwright and Brown, 1974). It will be instructive to compare the sequence organization of the 5S rRNA genes of the two genera, *Xenopus* and *Drosophila*.

Results

Recombinant DNA molecules comprised of segments of *Drosophila* DNA attached by the AT joining method to Col E1 DNA were prepared (Clarke and Carbon, 1976). Bacteria were transformed using selection for colicin E1 resistance. Quantitative studies were not made, but our qualitative observation was that the *Drosophila* inserts varied in length from about 1–15 kilobases (kb). A ^3H -labeled 5S rRNA probe was used to screen 9000 transformed colonies by colony filter hybridization (Grunstein and Hogness, 1975) for inserts carrying 5S DNA—that is, 5S rRNA genes. Two colonies, denoted JA199 (pCIT9) and JA199 (pCIT19), which gave rather strong scintillation autoradiographic responses were chosen for further study.

We observed a length of 6.66 ± 0.21 kb ($N = 54$) for Col E1 DNA. The main component of circular DNA, designated pCIT19, extracted from JA199

(pCIT19) cultures had a length of 8.30 ± 0.32 kb. Molecules of up to five integral multiples of this length were observed. Partial denaturation studies of the type reported below show that these larger circles are head-to-tail multimers of the pCIT19 plasmid.

Circular molecules extracted from strain JA199 (pCIT9) had a more complex length distribution. About 75% of all molecules larger than Col E1 fell into two abundant size classes with lengths 19.2 ± 1.4 and 8.9 ± 1.2 kb; there were, however, molecules with lengths ranging from approximately 19.2 kb to the Col E1 size of 6.7 kb. We designate the 19.2 kb class as pCIT9. Small circular molecules with a length of 350–425 ntp and molecules about twice this length were present in numbers comparable to those for the 19.2 and 8.9 kb classes. There were smaller numbers of molecules ranging in size from about 0.8 kb–6.7 kb. The evidence presented below shows that the molecules smaller than Col E1 consist solely of varying numbers of the *Drosophila* 5S gene plus spacer sequence of length approximately 0.4 kb.

Strain JA199 (pCIT9) was subcloned. Subcultures were grown to about 10^{11} bacteria, and the DNA was extracted. Some subcultures yielded plasmids of size 19.2 kb and smaller, as described above. Other subcultures contained an abundant species of a shorter length—for example, 13.2 kb or 8.9 kb—and smaller molecules.

Partial Denaturation Mapping

A regular pattern of alternating genes and spacers on the *Drosophila* 5S DNA segments was revealed by partial denaturation mapping. Nicked circular DNA molecules were subjected to partial denaturation conditions involving formaldehyde fixation of the denaturation loops and were then mounted for electron microscopy. An electron micrograph of a partially denatured pCIT9 molecule is shown in Figure 1a; a diagrammatic representation of the partial denaturation map is given in Figure 2a. A long region, denoted D5, of small regularly repeated denaturation loops is bounded by two larger loops, denoted AT1 and AT2, of unequal size. The remaining segment of the plasmids, denoted COL, is largely duplex but contains usually one (OL1) and sometimes a second (OL2) denaturation loop. Because of its asymmetric position in the COL region, OL1 provides a reference point that allows determination of polarity of the molecule.

The obvious interpretation of the pattern in Figure 2a is that the D5 region contains a set of tandemly repeated 5S rRNA genes. The duplex regions contain the G + C-rich 5S gene sequences; the denatured regions are A + T-rich spacer sequences. The data are presented in Table 1. When

Col E1 DNA was cleaved at its Eco RI endonuclease site and subjected to partial denaturation, molecules like that shown in Figure 1b were seen; a schematic representation of the denaturation pattern is given in Figure 2b. Length measurements for such molecules are presented in Table 1. It should be recalled that the junctions between the *Drosophila* insert and Col E1 are short A:T segments attached at the Eco RI site of Col E1. The Col E1/Eco RI denaturation pattern therefore shows that the Eco RI site lies within a large A + T-rich region of Col E1 and that several additional A + T-rich segments (the OL features) are asymmetrically distributed relative to the Eco RI site. The data in Table 1 then show that both the AT1 and the AT2 regions of pCIT9 consist of some *Drosophila* DNA, some Col E1 DNA and the short A:T junction segment.

A histogram (not shown) of the center-to-center distances of the loops in the D5 region displayed a major peak at 391 ± 39 nucleotides and a second much smaller peak at 787 ± 52 nucleotides. We therefore take 391 ± 39 as the repeat length. The major peaks of similar histograms showed the average duplex segment length in D5 to be 166 ± 48 ntp ($N = 548$) at 48% denaturation (of the D5 region) and 124 ± 8 ntp ($N = 585$) at 62% denaturation. The data are presented in Table 2.

That the insert of *Drosophila* DNA in pCIT9 consisted solely of repeating 5S DNA was confirmed by the existence of several partially denatured molecules having regularly spaced loops over the entire D5 region. From the length of the insert (12.9 kb) and the repeat length (0.39 kb), we calculate that the *Drosophila* insert could comprise a maximum of approximately 32 repeats of this length. This is precisely the maximum number of evenly spaced loops observed in such molecules. Thus no spacer DNA other than that of the repeating unit occurs within this stretch of about 33 genes. In most molecules, less than the maximum number of 32 loops were observed due either to failure of denaturation to occur to an observable extent in a particular repeat or to breakage of one arm of a denaturation loop. These breaks sometimes left single-strand tails which could be recognized in the electron microscope. Both breakage and lack of denaturation generated center-to-center distances that were multiples of the repeat length, within experimental error.

Examination of the total sample of partially denatured DNA extracted from strain JA199 (pCIT9) showed that the molecules with lengths between Col E1 and pCIT9 differed from pCIT9 only in that they contained a shorter length of the D5 region—that is, they had a smaller number of 5S DNA repeat units. The repeat length determined from such

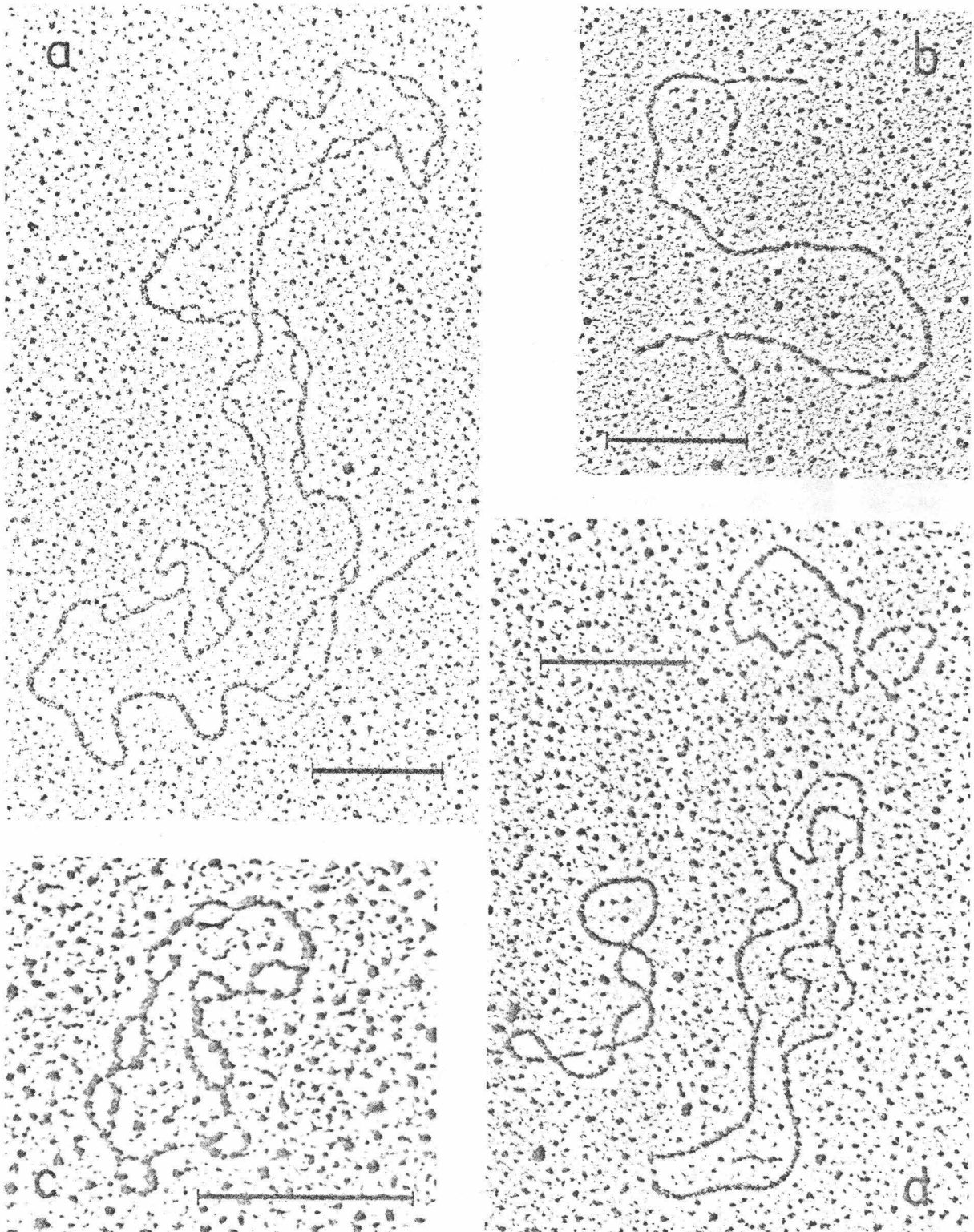


Figure 1. Partially Denatured DNA Molecules

(a) pCIT9; (b) Col E1/Eco RI; (c) circular molecule composed solely of 5S DNA; (d) pCIT19. Both a single- and a double-stranded (RFI) molecule of ϕ X174 are present in (d). Diagrammatic representations are given in Figure 2. Bars = 1 kb.

molecules was 396 ± 39 , identical within experimental error to that found for pCIT9. Thus the repeat length is not detectably altered by excision of *Drosophila* DNA. Excision appears to occur by removal of a block of from one to many repeats from the *Drosophila*.

The circles smaller than Col E1 appeared to consist entirely of tandem repeat units like the D5 region of Figure 2a. A micrograph of a molecule with nine loops is shown in Figure 1c. Denaturation loops were not observed in the circles of this type which consisted of only two or three repeats. These smaller molecules appear as almost perfect circles on the protein film. Probably the spreading forces in the film forced the two arms of the denaturation loop to lie together.

The partial denaturation pattern observed for the smaller plasmid pCIT19 is illustrated in Figure 2c. An electron micrograph is shown in Figure 1d. As in pCIT9, these molecules contain the AT1 and AT2 loops at the junctions between the *Drosophila* and Col E1 DNAs and the OL loops of the Col E1 DNA. There are quite reproducibly two loops, denoted S1

and S2, and three short duplex regions, denoted G1, G2 and G3, in the *Drosophila* insert bounded by the two loops AT1 and AT2. The average length of the three duplex regions after 59% denaturation was 128 ± 44 ntp ($N = 54$) and the center-to-center distance between the loops was 412 ± 58 nucleotides. The straight-forward interpretation of these data is that pCIT19 contains three gene sequences, two complete spacers and two additional spacers which may not be complete. Partial denaturation data for pCIT19 are presented in Tables 1 and 2.

Ferritin Mapping

The number and the positions of the 5S genes on pCIT19 have been directly determined by electron microscopic mapping of 5S rRNA:DNA hybrids on a single strand of the *Drosophila* DNA insert. These observations were made on heteroduplexes of pCIT19 with Col E1. The heteroduplex structure, which is depicted in Figure 3, consists of a circular duplex region corresponding to the Col E1 sequence, a duplex stem of length approximately 80 ntp due to the A:T junctions between the Col E1 vector and the *Drosophila* insert, and a single-stranded loop of length 1.40 ± 0.14 kb due to the *Drosophila* insert.

Ferritin mapping was carried out by a modification of the procedure of T.R. Broker et al. (manuscript in preparation) and Angerer et al. (1976). Biotin was conjugated to the 3' end of 5S rRNA via a cytochrome c bridge. RNA-biotin was hybridized to the heteroduplexes and then labeled with ferritin-avidin conjugates.

The heteroduplex structure (Figure 4) is symmetrical, and there is no reference marker to indicate polarity around the *Drosophila* insert. We label the two junction points of the A:T stem with the Col E1 duplex circle as j and j' . Most of the molecules used for mapping had only one ferritin label attached to the single-stranded loop of *Drosophila* DNA. The heteroduplex in Figure 4a has a ferritin label near the middle of the *Drosophila* DNA loop,

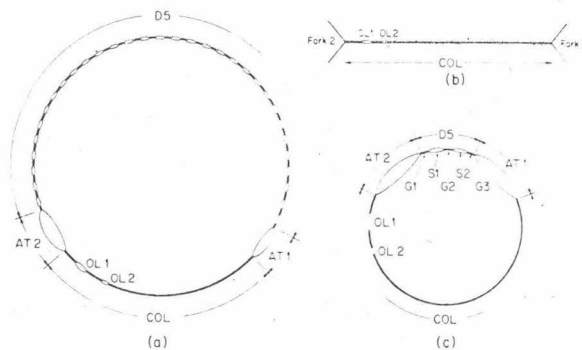


Figure 2. Diagrammatic Representation of Partially Denatured DNA Plasmid Molecules

(a) pCIT9; (b) Col E1/Eco RI; (c) pCIT19. Measurements of each segment of the D5 region of 18 pCIT19 molecules yielded the following lengths in nucleotides: G1, 127 ± 30 ; S1, 265 ± 44 ; G2, 138 ± 48 ; S2, 284 ± 50 ; G3, 118 ± 51 . For further explanation, see the text.

Table 1. Segment Lengths in Kilobases of Partially Denatured DNA Plasmid Molecules

Segment	pCIT9				pCIT19		Col E1/Eco RI			
	30 Min		50 Min		30 Min		30 Min		50 Min	
		N		N		N		N		N
D5	12.79 ± 0.76	24	12.99 ± 0.46	22	0.93 ± 0.08	18				
COL	5.35 ± 0.14	24	5.36 ± 0.20	22	5.11 ± 0.18	18	5.64 ± 0.28	33	5.49 ± 0.23	33
AT1	0.93 ± 0.12	45	0.91 ± 0.13	44	1.08 ± 0.13	36	0.51 ± 0.08	66	0.61 ± 0.11	66
AT2	1.15 ± 0.13	48	1.17 ± 0.15	44	1.07 ± 0.14	36	0.56 ± 0.10	66	0.66 ± 0.13	66
Total Length	19.73 ± 0.79	24	20.33 ± 0.61	22	8.24 ± 0.27	18	6.71 ± 0.31		6.76 ± 0.29	

For explanation of symbols, see Figure 2.

For Col E1/Eco RI, the entries under AT1 and AT2 are forks 1 and 2 (Figure 2b), respectively; those under "total length" are sums, not independent measurements.

Table 2. Characteristics of the D5 Region of Partially Denatured Recombinant DNA Plasmid Molecules

Plasmid	Denaturation Time (Min)	Number of Molecules Examined	Average Number of Loops per D5 Region	Mean (and Number) of Center-to-Center Distances between 280 and 500 ntp	Mean Length (and Number) of Duplex Segments between 0 and 330 ntp	% Denaturation
pCIT9	30	24	27.5 ± 2.0	391 ± 39 (545)	166 ± 48 (548)	48
	50	22	29.4 ± 1.9	392 ± 42 (559)	124 ± 48 (585)	62
Smaller Molecules of JA199 (pCIT9)	30	21	10	396 ± 39 (173)		52
pCIT19	30	18	2	412 ± 58 (18)	128 ± 44 (54)	59

The length of each double- and single-stranded segment of the D5 region was measured sequentially and converted to nucleotides or nucleotide pairs using the appropriate length standard. Values in this table were computed from these data. The limits given on the center-to-center distances and the duplex segment lengths represent the widths of the major peaks in appropriate histograms.

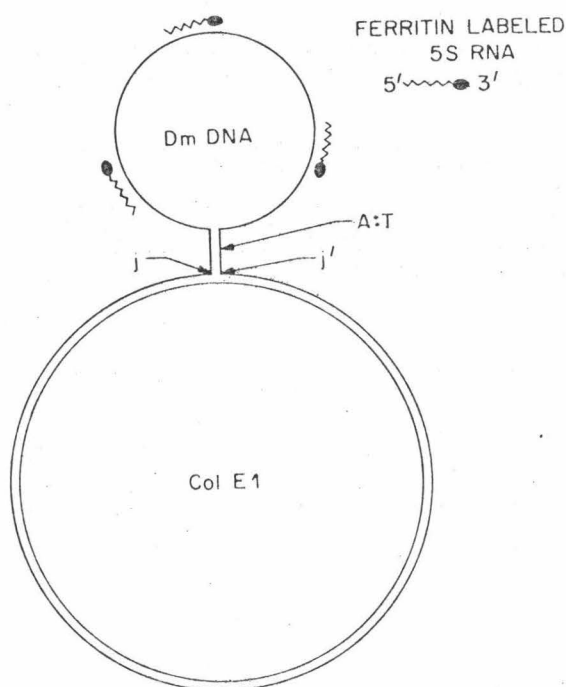


Figure 3. Diagrammatic Representation of the Heteroduplex between Col E1 and pCIT19 with Ferritin-Conjugated 5S rRNA Hybrids

For further explanation, see the text.

while the one in Figure 4b is labeled about 0.25 of distance around the Drosophila loop from the base of the A:T stem. Because polarity cannot be established for these structures, we measured and tabulated the shorter of the two distances from *j* or *j'* to the label. Thus all labels have a coordinate equal to or less than half the contour length from *j* to *j'*—that is, 0.78 kb. The resulting histogram (Figure 5) shows that there were 27 cases of a ferritin label observed at the 0.775 ± 0.030 kb position on the loop (that is, very close to the center) and 2.7 times as many cases of labels found at 0.386 ± 0.052 kb.

Note that the plotting procedure causes the smaller central peak to appear anomalously sharp and abruptly truncated.

Our interpretation of these results is that there are three 5S genes on the Drosophila insert of pCIT19. The 3' end of one gene sequence (the position of the ferritin label) is, within experimental error (approximately 0.06 kb), at the center of the insert. The 3' ends of the two other genes are approximately symmetrically placed, each 390 ± 80 nucleotides from the center and (fortuitously, we presume) 390 ± 80 nucleotides from *j* and *j'*.

The overall efficiency of labeling is estimated to be about 0.40 per site, so that the predicted probabilities of molecules with one, two and three labels are 0.43, 0.29 and 0.06, respectively. However, the ferritin-avidin labels contain 3–6 avidins per ferritin and are therefore multivalent for biotin binding. On many molecules with several 5S rRNA-biotin sites labeled, the two or three biotins are all bound to a single ferritin. These molecules tend to be rather tangled and difficult to trace and measure.

Overall, the ferritin mapping data agree with the partial denaturation data in showing that there are three genes on pCIT19, and that the two spacings between them are approximately equal at about 390 nucleotides.

Ferritin labeling experiments with pCIT9, which contains many 5S genes, gave highly tangled structures because of the multivalent nature of the ferritin-avidin label.

Restriction Endonuclease-Gel Electrophoresis Studies

The sequence of Drosophila 5S rRNA is known (Benhamou and Jordan, 1976). There are several specific restriction endonuclease sites in the corresponding 5S duplex DNA sequence, including an

↓
Hae III site, GGCC (Yang, Van de Voorde and Fiers,

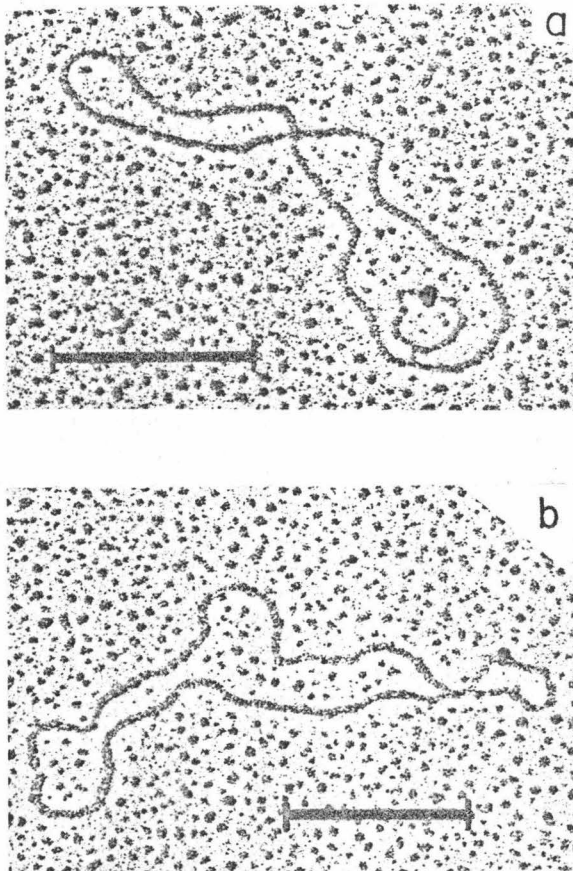


Figure 4. Heteroduplexes between Col E1 and pCIT19 Labeled with Ferritin-Avidin

(a) The 5S gene at or near the center of the *Drosophila* insert labeled with ferritin; (b) a gene about 0.25 of the distance around the *Drosophila* loop from the base of the A:T stem labeled with ferritin. Bars = 1 kb.

1976), at nucleotides 116-119 (with nucleotide 120
↓
being the 3' end) and an Hha I site, GCGC (Roberts et al., 1976), at nucleotides 66-69. If, for either enzyme, no additional sites occur within the spacer sequence, an endonuclease digest of a repeated gene sequence will yield fragments of length equal to the repeat length. The gel electrophoretic patterns of both Hae III and Hha I digests of Col E1 and of pCIT19 are shown in Figure 6a. There is a band in both Col E1 patterns, marked by J for Hae III and K for Hha I, which is absent in the pCIT19 pattern. In each case, this fragment must span the Eco RI site of Col E1; this site was cleaved and destroyed in generating the recombinant DNA molecules. For each enzyme, four bands are present in the digest of pCIT19 that are absent in the Col E1 digest. Two of these bands, R₁ and R₂, appear to contain equal amounts of DNA and have estimated molecular lengths of 370 and 390 nucleotides, respectively.

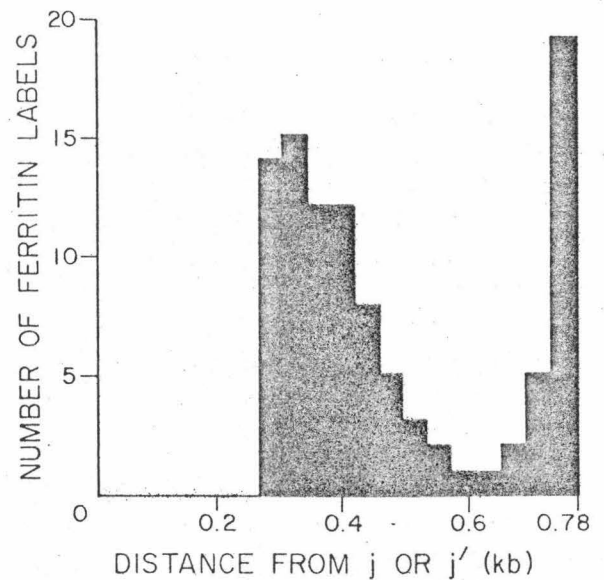


Figure 5. Histogram of the Ferritin Positions on the *Drosophila* DNA Insert of the Heteroduplex between Col E1 and pCIT19

Positions are the shorter of the two distances from j or j' to the ferritin label. Single-strand ϕ X174 served as the length standard for the *Drosophila* DNA.

These two bands are present at the same position and have comparable intensities in both the Hae III and Hha I digests. (The lengths of the fragments are estimated from the ϕ X174/Hae III calibration curve shown in Figure 7.) That the same two bands are observed in both enzyme digests strongly supports the interpretation that these bands arise from the two complete repeats of the 5S genes which are present in pCIT19, that they differ in length by about 20 ntp and that there are no other Hae III or Hha I sites in a 5S repeat unit. The two bands L₁ and L₂ in the Hae III digest and the two bands M₁ and M₂ in the Hha I digest must correspond to fragments extending from the enzyme sites within Col E1 to sites within the *Drosophila* insert.

Those fragments of the digest which contain 5S rRNA sequences were identified by a hybridization experiment of the type introduced by Southern (1976). DNA samples from enzyme digests were fractionated according to length by gel electrophoresis in 1% agarose. The fragments were denatured, transferred to nitrocellulose filters and incubated under hybridization conditions with ³H-labeled 5S rRNA. Scintillation autoradiography (Figure 8) showed that the bands R₁ and R₂ together (unresolved), the fragment L₁, but not L₂, of the Hae III digest, and both fragments M₁ and M₂ of the Hha I digest hybridized with 5S rRNA.

From the calibration curves of Figure 7, we deduce that the fragments L₁, L₂, M₁ and M₂ have respective lengths of about 1120, 710, 1500 and 1035 ntp. The distances from the nearest Hae III

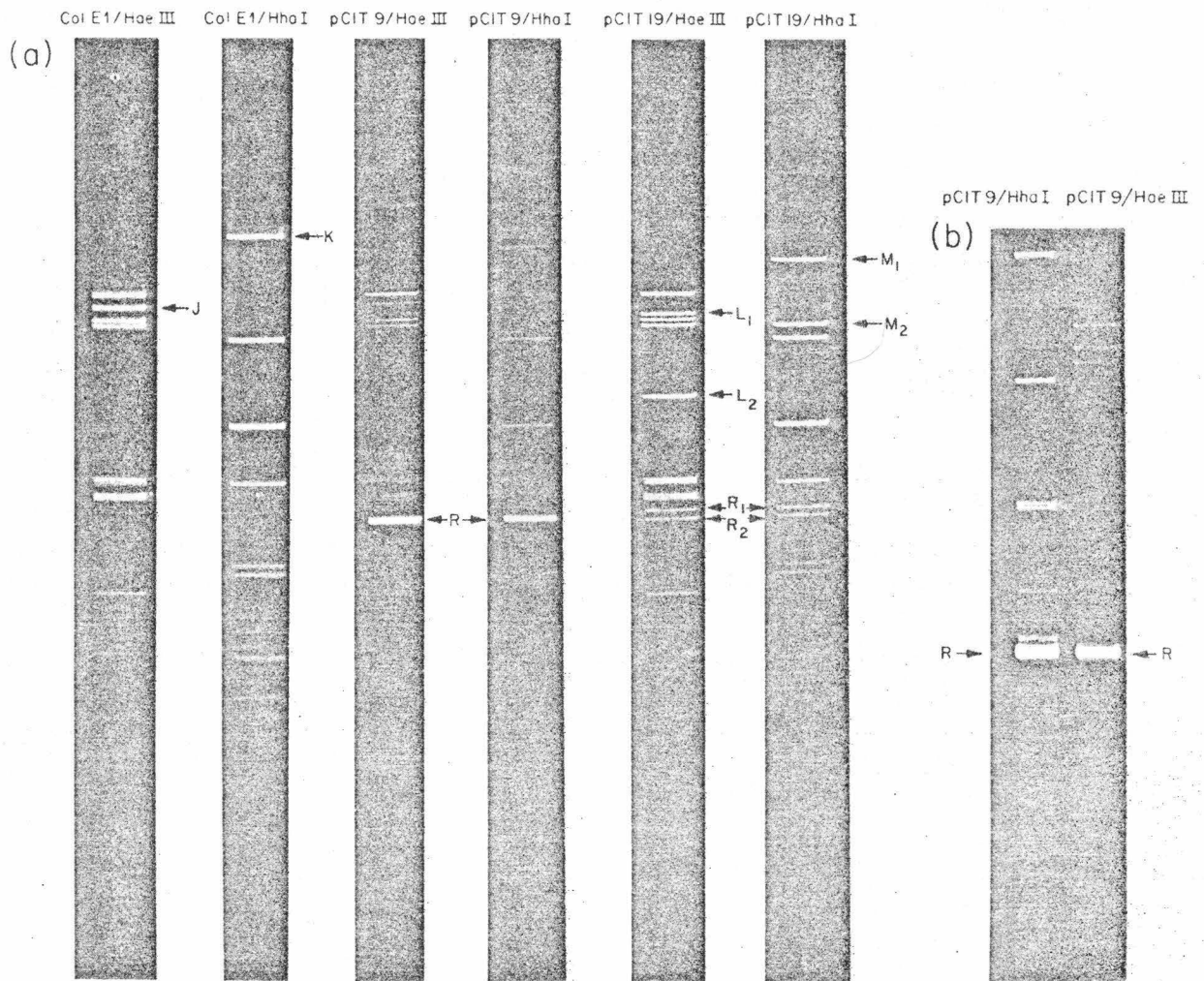


Figure 6. Polyacrylamide Gel Electrophoretic Analysis of Restriction Enzyme Digests of Plasmid DNA
 (a) 3-15% acrylamide gradient gel, 6 hr at 100 V; (b) 5-25% acrylamide gradient gel, 12 hr at 100 V.

and Hha I sites of Col E1 to the Eco RI site are shown in Figure 9. These values were determined by measuring fragment lengths of a combined digest of Col E1 with Hae III plus Eco RI or with Hha I plus Eco RI. Our lengths for the fragments produced by the combined digest using Hae III and Eco RI agree well with those of Oka and Takanami (1976). Since Hae III cleaves at a position three nucleotides from the 3' end of the RNA sequence, only one of the two junction fragments L₁ and L₂ is expected to hybridize to the ³H-labeled 5S rRNA probe, in agreement with experiment. Because Hha I cleaves close to the middle of the 5S gene, both junction fragments M₁ and M₂ are expected to hybridize. These facts lead to the map of the *coding strand* of pCIT19 shown in Figure 9. All the assignments in the map are unique, except that there is no determination of the relative positions of the 370 and 390 repeat units on the *Drosophila* insert. In

the ferritin labeling experiments, the ferritin is attached to the 3' end of the 5S rRNA. The positions observed for these labels are also depicted in Figure 9. There are small numerical differences in the repeat lengths determined by electron microscopy and by gel electrophoresis, but in all essential aspects, the two mapping experiments are in complete agreement.

Gel electrophoretic patterns of Hae III and Hha I digests of pCIT9 DNA are shown in Figures 6a and 6b. The outstanding feature is an intense band corresponding to a length of 360-370 nucleotides, which we interpret as the 5S repeat band. Its high intensity is expected because of the large number (up to 32) of 5S repeat units per circular DNA molecule. As may be seen in Figure 6a, the widths of these bands are less than the separation of the two repeat fragments R₁ and R₂ of pCIT19, which differ in length by about 20 nucleotides. The band widths

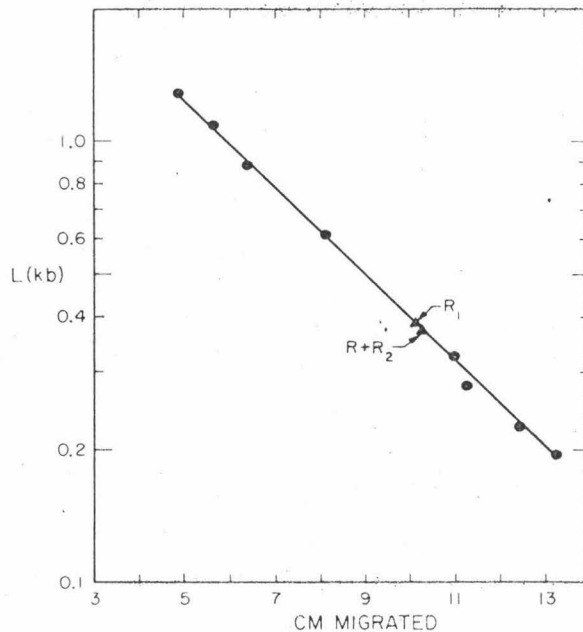


Figure 7. DNA Fragment Length Calibration Using a ϕ X174/Hae III Digest

Electrophoresis of a ϕ X174/Hae III digest was carried out on a 3–15% acrylamide gradient gel for 6 hr at 100 V. Distances on the abscissa were measured on a photograph. The lengths of ϕ X174/Hae III fragments are those of Morris (1976). The positions of the repeat bands in pCIT19 (R_1 and R_2) and pCIT9 (R) are indicated. The use of SV40/Hae III digest fragments as length standards (Subramanian et al., 1974) resulted in significantly shorter lengths for the recombinant plasmid fragments (data not shown).

in Figure 6b suggest that the fragment lengths could be heterogeneous to the extent of 10 nucleotides. Also visible above the repeat band in the Hha I digest of Figure 6b is a less intense band which we interpret as a joining fragment.

In Situ Hybridization

3 H-labeled complementary RNA was transcribed from pCIT9 DNA with *E. coli* RNA polymerase and hybridized in situ to preparations of *Drosophila* chromosomes. As shown in Figure 10, the probe hybridized to region 56F of chromosome 2R, a locus to which 5S rRNA hybridizes (Prensky et al., 1973; Grigliatti et al., 1973). Analysis of more than 100 nuclei in five different chromosome preparations showed that silver grains were localized only over region 56F, even when the autoradiographic exposure was 300 times longer than necessary for development of 10 grains at that locus, an exposure judged more than sufficient for definitive gene localization.

Length Heterogeneity in pCIT9

The results reported in several of the preceding sections suggest that pCIT9 contains 32 repeat units of the 5S gene in the *Drosophila* insert, and

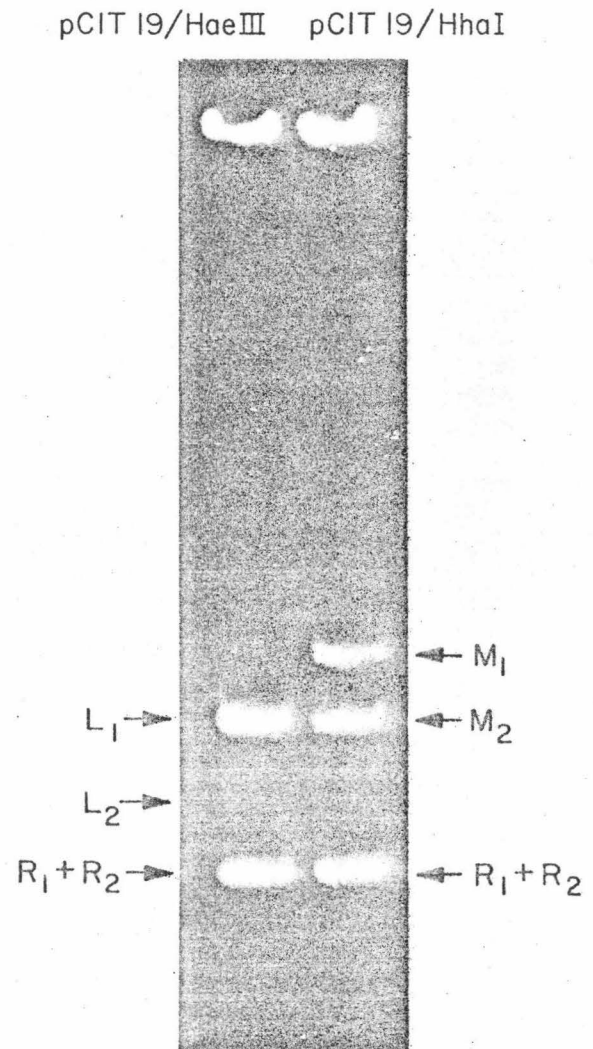


Figure 8. Hybridization of 3 H-5S rRNA to pCIT19 Restriction Enzyme Fragments

Approximately 1 μ g of pCIT19 DNA was digested with either Hae III or Hha I and subjected to electrophoresis on a 1% agarose gel at 80 V for 195 min. Fragments were transferred to nitrocellulose filters and hybridized with 3 H 5S rRNA as described in Experimental Procedures. The tops of the gel slots, which were marked with radioactive ink, appear bright on the photograph.

that shorter molecules are generated by a recombination event that causes excision of an integral number of copies of the repeat unit. We wished to determine whether the excision event is affected by the *recA* character of the bacterial cell and/or by chloramphenicol amplification. pCIT9 DNA was extracted and used to transform HB101, a *recA* strain of *E. coli*, to colicin resistance. Four transformed colonies, designated strains HB101-9-1, -9-2, -9-3 and -9-4, were isolated. The closed circular DNA extracted from these colonies had maximum lengths of about 8.0 ± 0.4 , 9.0 ± 0.3 , 19.0 ± 0.8 and 9.0 ± 0.3 kb, respectively.

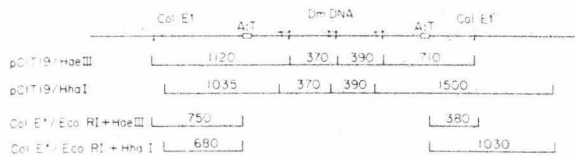


Figure 9. Restriction Enzyme Map of Coding Strand of *Drosophila* Insert in pCIT19

The positions of the 5S genes in pCIT19 are mapped relative to the adjacent Hae III and Hha I sites in Col E1. 5' → 3' = ferritin-labeled 5S RNA; ↓ = Hae III site; ξ = Hha I site. The relative positions of the 370 and 390 nucleotide repeats are not determined. The positions of the Hha I joining fragments are tentative, since both hybridize to ³H 5S rRNA.

An overnight culture of each subclone grown in the presence of colicin E1 was diluted 1:20 into two separate L broth cultures. Chloramphenicol (CAM) was added to one culture at a cell density of $3-5 \times 10^8$ cells per ml. This and the -CAM culture were grown overnight. Cleared lysates were prepared from each culture. Gel electrophoresis showed that plasmid DNA from the -CAM cultures was nearly all in the form of relaxed circles, as expected from the work of Clewell and Helinski (1969).

Both electron microscopy and gel electrophoresis showed that the predominant circular DNA extracted from -CAM cultures of strain HB101-9-3 had about the same length (19.0 kb) and the same partial denaturation pattern as pCIT9. DNA from the +CAM culture did not have a detectable amount of the full-length plasmid. Many smaller circular molecules were seen, including some of length less than Col E1.

Plasmid DNA extracted from -CAM cultures of strains HB101-9-1, -9-2 and -9-4 had molecular lengths of 8.0, 9.0 and 9.0 kb, respectively. DNA from +CAM cultures of strains HB101-9-1 and -9-2 also had lengths of 8.0 and 9.0 kb, with 5% or fewer of the molecules being shorter. However, about 30% of the circular molecules from +CAM HB101-9-4 were smaller than Col E1.

These results suggest that the excision of *Drosophila* DNA from the many tandemly repeated 5S gene units of pCIT9 is not dependent upon the *recA* character of the cell, but that the process occurs mainly during chloramphenicol amplification. The excision process is much less frequent in plasmids containing a smaller number of 5S repeat units.

Discussion

The plasmid pCIT9 contains 32 tandem repeat units of *Drosophila* 5S genes. Gel electrophoresis studies indicate that most of the repeat units in this plasmid have a length about 370 ntp, with a length heterogeneity of <10 ntp. The plasmid pCIT19 has two complete repeat units with lengths measured

as 370 and 390 ntp by gel electrophoresis. The partial denaturation measurements give a combined average length of 392 ntp for the repeat units in both plasmids, and ferritin mapping gives a length of 389 ntp for the two repeats in pCIT19. The electron microscope techniques are not sufficiently precise to give reliable information about small amounts of heterogeneity. The repeat lengths determined by all methods on either plasmid are the same within experimental error. Proconier and Tartof (1975) report that there are approximately 165 5S genes in the haploid *Drosophila* genome. The 32-34 units in the *Drosophila* inserts we studied therefore constitute about 20% of the total. Our first conclusion is that the average length of the repeat unit in this sample is between 370 and 390 ntp.

On the other hand, Proconier and Tartof (1976) estimate the size of the repeat unit as 290 ntp from hybridization to fragments extracted after gel electrophoresis of an Hae III digest of total *Drosophila* DNA. As noted in the legend to Figure 7, there is a 14% discrepancy between the length standards used by us and by these investigators. On our scale, the repeat length measured by Proconier and Tartof is revised to 331 ntp. The cause of the remainder of the discrepancy is unknown.

Proconier and Tartof (1976) suggest that the *Drosophila* 5S genes are arranged in two clusters, each containing 80 genes. They further suggest that two fragments of length about 23 kb corresponding to these two clusters are released from the genome by Eco RI digestion. If the length of a repeat unit is 370 ntp, then a cluster of 80 genes would have a length of 29.6 kb. In their gel electrophoresis system, the log (molecular length) against mobility curve is highly nonlinear in the 20-30 kb range. We therefore believe that their measurements do not exclude a value as high as 30 kb for the length of the fragments containing the 5S genes.

Gel electrophoresis of digests of pCIT9 gives one broad band for most of the genes, with a limited length heterogeneity of approximately 10 ntp or less. The heterogeneity in this band is much less than that for *Xenopus laevis* 5S genes (Carroll and Brown, 1976a), for which a number of bands covering a range of over 100 ntp are readily observed in a suitable gel electropherogram.

Two repeat units that differ in length by 20 ntp are present in pCIT19. It seems improbable that this length difference is an artifact introduced by the cloning procedure, since heterogeneity does not occur to this extent in pCIT9; however, this possibility is not excluded. Further studies are needed to explore the full range of spacer heterogeneity in the 5S genes of *Drosophila melanogaster*.

The 5S DNA of *Drosophila* has the same buoyant density as main band DNA (Proconier and Tartof,



Figure 10. Salivary Gland Chromosomes of *Drosophila* Hybridized to pCIT19 cRNA after Autoradiography

1976); it therefore must have the same G + C content of approximately 40% (Ritossa, Atwood and Spiegelman, 1966; Hastings and Kirby, 1966). The G + C content of the genes themselves is known from the sequence to be 57% (Benhamou and Jordan, 1976). The gene (0.12 kb) and spacer (0.25 kb) lengths then imply that the G + C content of the spacer is 32%. This high A + T content for the spacer is consistent with the partial denaturation mapping observations. The observation that the duplex regions in both partially denatured plasmids are about gene size also supports this model. Thus *Drosophila* 5S DNA resembles the 5S DNA of *Xenopus* (Brown and Sugimoto, 1973) and some of the 5S DNA of Chinese hamster (Stambrook, 1971) in having G + C-rich genes separated by A + T-rich spacers.

The hybridization of cRNA made from pCIT9 to band 56F, which is known from both genetic and in situ hybridization data to be the locus of the 5S RNA genes, is further proof that the DNA of the plasmid is indeed *Drosophila* 5S DNA. The absence of any detectable labeling in the nucleolus, the site of the 18S and 28S rRNA genes, or at any other site on the chromosomes shows that the 5S DNA spacer does not have a significant length of sequences in common with those of the larger rDNA genes or with other sequences in the genome.

Bacteria grown in the presence of chloramphenicol and containing some pCIT9 full-length plasmids also contain some smaller plasmids that have a full-length Col E1 vector but a smaller number of 5S repeat units. Still smaller circular molecules containing only *Drosophila* 5S DNA tandem repeats in amounts from one to many units are also present. These observations suggest that integral numbers of 5S repeats can form circles by excision from the parental plasmid. However, subcloned bacteria never give rise upon growth to circular DNA molecules containing a greater number of repeats than that present in pCIT9. Thus there seems to be no corresponding process for increasing the number of genes in the plasmid. It may also be noted that plasmids containing smaller numbers of repeat units are relatively stable.

The excision process is not *recA*-dependent. On the other hand, excision is greatly reduced, although not eliminated, by omission of chloramphenicol treatment. In experiments in which chloramphenicol amplification was not used, other tandemly repeated sequences have been cloned using a different vector (pSC101) without the excision complication encountered here (Carroll and Brown, 1976b; Wellauer et al., 1976). Thus for inserted repeats of 400 ntp or greater, as in the present case, chloramphenicol amplification of the Col E1 vector may be the principal cause of plasmid

size changes during growth. It should be noted, however, that some highly repeated *Drosophila* satellite sequences are unstable due to segregation even in hybrid molecules containing pSC101 when grown in both *recA* and *recBC* hosts (Brutlag et al., 1977).

Experimental Procedures

Construction of Hybrid Col E1-*Drosophila* DNA

Hybrid Col E1-*Drosophila* DNA was constructed by the procedures of Clarke and Carbon (1976) using the poly(dA:dT) connector method (Jackson, Symons and Berg, 1972; Lobban and Kaiser, 1973). High molecular weight *Drosophila* DNA, prepared from *Drosophila melanogaster* Oregon R pupae by the method of Manning, Schmid, and Davidson (1975), was sheared by stirring to a mean length of 15 kb. 3' single-strand ends were exposed by digestion with lambda exonuclease. Poly(dA) extensions of average length 133 nucleotides were added to the 3' ends using calf thymus deoxynucleotidyl terminal transferase in the presence of dATP. Similarly, Col E1 DNA was digested to completion with Eco RI restriction endonuclease, treated with lambda exonuclease and then with terminal transferase and dTTP to add poly(dT) tails of average length 154 nucleotides to the 3' ends. The two tailed DNAs were annealed on an equimolar basis. Electron microscopy of the annealed mixture revealed approximately 11% circular hybrid DNA molecules in the final preparation (200 molecules scored).

Construction of Col E1-*Drosophila* Hybrid Plasmid Colony Bank

The method of Clarke and Carbon (1976) was used with slight modification to prepare a Col E1-*Drosophila* hybrid plasmid colony bank. Prior to transformation, recipient *E. coli* cells (strain JA199 *hsdR⁻ hsdM⁻ lacY⁻ leuB6 ΔtrpE5/F⁺*) were grown in Luria broth (L broth) in the presence of 0.05% deoxycholate to reduce the frequency in the culture of cells resistant to colicin (Davies and Reeves, 1975). After being washed with an equal volume of L broth, 6×10^{10} cells in a final volume of 7.5 ml were transformed as previously described with 25 μg of annealed hybrid DNA, grown for 1 hr in L broth, then plated on L agar plates in the presence of colicin E1. A total of 227,000 transformed clones were obtained with a 5% background of colicin resistance in a mock-infected culture, corresponding to an efficiency of transformation of 8600 clones per μg DNA. Of 20 transformed clones picked at random, 18 were resistant to colicin E1 and sensitive to colicin E2—that is, approximately 90% of the clones harbored a Col E1 plasmid. Furthermore, screening of hybrid plasmid DNA molecules isolated by the Triton lysis method (Clarke and Carbon, 1976) from eight of the clones revealed that all eight contained plasmids larger than Col E1.

5S rRNA Preparation

D. melanogaster tissue culture cells (Schneider's line 2) were grown, labeled with ³H-uridine and lysed according to the procedure of McKenzie, Henikoff and Meselson (1975), except that 0.004 M dithiothreitol (DTT) were used in place of heparin in the lysis buffer (TMK buffer). To prepare 5S rRNA, the postnuclear supernatant was centrifuged for 3 hr at 84,000 g (30K rpm in an SW50.1 rotor) at 4°C to pellet the ribosomes. The ribosomal pellet was resuspended in TMK, 0.25 M sucrose and recentrifuged through TMK, 1.0 M sucrose at 32K rpm for 2.5 hr at 4°C. The ribosomal pellet was resuspended in a minimal volume of 0.1 M NaAc, 0.5 M NaCl, 0.005 M MgCl₂, 0.004 M DTT, 0.006 M mercaptoethanol (pH 5.0). SDS was added to 1%, and the RNA was extracted with a mixture of phenol:chloroform:isoamyl alcohol (50:48:2). High molecular weight RNA was precipitated by stirring overnight at 4°C in 1.5 M NaCl and then removed by centrifugation. Ethanol was added to the remaining solution, and the precipitate was collected. This precipitate was resuspended in 0.1 M NaCl, 0.005

M EDTA (pH 8.4), and loaded on a 95×2 cm Sephadex G-100 column to separate the 5S rRNA from remaining rRNA and 4S rRNA. The specific activity of this RNA was $1.5\text{--}2 \times 10^6$ cpm/ μ g.

To prepare cold 5S rRNA for ferritin labeling, *Drosophila* larvae were ground with mortar and pestle in TMK, 0.35 M sucrose, 1% Triton X-100. The homogenate was filtered through four layers of sterile cheesecloth. 5S rRNA was then prepared as described above.

Screening of Clones for the Presence of Plasmids Containing *Drosophila* 5S Genes

Approximately 9000 colonies from the original collection of transformed clones were transferred in 48 colony grid arrays (Clarke and Carbon, 1976) to L agar plates overlaid with 0.8% agar containing colicin E1. After growth at 37°C, these colonies were transferred to Millipore filters using a block containing 48 needles in the same grid arrangement. By shifting the grid pattern slightly, four transfers could be made onto a single filter so that each filter carried 192 colonies. The Millipore filters were placed on L agar plates and incubated overnight at 37°C. Colonies were then screened by a modification of the colony filter hybridization technique (Grunstein and Hogness, 1975) using film sensitization (Lasky and Mills, 1975) to detect clones containing *Drosophila* 5S genes by scintillation autoradiography (Bonner and Laskey, 1974). Hybridizations were performed under the following conditions: 50% formamide, $2 \times$ SSC, 1 mM EDTA, 0.1% SDS, 43°C, 36 hr. ^3H -labeled 5S rRNA was present at 0.1 μ g/ml with an excess of unlabeled *Drosophila* rRNA (16.7 μ g/ml).

Plasmid DNA Isolation

Plasmid DNA from the clones was isolated by a slight modification of the procedure of Clarke and Carbon (1976). The RNAase treatment was omitted. The lysis supernatant from the low-speed centrifugation was adjusted to a density of 1.55 g/cc with CsCl and allowed to sit overnight at 4°C. A protein layer which formed at the top of the solution was removed. The plasmid DNA was then purified by ethidium bromide-CsCl density gradient centrifugation at 40K rpm for 48 hr in a 60Ti rotor as described previously (Clarke and Carbon, 1976).

Partial Denaturation

Partial denaturation was carried out by the alkaline formaldehyde procedure of Inman and Schnöds (1970) as modified by Carroll and Brown (1976a), with the following additional minor changes. The denaturation reaction was stopped by adding a portion of the reaction mixture to an equal volume of cold 10% formaldehyde, 0.2 M EDTA (pH 8.0). The sample was diluted with 3 vol of cold 50% formamide, 0.1 M Tris, 0.01 M EDTA (pH 8.5), then briefly dialyzed against this same mixture at 4°C before being spread for electron microscopy by the formamide modification of the Kleinschmidt technique (Davis, Simon and Davison, 1971). Single-stranded and duplex (RFII) circular ϕ X174 DNA were included in the denaturation mixture as length standards of 5370 ± 20 bases or base pairs (Barrell, Air and Hutchison, 1976). At long denaturation times when the internal double-stranded standard was considerably denatured, additional duplex ϕ X174 DNA was added just before spreading. A Philips 300 electron microscope was used to take 35 mm photomicrographs, from which molecule lengths were measured by overhead projection using a Hewlett-Packard 9280A calculator and a Hewlett-Packard digitizer.

Restriction Enzyme Digestions

Restriction enzyme digestions were routinely carried out overnight (12–16 hr) at 37°C in the assay buffer recommended by the supplier. Commercial enzymes were purchased from both New England Biolabs and Bethesda Research Co.

Gel Electrophoresis

Slab gels with dimensions $18 \times 14 \times 0.4$ cm were used. The buffer

in both gel and reservoirs, except where noted, was E buffer [0.04 M Tris, 0.005 M Na acetate, 0.001 M Na_2EDTA (pH 7.4)]. Gels were run at constant voltage for the indicated time in holders equipped with cooling fans to prevent overheating during extended runs at high voltage. In most cases, the buffer was recirculated between the two reservoirs during the run.

To achieve high resolution, acrylamide linear gradient gels were used. Acrylamide gradients were formed by the method usually used for sucrose gradients. The solution from the gradient mixer was pumped, low concentration first, into the bottom of the gel mold with a slow steady flow to minimize turbulence. In some trials, a 0–5% sucrose gradient was present to stabilize further the acrylamide gradient, but this precaution seemed unnecessary. A 1 cm layer of water, placed in the bottom of the mold before introduction of the acrylamide gradient, was carried above the acrylamide during the filling of the mold, and when removed, left a flat even surface at the top of the gel. To insure that polymerization proceeded from the top to the bottom of the gel, the bis:acrylamide ratio was different in the two solutions (bis:acrylamide high concentration, 1:59; low concentration, 1:29). For each 60 ml of solution, 100 μ l of 10% ammonium persulfate and 20 μ l TEMED were added. Sample wells were formed in 0.5% agarose (Marine Colloids, Inc.) poured over the acrylamide gel. Less than 1 cm of agarose separated the bottom of the sample wells from the start of the acrylamide gel.

Identification of Restriction Enzyme Fragments Containing 5S rRNA Genes

Elutions from agarose gels onto nitrocellulose filters were performed by the method of Southern (1975). Hybridization conditions were identical to those in the colony screening procedure with the following exceptions: the concentration of ^3H -labeled 5S rRNA was 0.5 μ g/ml, *Drosophila* rRNA was omitted and *E. coli* 23S rRNA was added to 2 μ g/ml.

cRNA Preparation

^3H -labeled cRNA was synthesized from supercoiled plasmid DNA by the procedure of Wensink et al. (1974) with the following modifications. 2.5 μ g of plasmid DNA were incubated with 50 μ Ci each of ^3H -ATP, -CTP, -GTP and -UTP (17, 24.7, 12, and 16 Ci/mmol, respectively) and approximately 5 units of *E. coli* RNA polymerase (Boehringer-Mannheim; 526 units per mg) for 75 min at 37°C.

Heteroduplex Formation and Ferritin Labeling

5S rRNA was conjugated to biotin, using a cytochrome c bridge instead of the pentane diamine bridge described by Angerer et al. (1976). Details of this preparation will be described separately (A. Sodja, personal communication).

Open circular Col E1 DNA and pCIT19, prepared from the closed circular forms by X-ray nicking and EtBr-CsCl centrifugation, were mixed, denatured in 0.2 M NaOH, 8 mM EDTA for 10 min at room temperature and neutralized with 2.5 M Tris-HCl. 5S rRNA-cytochrome c-biotin and 40 μ l of 3 times-recrystallized formamide were added to give a final volume of 100 μ l. Concentrations in this solution were: Col E1 DNA, 2 μ g/ml; pCIT19 DNA, 0.5 μ g/ml; 5S rRNA-cytochrome c-biotin 5 μ g/ml. This solution was dialyzed for about 1 hr at 52°C against 40% formamide 0.3 M NaCl, 0.1 M Tris, 0.001 M Na_2EDTA (pH 8.0) to allow renaturation to occur. Subsequent manipulations were performed as described by Angerer et al. (1976).

In Situ Hybridization

Salivary chromosome squashes from late third instar larvae of *Drosophila melanogaster* were prepared on glass slides following the method of Atherton and Gall (1972). Larvae used were *giant* mutant heterozygotes of genotype gt^w/gt^{137} (Kaufman, 1971, 1973). After ribonuclease treatment of the squash preparations (100 μ g/ml, $2 \times$ SSC, 37°C, 1 hr), DNA was denatured by incubat-

ing the slides at 65°C for 2.5 hr in 0.1 × SSC, 90% formamide (Alonso, 1973). The hybridization solution containing 50% twice-recrystallized formamide, 6 × SSC, 40 µg/ml *E. coli* ribosomal RNA and pCIT9 ³H-labeled cRNA (5 × 10⁷ cpm/µg; estimated *Drosophila* sequence cRNA concentration 0.02 µg/ml) was heated at 85°C for 5 min; then 50 µl of the solution were layered over each squash. The slides were placed in a closed container over 6 × SSC, 50% formamide, incubated at 52°C for 40 hr and again treated with ribonuclease (20 µg/ml, 2 × SSC, 37°C, 30 min). The preparations were thoroughly rinsed and dehydrated in ethanol. Autoradiography was performed with Kodak NTB-2 liquid emulsion for 21 days at 4°C. The developed slides were stained with Giemsa blood stain (Harleco).

Retransformation into Strain HB101

Transformation of strain HB101 (*thr leu pro hsdM⁻ hsdR⁻ recA supE*) with purified pCIT9 DNA was performed by the method of Wensink et al. (1974) with the following modifications. Cells were diluted with 1 ml of L broth and grown for 30 min before the addition of 10 µl colicin E1. 2.5 ml of soft agar were then added to the cells, and the mixture was poured onto LB plates plus 0.2% deoxycholate. 5 ml cultures of purified transformants were grown with or without CAM treatment, and cleared lysates were prepared by the method of Clewell and Helinski (1969), except that chromosomal DNA was removed by centrifugation for 10 min at 30 psi in a Beckman Airfuge (approximately 135,000 g). The resulting supernatant solutions were extracted twice with phenol:chloroform:isoamyl alcohol (50:48:2) and 3 times with ether. DNA was precipitated with 1 vol of isopropanol and resuspended in an appropriate solution for gel electrophoresis or electron microscopy.

Containment Conditions

All plasmids containing *Drosophila* segments were propagated under P2 physical containment as defined and recommended for this class of experiments by the Guidelines for Research Involving Recombinant DNA Molecules issued in June, 1976 by the NIH. Before issuance of these guidelines, an F⁺ host (strain JA199) was in use. Recombinant DNA molecules have since then been used to transform strain HB101 to give a host-vector system defined as EK1 under the Guidelines. The F⁺ strains carrying recombinant plasmids have been destroyed.

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Chapter 2

Sequence Arrangement of tRNA Genes
on a Fragment of Drosophila melanogaster
DNA Cloned in E. coli

Pauline H. Yen, Ann Sodja, Maurice Cohen, Jr.,
Susan E. Conrad,* Madeline Wu and Norman
Davidson

Department of Chemistry and
*Division of Biology
California Institute of Technology
Pasadena, California 91125

Christine Ilgen†

Section of Biochemistry and Molecular Biology
Department of Biological Sciences
University of California
Santa Barbara, California 93106

Summary

A plasmid with the vector Col E1 attached to an insert of *Drosophila melanogaster* DNA carrying four tRNA genes has been cloned in *E. coli*. Some features of the sequence arrangement and the positions of the tRNA genes have been determined by electron microscopic methods and by restriction endonuclease mapping. tRNA genes were mapped at 1.4, 4.7, 5.9 and 8.6 kb from one of the *Drosophila*/Col E1 junctions in the *Drosophila* insert of total length 9.34 kb. There are several secondary structure features consisting of inverted repeat sequences of length about 70–100 nucleotide pairs, some with and some without intervening loops, irregularly distributed on the insert. Cross-hybridization of tRNAs isolated by hybridization to separated restriction fragments indicate that the tRNA genes at 4.7, 5.9 and 8.6 kb are identical and differ from the one at 1.4 kb. Thus the positions of the genes, of the secondary structure features and of the restriction endonuclease sites all indicate that the spacers between the genes are not identical tandem repeats. In situ hybridization with cRNA transcribed from the plasmid showed localization at region 42A of chromosome 2R.

Introduction

Relatively little is known about the sequence organization of tRNA genes in eucaryotes. There are 600–800 tRNA genes in the haploid genome of *Drosophila melanogaster* (Ritossa, Atwood and Spiegelman, 1966; Tartof and Perry, 1970; Weber and Berger, 1976). On RPC-5 columns, *Drosophila* tRNA can be resolved into 56 major and 33 minor peaks (White et al., 1973). From an analysis of the kinetics of RNA:DNA hybridization on membrane filters, Weber and Berger (1976) estimate that *Dro-*

sophila tRNA is made up of about 59 families of kinetically different sequences. All these data suggest that there is an average of about 10 genes for each tRNA sequence. In situ hybridization experiments, total unfractionated tRNA hybridized to a multitude of sites on the *Drosophila* polytene chromosomes (Steffensen and Wimber, 1971). A few purified tRNAs have been shown to hybridize to single small chromosomal regions, thus indicating that the genes of each of these families are clustered (Grigliatti et al., 1974; Delaney et al., 1976; O. Uhlenbeck and R. Elder, personal communication).

We have initiated a study of the fine structure of the tRNA gene arrangement in *Drosophila melanogaster* DNA using recombinant DNA techniques. This paper reports the properties of one plasmid with a *Drosophila* insert containing four tRNA genes.

The only other higher eucaryote for which there is any information about the organization of tRNA genes is *Xenopus laevis* (Clarkson, Birnstiel and Purdom, 1973; Clarkson and Kurer, 1976). The properties observed in this case will be compared with those of *Drosophila* in the Discussion.

Results

Isolation and General Characterization of pCIT12 DNA

The preparation of recombinant DNA molecules containing segments of *Drosophila* DNA joined to the plasmid Col E1 by the AT tailing method (Jackson, Symons and Berg, 1972; Lobban and Kaiser, 1973) has been previously described (Hershey et al., 1977). The colicin-sensitive bacterial strain JA199 was transformed with this preparation of DNA. Clones containing *Drosophila* tRNA genes were selected by colony filter hybridization (Grunstein and Hogness, 1975) using a ³H-tRNA probe. Of the 9000 colonies screened, four gave a positive hybridization signal. Membrane filter hybridization experiments performed with plasmid DNA extracted from these cultures indicated that only one plasmid, designated pCIT12, contained more than one tRNA gene.

The first preparations of pCIT12 DNA also contained a second plasmid consisting of Col E1 and a short *Drosophila* insert which is not related to that of pCIT12. In later preparations, this contaminant was removed by subcloning the bacteria. The lengths of pCIT12, the contaminant plasmid and Col E1 were measured by electron microscopy to be 16.00 ± 0.32 , 7.50 ± 0.25 and 6.66 ± 0.21 kb, respectively [relative to open circular ϕ X174 RF DNA, taken as 5.37 kb (Barrell, Air and Hutchison, 1976)]. Thus the length of the *Drosophila* insert in pCIT12 is 9.34 ± 0.4 kb, while that of the insert in

† Present address: Department of Biochemical Sciences, Princeton University, Princeton, New Jersey 08540.

the contaminant plasmid is 0.84 kb. Heteroduplexes of pCIT12 with Col E1 DNA and with the contaminant plasmid were prepared; a schematic representation of these structures is depicted in Figure 1. Representative electron micrographs are shown in Figure 2. In the Col E1 heteroduplex, there is a duplex circle due to the full Col E1 genome and a single-stranded loop of length 9.34 kb due to the *Drosophila* insert; these two circles are joined by a duplex stem of length 80 ntp (nucleotide pairs) due to the A:T junctions of Col E1 with *Drosophila* DNA. In the heteroduplex with the contaminant plasmid, there is the same Col E1 duplex circle, two A:T stems coming from the same point on the circle, and separate single-stranded loops of length 9.34 kb and 0.84 kb.

There are several secondary structure features in the single strand of the *Drosophila* insert of pCIT12. These features, which have been very useful as reference markers in the mapping studies, are depicted schematically in Figure 1 and are illustrated in the several electron micrographs in Figure 2. One of the two most commonly observed structural features, a loop of length 1.0 kb, bounded by short inverted repeat sequences *a* and *a'*, lies approximately in the middle of the insert. At a reproducible position within the loop, there is a small foldback structure, which is labeled *f* in Figures 1 and 2. The other frequently observed feature is a foldback structure denoted *b₁b'* with no observable loop in

cytochrome spreads. As shown in Figure 6, we have assigned coordinates to the *Drosophila* insert, with the origin at the beginning of the A:T stem, and 9.34 kb at the end. The foldback structure *b₁b'* lies at about 9 kb. The *aa'* and *b₁b'* structures are observed in about 90% and 95% of all molecules, respectively. There is another feature, observed in about 10% of all molecules, consisting of an inverted repeat stem *cc'* separated by a rather long loop (3.4 kb). From structures such as that shown in Figure 2d, it is clear that the sequence, *c*, lies slightly to the left of the sequence *a*. A fourth inverted repeat, which we denote as *b₂b'*, is observed at the rather low frequency of 2–5%. The structures *b₂b'* and *b₁b'* were never observed in the same molecule. The best quantitative interpretation of the mapping data is that these structures involve the same sequence *b'* joined to either *b₂* or *b₁*; an alternative interpretation, which is within the experimental error of the measurements, is that *b₂* is the same as *b'*, so that the low frequency structural feature is *b₁b₂*. All the inverted repeat stems, *aa'*, the two *bb'* and *cc'* have lengths of about 70–100 base pairs. The structure *f* has an estimated duplex length of 40–50 base pairs.

Mapping tRNA Genes on pCIT12

The positions of tRNA genes on pCIT12 were mapped by the ferritin-avidin, tRNA-cytochrome *c*-biotin labeling technique (Angerer et al., 1976; Hershey et al., 1977; A. Sodja, manuscript in preparation). Total pupal *Drosophila* tRNA was covalently coupled to the ligand biotin via a cytochrome *c* bridge. This tRNA was then hybridized to the plasmid heteroduplexes, and the positions of the tRNA hybrids on the single-strand of *Drosophila* DNA were identified with ferritin-avidin labels. A typical electron micrograph showing two tRNA genes labeled is shown in Figure 3a. The histogram of tRNA gene positions determined by these experiments, shown in Figure 4, indicates that there are three tRNA genes, denoted tRNA-1, tRNA-2 and tRNA-4, at coordinate positions 1.44 ± 0.1 kb ($N = 61$), 4.72 ± 0.19 kb ($N = 45$) and 8.57 ± 0.13 kb ($N = 101$) along the *Drosophila* insert. The average labeling efficiency was about 40% per site. Molecules with all three possible combinations of two labels were seen; thus the coding sequences of the three tRNA genes are on the same strand of DNA.

The restriction enzyme mapping experiments described below indicate that there is a fourth tRNA gene rather close to the inverted repeat stem *a'*. This gene was not labeled in the ferritin mapping experiments. One possible reason for this apparent failure of the ferritin labeling method is degradation of the particular tRNA by the chemical modification procedures involved in attaching cytochrome *c* and biotin. Another is that the relative

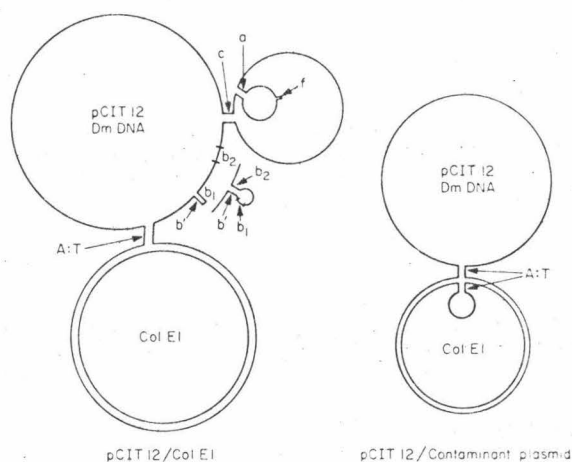


Figure 1. Schematic Representation of Heteroduplexes of pCIT12 with Col E1 and with the Contaminant Plasmid

The secondary structure features on the *Drosophila* insert of pCIT12 are indicated for the former case, but not for the latter. It is to be understood that sequences *a* and *c* are paired to *a'* and *c'* (not indicated by letters), respectively. The positions of the inverted repeat sequence in the pCIT12 *Drosophila* in units of kilobases (kb) from the counterclockwise junction of the A:T stem with the Col E1 duplex circle are as follows: (A:T) 0.00–0.07, 9.29–9.34; (*aa'*) 4.85–4.92, 5.92–5.99; (*b₁b'*) 8.91–9.00, 9.00–9.10; (*b₂b'*) 8.54–8.62, 9.01–9.10; (*cc'*) 4.91–5.01, 8.41–8.50. The feature *f* is located about one third the *aa'* loop length from *aa'*. Standard deviations for the measurements are all about ± 0.15 kb.

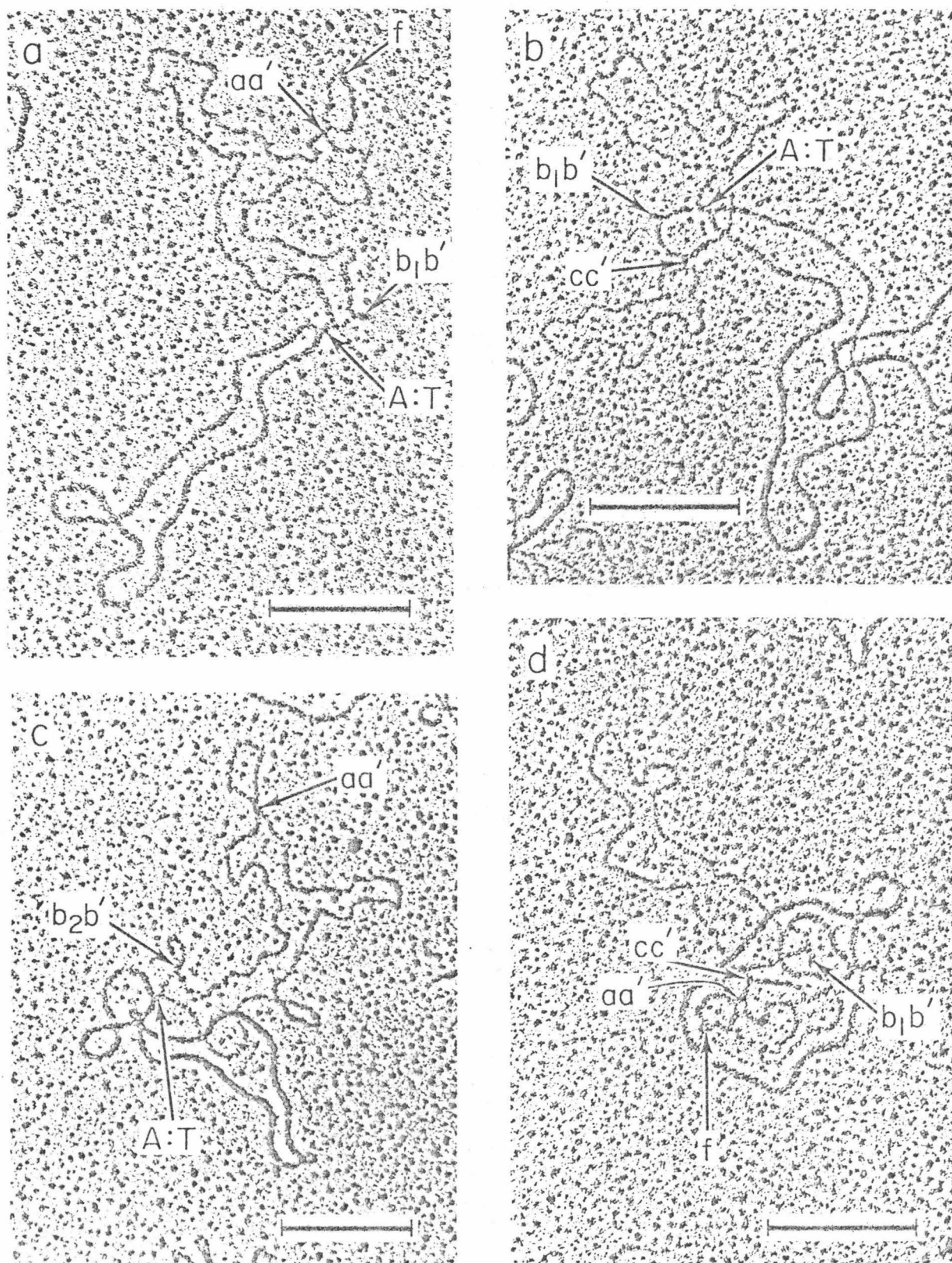


Figure 2. Electron Micrographs of pCIT12/Col E1 Heteroduplexes Showing Several Secondary Structure Features
Scale bars = 1 kb.

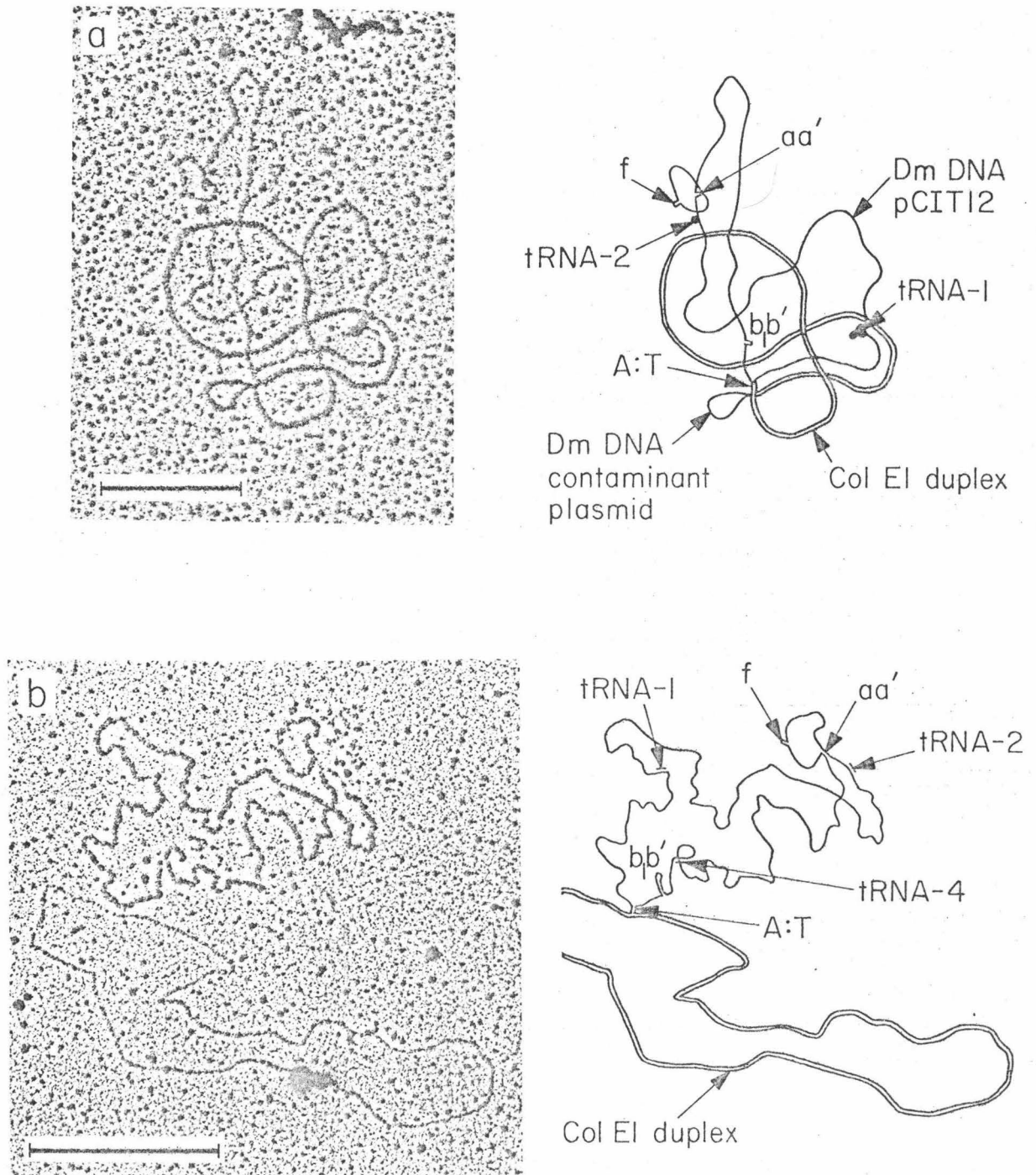


Figure 3. Electron Microscopic Mapping of the tRNA Gene Positions on the *Drosophila* Insert of pCIT12

(a) A ferritin-avidin labeling experiment with a pCIT12/contaminant plasmid heteroduplex mounted by the conventional cytochrome c-formamide technique. tRNA-1 and tRNA-2 are labeled in this experiment.

(b) A gene 32-ethidium bromide spread of a pCIT12/Col E1 heteroduplex, with tRNA-1, -2 and -4 hybridized and identified. In this mounting method; DNA:DNA duplex and tRNA:DNA hybrid regions are thin; single-strand DNA regions are thick. Scale bars = 1 kb.

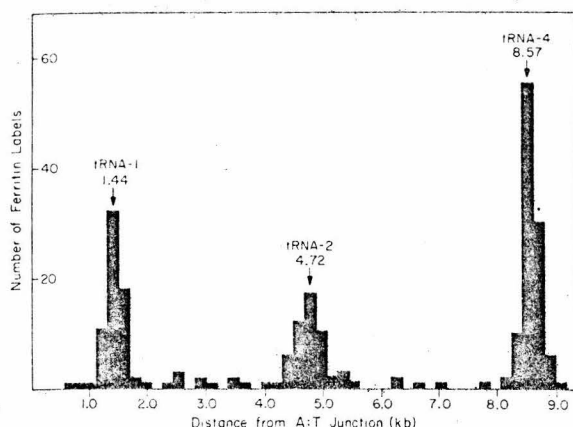


Figure 4. Histogram of Ferritin Label Positions on the pCIT12 *Drosophila* Insert for the tRNA Ferritin-Avidin Mapping Experiments

Label positions are measured relative to the counterclockwise junction of the A:T stem with the Col E1 duplex as in Figure 1.

abundance of this tRNA in pupal tRNA is too low for efficient labeling, whereas it is sufficiently high to give hybridization in the Schneider cell tRNA sample used for the restriction enzyme hybridization experiments. We have therefore made a brief electron microscope mapping study, using Schneider cell tRNA, by the T4 gene 32-ethidium bromide procedure (Wu and Davidson, 1975) which does not require chemical modification of the tRNA. In this technique, unlabeled tRNA is hybridized to the single-stranded DNA part of the heteroduplexes, and the single-stranded portions of the resulting molecules are stained with T4 gene 32 protein. When mounted for electron microscopy, the single-stranded regions are thick and the duplex regions are quite thin. As reported previously (Wu and Davidson, 1975), it is possible, but difficult, to recognize short duplex regions such as a tRNA:DNA hybrid (~80 ntp). In the present instance, however, we knew the position of the fourth gene from the restriction enzyme analysis. We did observe labeling at a high efficiency at the positions of the three tRNA genes identified by the ferritin studies; Figure 3b shows a gene 32 spread in which all three of these gene positions show short tRNA:DNA hybrid regions. However, careful examination of the region around the fourth gene did not reveal any significant number of short duplex segments.

Restriction Endonuclease Mapping

To verify the electron microscope mapping data by an independent method and to investigate the relations of one tRNA gene to another, we have mapped a number of restriction endonuclease sites on the plasmid. Several enzymes recognizing hexanucleotide sequences and one (Hae III) recogniz-

ing a tetranucleotide sequence have been studied. Col E1 itself has one Eco RI site which was destroyed during the construction of the recombinant plasmids. Col E1 has no sites for Hind III, Bam I, Sal I or Hpa I (W. Brown, personal communication). There is one site for Sma I which we find by double digestion to be located 1.15 kb from the Eco RI site. pCIT12 was not cleaved by Bam I and Sal I. Gel electropherograms after digestions with Hind III, Eco RI and Hpa I, Hpa I plus Eco RI, and Hpa I plus Hind III are shown in Figure 5. Fragment sizes from some single and double enzyme digests are given in Table 1.

The final restriction endonuclease map is shown in Figure 6. It was constructed by a combination of electron microscopic studies, partial digestions and double digestions. There is no Hind III, Eco RI or Hpa I site in the Col E1 region of pCIT12, and in each digest, only the largest fragment (A) is larger than Col E1. This fragment must therefore contain the intact Col E1 part of pCIT12. Heteroduplexes of Col E1 with an enzyme digest of the plasmid were prepared. In heteroduplexes of fragment A with Col E1 (Figure 7), there is a duplex circle of Col E1 sequences, a short A:T stem leaving the duplex circle and two single-stranded arms due to those parts of the *Drosophila* insert remaining in the A fragment. The two arms can be distinguished because one contains the secondary structure feature b,b'. Thus for each enzyme, the two restriction sites proximal to the A:T stem were mapped. The Hpa I-B fragment when mounted for electron microscopy as a single strand contains the aa' secondary structure feature (Figure 7d). The lengths of the two arms extending from a and a' provide coordinates for two sites; there is no ambiguity as to the orientation because there is only one way for these lengths to fit in with the previously mapped positions at the ends of the A fragment. The sizes of the remaining gaps require the unambiguous assignment of fragment C as indicated in Figure 6. Fragment D of length 0.27 kb must then lie in the interval from 8.7–9.2 kb. The length of fragment Hpa I-E is 0.15 kb, which is approximately the same as the experimental error in the electron microscope measurements; its position could therefore not be determined by these experiments. Further mapping information was obtained by subjecting restriction enzyme fragments eluted from gels to digestion by a second enzyme, followed by gel electrophoretic analysis. These results are presented in Table 2. Some of the fragments are very similar in size; the complete mixed enzyme digest was therefore always present in the same gel to insure correct assignment of the fragments. Table 3 presents the results of partial digestion mapping experiments.

The maps in Figure 6 were obtained by analysis

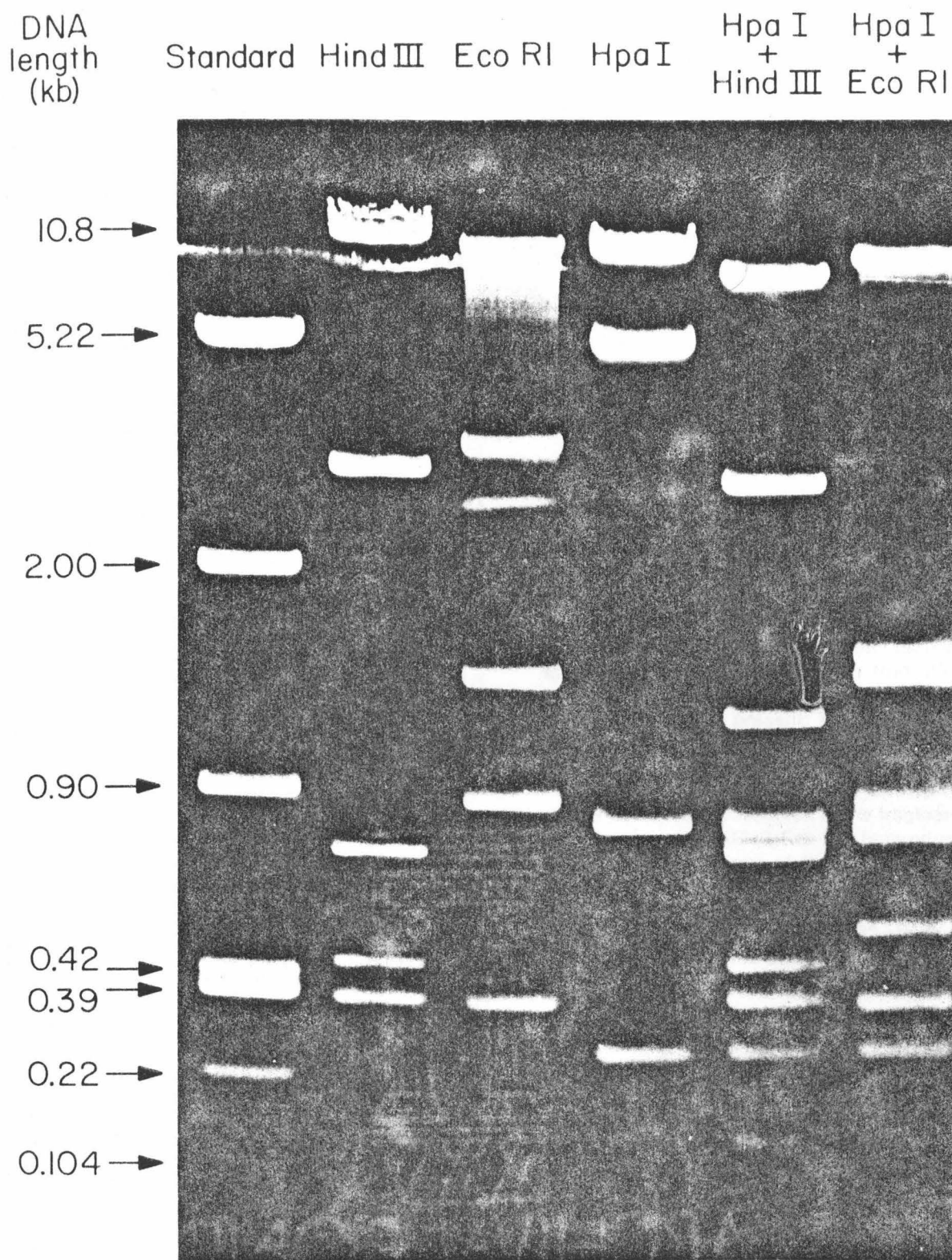


Figure 5. Gel Electrophoresis Patterns in 1% Agarose of Restriction Endonuclease Digests of pCIT12 DNA
Length standards are Hind III fragments of PM2 DNA. The length of Hind III fragment A was determined by electron microscope to be 10.8 kb. The DNA is visualized by ethidium bromide fluorescence.

Table 1. Restriction Fragments of pCIT12 DNA

Restriction Enzyme	Fragment Length (kb)										
	A	B	C	D	E	F	G	H	I	J	K
Hpa I	9.5*	5.00*	0.78	0.27	0.15						
Hind III	10.8*	2.96*	0.69	0.43*	0.35	0.13					
Eco RI	9.4*	3.25*	1.33	0.83*	0.34	0.12					
Sma I	14.0*	1.37	0.28	0.20							
Hpa I + Hind III	7.5	2.81*	1.17*	0.78	0.78	0.68	0.43*	0.37	0.27	0.15	0.13
Hpa I + Eco RI	8.9*	1.47*	1.35	0.85*	0.77	0.75	0.50*	0.36	0.27	0.15	0.12
Hind III + Eco RI†	8.8*	2.90*	1.30	0.49	0.45*	0.42*	0.37	0.36	0.15	0.14	0.12
Sma I + Hind III	6.0	2.95*	2.75*	1.36	0.70	0.42*	0.35	0.28	0.20	0.13	
Sma I + Eco RI	7.2*	3.20*	1.40	1.35	0.84*	0.45*	0.32	0.28	0.20	0.12	

* Fragment containing tRNA gene(s), by Southern elution and hybridization.

† The expected additional small fragment extending between the right ends of Hind III-D and Eco RI-A (Figure 6) was not detected, we estimate that this fragment is <70 ntp in length.

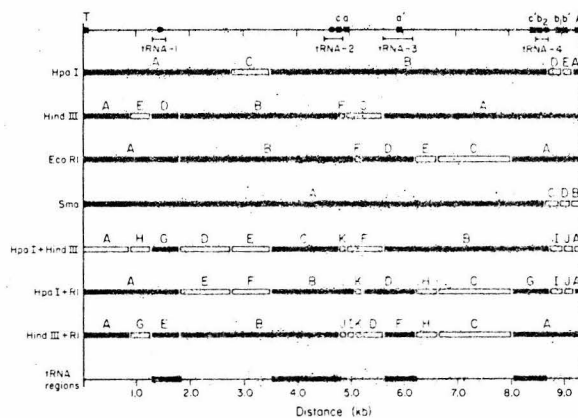


Figure 6. Map of the pCIT12 Plasmid Showing the Locations of the Restriction Sites, the Inverted Repeats and the tRNA Genes. Only the *Drosophila* region is presented. The bars for the genes for tRNA-1, -2, and -4 represent the standard deviation of electron microscope measurement. The tRNA-3 gene has been identified and mapped only by hybridization to restriction fragments. Restriction fragments with tRNA genes are indicated as solid bars. There is one Sma I site 1.15 kb to the right of the map in the Col E1 region not shown here.

of all the data discussed above. Several uncertainties in the final maps should be noted. It is not certain whether it is Hpa I-D or Hpa I-E that is next to Hpa I-B. These two fragments differ in length by only 0.1 kb and are next to fragments larger than 5 kb. In that region of the gel, it is difficult to say whether the partial digestion fragment of length 5.2 kb consisted of Hpa I-B plus Hpa I-D or Hpa I-E. The accuracy of length measurements by electron microscopy and by gel electrophoresis is about 0.15 kb and 5% of fragment length, respectively. There are some discrepancies in maps in which data from the two methods were combined. When

one large fragment (for example, Hind III-B) was treated with a second enzyme, the lengths of the small fragments did not always add up to the length of the original fragment. For example, the total length of pCIT12 was measured as 16.0 ± 0.32 kb; the sums of fragment lengths of Hpa I, Hind III and Eco RI digests were 15.7, 15.27 and 15.35 kb, respectively. There is no indication from the band intensities however, that two copies of any of the fragments might be present. The map in Figure 6 was constructed to give a best fit to all the data, assuming that each fragment is present only once.

The positions of Eco RI-B and Eco RI-D were further confirmed by isolation of the fragments and hybridization to pCIT12/Col E1 heteroduplexes. The observed positions of the duplex regions as determined by electron microscopy [by conventional formamide spreading (Davis, Simon and Davidson, 1971) and by the gene 32-ethidium bromide method (Wu and Davidson, 1975)] are consistent with the map in Figure 6.

Restriction Enzyme Mapping of tRNA Gene Positions

The restriction fragments containing tRNA genes were identified as follows. Plasmid DNA was digested with a suitable enzyme. The fragments were separated by gel electrophoresis, denatured, transferred to nitrocellulose filters (Southern, 1975a) and hybridized with ^3H -tRNA. The bands to which ^3H -tRNA hybridized were localized by scintillation autoradiography. The results are shown in Table 1. After digestion with Hpa I plus Eco RI, fragments A, B, D and G (Figure 6) showed positive hybridization signals, although the electron microscope mapping identified genes only in fragments A, B and G. Several other digests gave concordant results, in-

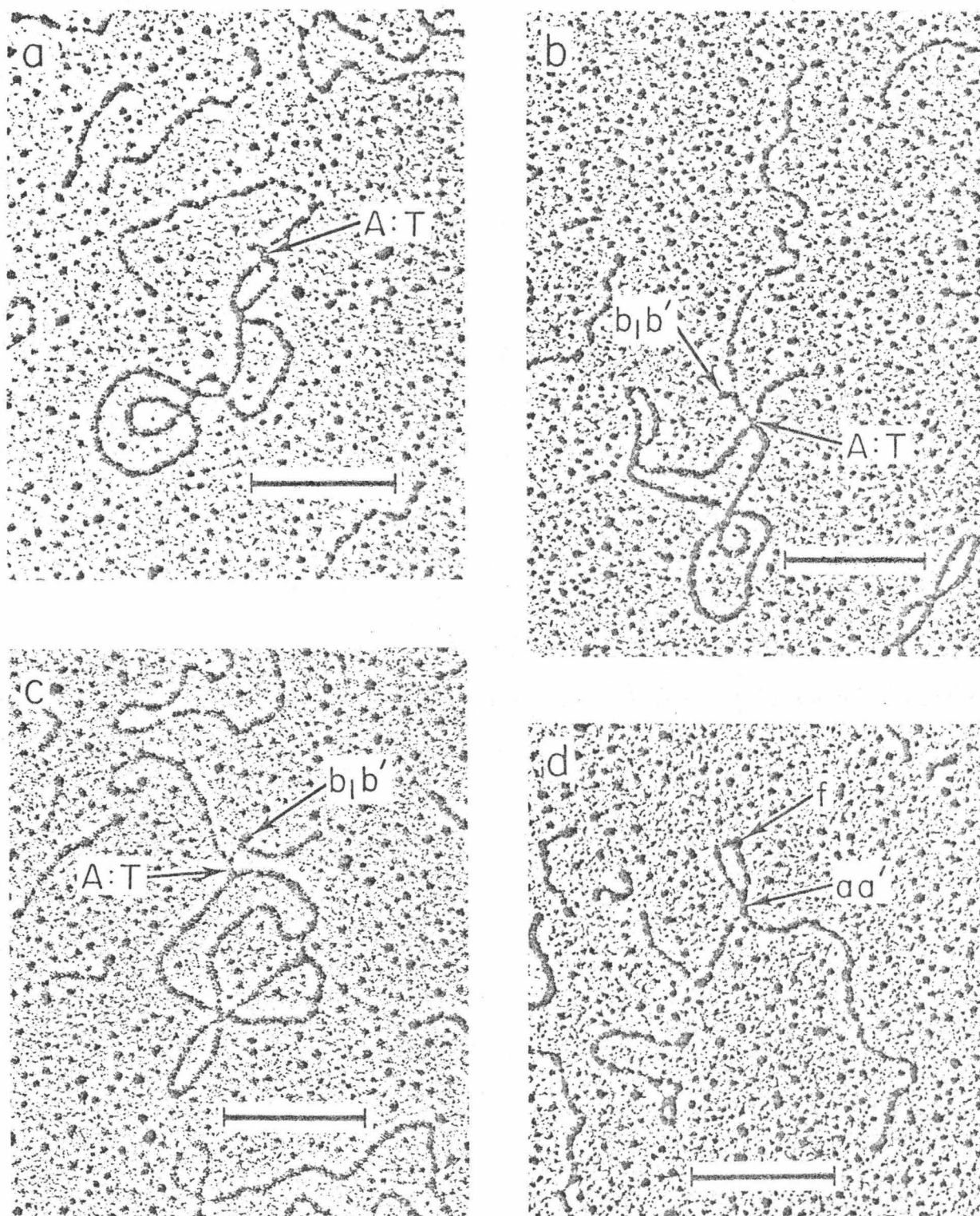


Figure 7. Electron Microscopic Mapping of Some Restriction Endonuclease Sites on pCIT12

Heteroduplexes of Col E1 with restriction endonuclease fragments are shown. (a) Hpa I-A; (b) Hind III-A; (c) Eco RI-A; (d) restriction fragment Hpa I-B with the aa' loop and the feature f. Scale bar = 1 kb.

Table 2. Digestion of Restriction Fragments with a Second Enzyme

Enzyme I Fragment	Enzyme II	Products*
Hind III-A	Hpa I	A, B, I(Hpa I-D), J(Hpa I-E)
Hind III-B	Hpa I	C, D, E(Hpa I-C)
Hpa I-B	Hind III	B, C, F(Hind III-C), K(Hind III-F)
Hpa I-A	Hind III	A, D, H(Hind III-E), G(Hind III-D)
Eco R1-B	Hpa I	B, E, F(Hpa I-C)
Hpa I-A	Eco R1	A, E
Eco R1-B	Hind III	B, I, J(Hind III-F)
Eco R1-A	Hind III	A, G(Hind III-E), E(Hind III-D)
Hind III-A	Eco R1	A, C(Eco R1-C), H(Eco R1-E), F

* The capital letter designations of the fragments are those given in Table 1 for digestion by the two enzymes involved. When the same fragment is produced by digestion with just one of the enzymes, that identification is shown in parentheses; for example, the I fragment from digestion of the Hind III-A fragment by Hpa I is the fragment D from digestion by Hpa I alone (see Figure 6).

dicating that there is a tRNA gene, which we denote as tRNA-3, in the map region from 5.6-6.2 kb (Figure 6). It should be reemphasized that this gene position was not identified by either of the two electron microscope mapping procedures used. A similar experiment was performed with the restriction endonuclease Hae III, even though a map of the sites for this enzyme has not been constructed. In this experiment, four gel bands gave positive hybridization signals, again supporting the idea of at least four tRNA genes.

Are tRNA-1, tRNA-2, tRNA-3 and tRNA-4 Identical?

To study this question, the individual tRNAs were isolated by hybridization to the appropriate restriction fragments. Plasmid DNA was digested with Eco RI plus Hpa I endonucleases. Fragments A, B, D and G (which contain the genes denoted tRNA-1, tRNA-2, tRNA-3 and tRNA-4, respectively) were separated by gel electrophoresis, eluted from the gels and transferred to separate nitrocellulose filters. ³H-tRNA was hybridized to these fragments on the filters. The hybridized tRNA was eluted and counted. Previous experiments on hybridization of ³H-tRNA to total *Drosophila* DNA fixed to nitrocellulose filters gave a Rot_{1/2} value of 8.6×10^{-2} sec-mole/l. In the present experiments, hybridization was carried out to a Rot of 0.734 sec-mole/l, which should give saturation. The total amount of pCIT12 DNA applied to the gel and the specific activity (1.0×10^6 cpm/ μ g) of the tRNA were known. The total amounts of tRNA eluted from the different fragments are shown in Table 4. These amounts were about 20-40% of those ex-

Table 3. Partial Digestion Products of Several Restriction Enzymes

Enzyme	Fragment (kb)	Interpretation
Hpa I	0.44	Hpa I-D + Hpa I-E
Hind III	0.80	Hind III-C + Hind III-F or Hind III-D + Hind III-E
	3.45	Hind III-B + Hind III-D
Eco R1	0.98	Eco R1-D + Eco R1-F
	1.27	Eco R1-D + Eco R1-E
Sma I	0.49	Sma I-C + Sma I-D
	1.60	Sma I-B + Sma I-D
	1.95	Sma I-B + Sma I-D + Sma I-C

pected (2.3×10^5 cpm per fragment), assuming that each fragment contains one tRNA gene and that the transfer of the DNA to the nitrocellulose filter as well as the hybridization are quantitative.

The following cross-hybridization experiment was performed to determine whether or not the tRNA genes on the different fragments are similar in sequence. The tRNAs eluted from Hpa I plus Eco RI fragments A, B, D and G (denoted tRNA-1, tRNA-2, tRNA-3 and tRNA-4, respectively) were hybridized to the individual restriction fragments on nitrocellulose filters with fragment H used as a control. The concentration of each tRNA in a hybridization experiment was $4-14 \times 10^{-2}$ μ g/ml, and hybridization was carried out to a Rot value of $1.6-5.7 \times 10^{-2}$ sec-mole/l. If we assume that the family number for a *Drosophila* tRNA species is 60 (Weber and Berger, 1976) and that all kinetic species in the population are present in approximately equal amounts, and if we accept our experimentally determined Rot_{1/2} value for total *Drosophila* tRNA of 0.086 sec-mole/l, the hybridization conditions correspond to 11-40 times the Rot_{1/2} value of an individual tRNA. The results of these cross-hybridization experiments are shown in Table 4. As explained in the legend to the table, the results are normalized for the relative amounts of DNA of the different fragments retained on the filter. The result in Table 4 clearly indicate that tRNA-2, tRNA-3 and tRNA-4 are kinetically identical (that is, identical or at least sufficiently similar in sequence to cross-hybridize) and are different from tRNA-1.

Gel Electrophoretic Separation of pCIT12 tRNAs

Further information on the relations between the tRNAs that hybridize to the different fragments were obtained by gel electrophoretic fractionation of the tRNAs. Figure 8 shows a scintillation autoradiogram of total *Drosophila* tRNA fractionated on a one-dimensional 10% polyacrylamide gel. Similar fractionations were performed with the tRNA spe-

Table 4. Isolation of pCIT12 tRNAs and Their Cross-Hybridization with the Restriction Fragments

Eco RI + Hpa I Fragments	cpm ($\times 10^{-4}$) Isolated		cpm Rehybridized†			
	First Preparation	Second Preparation	tRNA-1	tRNA-2	tRNA-3	tRNA-4
A	3.1 (29)*	4.8 (47)	1496	748	551	363
B	10.5 (100)	10.4 (100)	174	2990	2673	1989
D	7.3 (70)	8.6 (83)	203	2897	2850	2721
G	6.5 (62)	6.5 (62)	337	2569	2793	2882

* Numbers in parentheses are the relative amounts of counts isolated from each fragment, taking fragment B as 100. These numbers are taken as an indication of the relative molar amounts of DNA fragments on the filters.

† The counts have been corrected for the background binding and normalized for the relative amounts of the different DNA fragments on the filters, estimated as described above.

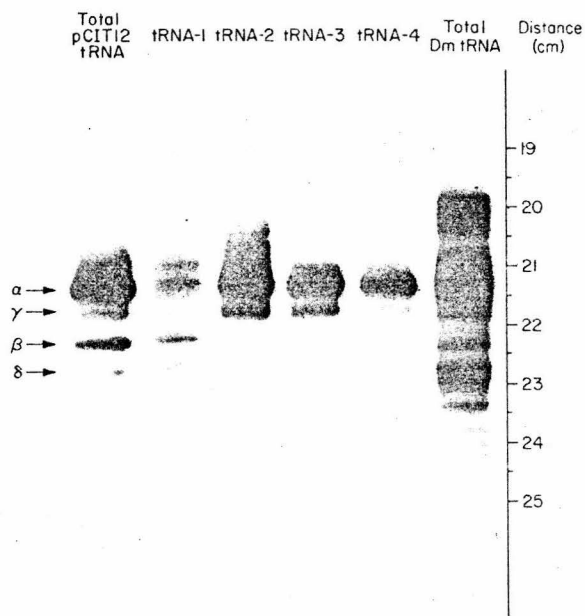


Figure 8. Fluorograph of a Gel Electrophoretic Analysis of Total *Drosophila* tRNA and of the tRNAs Purified by Hybridization to and Elution from pCIT12 and from Separated Restriction Fragments

10% polyacrylamide gel, 300 V, 20 hr, 4°C.

cies eluted after hybridization to pCIT12 DNA on a nitrocellulose filter and with the tRNAs that bound to the separated Hpa I plus Eco RI fragments A, B, D and G.

In a 30 cm gel (10% acrylamide, 4 M urea), total *Drosophila* tRNA was separated into about 15 bands. Total pCIT12 tRNA gave rise to two major bands α and β , and two minor bands γ and δ . When the gel was sliced, the ratio of radioactivity in band α to β was 2.77. In experiments with the tRNA eluted from the separated restriction fragments, tRNA-1 gave mainly bands β and δ , while tRNA-2, tRNA-3 and tRNA-4 all gave bands α and γ . We interpret these experiments to indicate that bands γ

and δ represent breakdown products of the tRNAs in bands α and β , that band α from the tRNA eluted from total pCIT12 DNA contains 3 times as much tRNA as does band β , and that the three tRNAs eluted from fragments B, D and G are identical in electrophoretic mobility, corresponding to band α , whereas that eluted from fragment A (tRNA-1) is different, corresponding to band β . These experiments are therefore consistent with the cross-hybridization experiments which indicate that tRNA-2, 3 and 4 are similar or identical, but tRNA-1 is different.

In Situ Hybridization

A ^3H -cRNA probe was transcribed from pCIT12 DNA with *E. coli* RNA polymerase and hybridized in situ to *Drosophila* salivary gland chromosomes. In the autoradiogram shown in Figure 9, it can be seen that the radioactivity is located in the puff in region 42A of chromosome 2R. Localization is to the left of and distinct from the doublet bands in 42B₁₋₄. Although silver grains in the puff were often not clustered, several nuclei were observed in which grains were localized in two distinct groups within the puff. Frequently the grains extended from the puff well into the nucleoplasm. Because of the diffuse nature of this puff, individual bands within the puff could not usually be recognized.

Discussion

Our principal findings are that the 9.3 kb insert of *Drosophila* DNA present in pCIT12 contains four tRNA genes denoted, in linear order, as tRNA-1, tRNA-2, tRNA-3 and tRNA-4, with respective intergene spacings of about 3.3, 1.2 and 2.7 kb. In the spacer sequences, there are several secondary structure features which are irregularly distributed, confirming the general picture that the spacers are not identical tandem repeats. Cross-hybridization experiments and gel electrophoretic fractionations both show that tRNA-1 is different from 2, 3 and 4,

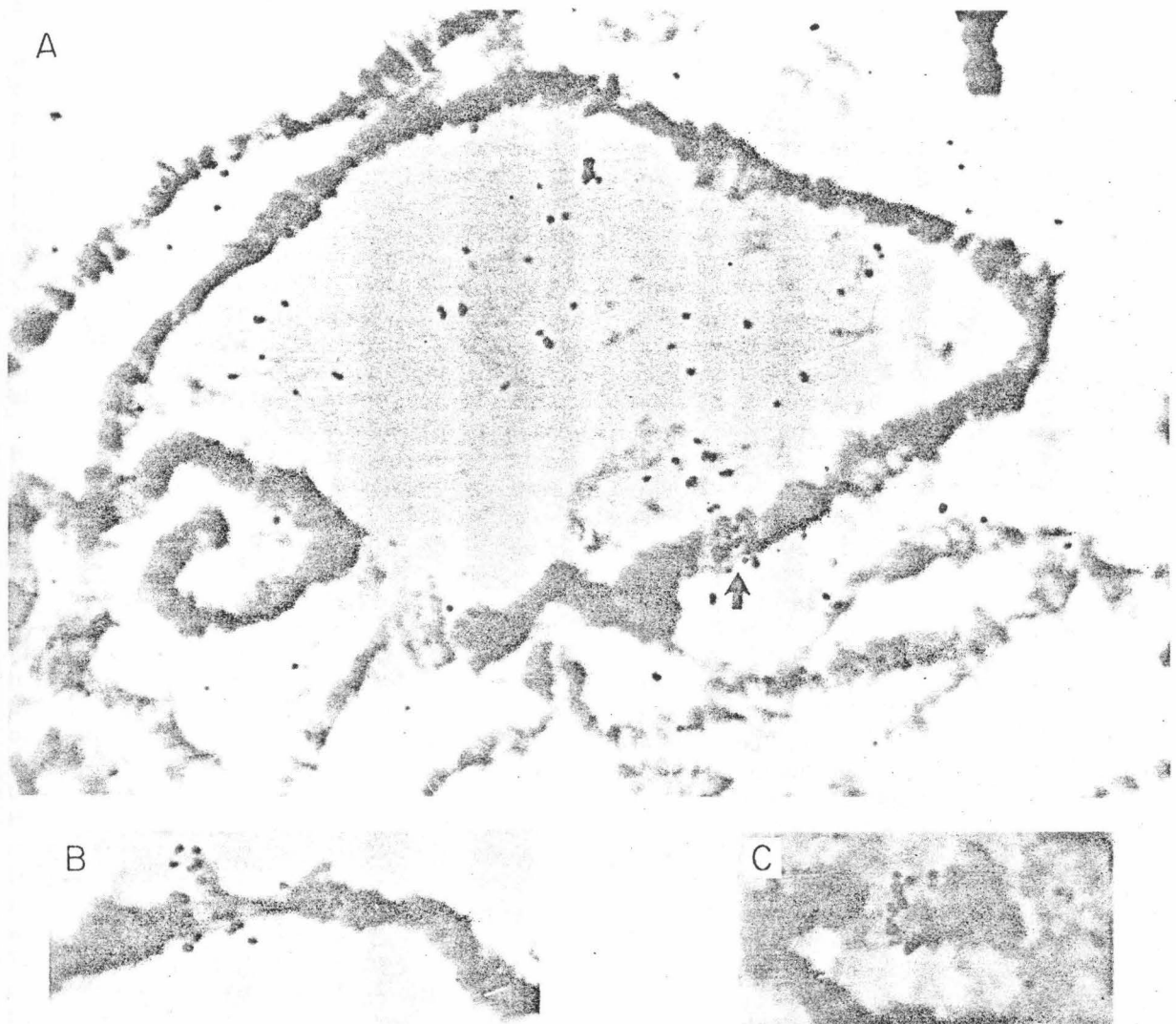


Figure 9. Autoradiograph of *Drosophila* Salivary Gland Chromosomes after in Situ Hybridization with pCIT12 ^3H -cRNA (see Text)
 (a) An arrow marks the site of hybridization in region 42A at the base of chromosome 2R. (b) and (c) Autoradiograms show localization of silver grains in 42A as two distinct clusters.

and suggest that the latter three are identical in sequence. Genes 1, 2 and 4 have the same coding strand. The coding strand for gene 3 has not yet been determined. The *Drosophila* DNA segment studied is localized to 42A of chromosome 2R.

We should first consider several possibilities for error in these conclusions. It is conceivable that there have been some sequence rearrangements due to the chemical and biological manipulations in the construction of the recombinant DNA molecules. However, there is no obvious mechanism in these procedures for generating a major sequence rearrangement. This is supported by the fact that another plasmid prepared at the same time as pCIT12 contains approximately 12 kb of *Drosophila*

DNA consisting of a set of 32 tandem repeats of 5S rRNA gene plus spacer regions but with no detectable irregularity (Hershey et al., 1977). In the procedure used for constructing the recombinant DNA, poly(dA) extensions were added to the exposed 3' single-stranded ends of the *Drosophila* DNA with the enzyme terminal transferase. It is conceivable that the terminal transferase reaction could have generated short poly(dA) stretches at internal nicks in the *Drosophila* DNA. Subsequent in vivo processes in *E. coli* could have generated new internal inverted repeats, not present in uncloned *Drosophila* DNA. The inverted repeat b,b' could conceivably be such an artifact. It seems less probable that an inverted repeat pair separated by a loop (as for the

aa', cc' and b₂b' features) could be an artifact.

tRNAs-2, 3 and 4 hybridize with all three genes under the hybridization conditions used. These conditions would permit 10 or 20% sequence mismatch, so that we can not assert that the three genes are identical in sequence; nevertheless, this is the simplest and most plausible interpretation of the results. The gene for tRNA-1 is clearly different from the other three genes. The cross-hybridization experiments are consistent with gel electrophoresis experiments on the tRNAs. These fractionations show the tRNA-1 is clearly different from 2, 3 and 4, but do not exclude the possibility that 2, 3 and 4 are different, even though not separated by the gel electrophoresis procedure used.

We are puzzled by the fact that the gene for tRNA-3 was not identified by the electron microscope hybridization experiments, since it was identified by hybridization to DNA fixed to a membrane filter. This gene is very close to the arm a' of the inverted repeat stem aa'. The estimated length of a' is 70 nucleotides. We can suppose that part of the tRNA sequence is included within a', and that rapid formation of the inverted repeat stem aa' inhibits hybridization of the tRNA under the conditions of the electron microscope experiment, although not on a membrane filter. If a and a' were perfectly matched sequences, one would suspect that a tRNA gene would also be present at sequence a. However, a and a', like many other inverted repeats in the *Drosophila* genome (Schmid, Manning and Davidson, 1975), may not be perfectly matched. It should be noted that the ferritin labeling studies were carried out with tRNA extracted from pupae. The chemical modification procedures might cause cleavage of some tRNAs. The tRNAs extracted from tissue culture cells which were used in the membrane filter hybridization experiments might have different relative amounts of some species. However, if tRNA-2, 3 and 4 cross-hybridize, artifacts due to chemical modification or relative abundance should not cause selective labeling of the different genes. Furthermore, the gene 32 electron microscope experiments, which were conducted with unmodified Schneider cell tRNA, gave the same results as the ferritin mapping. We therefore suspect that there is a difference between solution and membrane filter hybridization in the efficiency of hybridization of tRNA-3 to its site.

It is conceivable that there is one or several additional tRNA genes that we have missed. Just as for tRNA-3, other genes might not be identified by electron microscope ferritin mapping. The restriction endonuclease-gel electrophoresis-hybridization experiments could miss a gene that occurred on a very small restriction fragment. We believe that only further studies can resolve these uncertainties.

It should be noted that we have not identified the tRNAs nor have we studied the question of whether there are any sequence homologies between the several spacer regions.

The in situ experiment indicates that a probe prepared to the entire insert hybridized only to region 42A. This region must have some general significance for the pattern of development in *Drosophila*, since it is puffed throughout the larval third instar and most of the prepupal period (Ashburner, 1969). 42A was the single most prominent chromosomal site of hybridization in the experiments of Steffensen and Wimber (1971), in which total labeled *Drosophila* tRNA was hybridized to polytene chromosome squashes. The evidence cited in the Introduction suggests that the average number of genes per kinetic family is approximately 10. It is probable that 42A contains more genes than the average tRNA gene cluster, and thus that the segment present on pCIT12 is only a fraction of the entire tRNA gene cluster(s) in 42A. Our in situ hybridizations suggest that the pCIT12 probe hybridizes to two slightly separated regions in the puff. We hope in future experiments to isolate longer pieces of DNA extending outward in both directions from the sequence present on pCIT12 and to obtain further information about the organization of tRNA genes in this region. It will be of interest to determine whether the genes for the sequence tRNA-1 and those for the sequence tRNA-2, 3 and 4 (assuming they are identical) are interspersed, or whether pCIT12 fortuitously happens to include the junction between two distinct tRNA gene clusters, each containing only one kind of gene.

It should be noted that Grigliatti et al. (1974) have shown that tRNA^{lys} hybridizes to 48F-49A of chromosome 2R, a site not significantly labeled in the experiments of Steffensen and Wimber (1971). The same laboratory (Delaney et al., 1976) has now found that tRNA^{val} hybridizes to bands 84D3-4 of chromosome 3R.

Studies by Clarkson et al. (1973) suggest that identical tRNA genes of *Xenopus laevis* are clustered. Later studies by Clarkson and Kurer (1976) indicate that there is one cluster in *Xenopus laevis* in which the tRNA^{met} genes are rather regularly spaced with a repeat interval of 3.1 kb, and that this tRNA gene is interspersed with at least one other kind. The regularity observed in the *Xenopus* case is not observed in the small sample of *Drosophila* tRNA genes present on the *Drosophila* segment of pCIT12.

Experimental Procedures

DNAs and RNAs

Drosophila tRNA was purified from the post-ribosomal fraction from pupae as described by Twardzik, Grell and Jacobson (1971).

After the DEAE-cellulose chromatography, the tRNA was further purified by a Sephadex G-100 column. One such preparation was a gift from Dr. T. A. Grigliatti; a similar preparation was carried out by us. ^3H -labeled tRNA was prepared by the same procedure from *Drosophila* tissue culture cells (Schneider's line 2) labeled with ^3H -uridine as described previously (Hershey et al., 1977). A specific activity of $1\text{--}2 \times 10^6$ cpm/ μg was obtained. Plasmid DNA was isolated as described previously (Hershey et al., 1977), except that two EtBr-CsCl density gradient centrifugations were used: Open circular Col E1 and pCIT12 were prepared from the closed circular forms by X-ray nicking (Sharp et al., 1972) and EtBr-CsCl centrifugation.

Construction of Col E1-*Drosophila* Hybrid Plasmids and Screening of Clones Containing *Drosophila* tRNA Genes

The procedures for construction of hybrid Col E1-*Drosophila* DNA, its transformation into *E. coli* cells and screening for clones with *Drosophila* tRNA genes were described previously (Hershey et al., 1977). The hybridization solution for colony filter selection contained ^3H -tRNA at a concentration of 1.25 $\mu\text{g}/\text{ml}$. Unlabeled *Drosophila* 5S rRNA at 4 $\mu\text{g}/\text{ml}$ and rRNA at 155 $\mu\text{g}/\text{ml}$ were also present. Hybridization was carried out at 43°C for 36–45 hr in a solvent of 50% formamide, 2xSSC, 1 mM EDTA, 0.1% SDS.

Heteroduplex Formation

Heteroduplex formation between pCIT12 DNA or a restriction fragment thereof and Col E1 was carried out in 70% formamide, 0.25 M NaCl, 10 mM EDTA, 0.1 M Tris (pH 8.5) at room temperature for 2 hr at a DNA concentration of 0.5 $\mu\text{g}/\text{ml}$. The solution was then dialyzed against 50% formamide, 0.1 M Tris, 0.01 M EDTA (pH 8.5), and spread according to Davis et al., (1971).

For preparing heteroduplexes between the restriction fragment and Col E1, the whole digestion mixtures were used without isolation of the fragments. The presence of Mg^{++} in the digestion mixture prevented denaturation of the DNA by NaOH, so EDTA was added to a final concentration of 30 mM before the addition of NaOH.

Ferritin Labeling of the tRNA Genes on pCIT12

Total tRNA from pupae was conjugated to biotin by cytochrome c bridge by the procedure developed by A. Sodja (manuscript in preparation). Ferritin-avidin was prepared as described by Angerer et al. (1976).

The first preparation of pCIT12 which was contaminated with another small plasmid was used for the ferritin labeling experiment. Open circular DNA was denatured with 0.3 M NaOH at room temperature for 10 min and neutralized with 2.5 M Tris-HCl. Formamide and tRNA-cytochrome c-biotin were then added. The final mixture contained DNA at 1 $\mu\text{g}/\text{ml}$ and tRNA-cytochrome c-biotin at 30 $\mu\text{g}/\text{ml}$. The solution was dialyzed against 40% formamide, 0.3 M NaCl, 0.1 M Tris, 1 mM EDTA (pH 8.0) at 40°C for 1.5 hr to allow renaturation to occur. Subsequent manipulations were performed as described by Angerer et al. (1976).

Gene 32-Ethidium Bromide Method

Samples were hybridized as described above, except that unmodified tRNA from tissue culture cells was used. Hybrids were mounted for electron microscopy as described by Wu and Davidson (1975).

Restriction Enzyme Digestion

Restriction enzymes were purchased from New England Biolabs. Digestions were routinely carried out overnight at 37°C with a DNA concentration of 30 $\mu\text{g}/\text{ml}$ and enzyme concentration of 2 units per μg of DNA in the assay buffer recommended by the supplier. Partial digestions were carried out for 20 min and terminated by the addition of EDTA to a final concentration of 30 mM. For double enzyme digestions, the enzyme with a low salt requirement was used first, and completion of digestion was checked by gel electrophoresis. The reaction mixture was then brought up to the salt concentration recommended for the second enzyme, and

incubation was continued overnight. The order was as follows: Hpa I or Sma I, Hind III and Eco RI.

Gel Electrophoresis of the Restriction Fragments

4 mm thick slab gels were used. Usually 1 μg of a DNA sample in a volume of 25 μl containing 2% Ficoll, 0.005% bromophenol blue was used per slot. Three different gels were used to determine the lengths of the fragments. For fragments >5 kb, 0.7% agarose was used with either the Hind III or Eco RI fragments of λ DNA as length standards (Wellauer et al., 1974; Thomas and Davis, 1975). For fragments between 0.3 and 5 kb, 1% agarose was used. The lengths of the small fragments were determined with a 5% polyacrylamide gel. In the latter two cases, Hind III fragments of PM2 DNA were used as standards (Parker, Watson and Vinograd, 1977). The gels were run in buffer E [40 mM Tris, 5 mM NaOAc, 1 mM EDTA (pH 7.4)] at room temperature for 3 hr at 80 V for agarose gels, or for 6 hr at 60 V for acrylamide gels. The gels were then stained with EtBr (0.5 $\mu\text{g}/\text{ml}$ in H_2O) for 10 min, destained with H_2O for another 10 min and photographed.

For identification of those restriction enzyme fragments that contained tRNA genes, DNA bands fractionated by gel electrophoresis were denatured and transferred to a nitrocellulose membrane filter overnight by the Southern method (1975a), with the following modifications (Hutchison, 1977). The hybridization conditions for ^3H -tRNA were 50% formamide, 2 x SSC, 1 mM EDTA, 0.1% SDS and ^3H -tRNA at a concentration of 2 $\mu\text{g}/\text{ml}$. Incubation was carried out at 43°C for 35–40 hr. After extensive washing and an RNAase digestion, the filter was treated with 20% PPO in toluene (Southern, 1975a) and exposed to sensitized X-ray film (Lasky and Mills, 1975).

Isolation of Restriction Fragments

Restriction fragments were separated by 1% agarose gel electrophoresis and isolated either by dissolving the gel in saturated KI and banding in a potassium iodide gradient (Blin, Gabain and Bujard, 1975) or by the NaClO_4 -hydroxyapatite method described by Southern (1975b) with the following modifications (W. Bender, personal communication). About 20 μg of DNA fragments were applied to a preparative gel. After electrophoresis, the gel was stained with EtBr and the DNA bands were cut out. 2 vol of 8 M NaClO_4 were added to dissolve the gel, and the solution was applied to a short HAP column. The column was then washed sequentially with 5.3 M NaClO_4 , 0.05 M sodium phosphate (pH 6.8) and with 0.12 M sodium phosphate. The double-stranded DNA was eluted with 0.5 M sodium phosphate. EDTA was added to 30 mM, and the solution was passed through a small Bio-Gel P-2 column. This step, which presumably removed Ca^{++} ions, was necessary to render the DNA susceptible to further restriction enzyme digestion.

Isolation of pCIT12-Coded tRNAs

For isolation of total tRNAs coded by pCIT12, 50 μg of open circular pCIT12 DNA were denatured, neutralized and immobilized on a nitrocellulose membrane filter according to Birnstiel, Sells and Purdon (1972). Conditions for hybridization were identical to those used to identify restriction fragments carrying tRNA genes. After the hybridization, the filter was incubated under hybridization conditions without ^3H -tRNA for 1 hr and rinsed twice with the same solution. No RNAase treatment was used. The tRNA was then eluted with 1 ml of 90% formamide, 10 mM Tris-HCl (pH 7.4) at 46°C for 1 hr.

For isolation of individual pCIT12 tRNAs, 120 μg of Eco RI plus Hpa I fragments were separated by electrophoresis on an 8 mm thick by 20 cm long preparative gel. The fragments were denatured and transferred to a large sheet of nitrocellulose membrane filter as before. Five bands, each about 1 cm in length in the direction of electrophoresis and 12 cm wide, corresponding to the different restriction fragments of interest (A, B, D and G, with H as a control) were cut out from the filter paper. Four strips, 6 mm x 1 cm, were cut out from each such band (in the same vertical

position for the different bands) for the cross-hybridization experiments described below. The remaining portion of each band was used for isolating the individual tRNAs as described above. After elution of tRNA, the filters were reused for isolation of more tRNA.

Cross-Hybridization of pCIT12 tRNAs with Different Restriction Fragments

To the tRNA solutions in 90% formamide, an equal volume of H₂O and carrier E. coli rRNA to a final concentration of 30 µg/ml was added. tRNA was then precipitated from each solution with 3 vol of EtOH at -20°C overnight. Each precipitate was dissolved in 0.5 ml of the hybridization solution. To each of the solutions, five 6 mm × 1 cm strips of the millipore filters, one of each restriction fragment, were added. Hybridization was carried out as before. At the end of the incubation, the filters were washed thoroughly with 2 × SSC, treated with RNAase, dried and counted.

Gel Electrophoresis of pCIT12 tRNAs

Gel electrophoresis of *Drosophila* and pCIT12 tRNAs on 10% polyacrylamide was carried out according to Fradin, Gruhl and Feldmann (1975), with the following modifications. Samples were applied in 30% sucrose instead of 60%, and 0.09 M Tris-borate buffer (pH 8.3) containing 4 M urea was used. The gels were 30 cm long and 1 mm thick. Usually the sample contained 10⁴ cpm of ³H-tRNA with 2-5 µg of total *Drosophila* tRNA as carrier. After electrophoresis, the positions of the bands were detected either by EtBr staining (0.5 µg/ml in H₂O for 10 min) or by scintillation autoradiography (Bonner and Laskey, 1974) using film sensitization (Laskey and Mills, 1975).

In Situ Hybridization

Salivary chromosome squashes from late third instar larvae of *Drosophila* were prepared on glass slides following the method of Atherton and Gall (1972). Larvae used were *giant* mutant heterozygotes of genotype *gt^{1w}/gt¹³²* (Kaufman, 1971, 1973). After ribonuclease treatment of the squash preparations (100 µg/ml, 2 × SSC, 37°C, 1 hr), DNA was denatured by incubating the slides at 65°C for 2.5 hr in 0.1 × SSC, 90% formamide (Alonso, 1973). cRNA was prepared as described by Hershey et al. (1977). The hybridization solution containing 50% formamide, 6 × SSC, 50 µg/ml E. coli ribosomal RNA and pCIT12 ³H-labeled cRNA (3 × 10⁷ cpm/µg; estimated *Drosophila* sequence cRNA concentration 0.42 µg/ml) was heated at 85°C for 5 min; 50 µl of the solution were then layered over each squash. The slides were placed in a closed container over 6 × SSC, 50% formamide, incubated at 48°C for 18 hr and again treated with ribonuclease (20 µg/ml, 2 × SSC, 37°C, 45 min). The preparations were thoroughly rinsed and dehydrated in ethanol. Autoradiography was performed with Kodak NTB-2 liquid emulsion for 89 days at 4°C. The developed slides were stained with Giemsa blood stain (Harleco).

Containment Procedures

All plasmids containing *Drosophila* segments were propagated under P2 physical containment as defined and recommended for this class of experiments by the Guidelines for Research Involving Recombinant DNA Molecules issued in June 1976 by the National Institutes of Health. Before issuance of the Guidelines, an F⁺ host (strain JA199) was used. Recombinant DNA molecules have since been used to transform strain HB101 to give a host-vector system defined as EK1 under the Guidelines. The F⁺ strains carrying recombinant plasmids have been destroyed.

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Part II

In vitro Studies on Replication
of Plasmid DNAs

Introduction

Part II of this thesis reports work done in Dr. Judith Campbell's laboratory. We have developed an improved system for the in vitro replication of Escherichia coli plasmids. Two systems had previously been developed for studying the replication of covalently closed circular, double stranded plasmid DNAs (Sakakibara and Tomizawa, 1974; Staudenbauer, 1976). We have refined Staudenbauer's system, which gives higher levels of synthesis, in order to make replication completely dependent upon the addition of exogenous plasmid DNA. We have characterized this improved system, and shown that it carries out all stages of DNA replication (i.e., initiation, elongation and termination), but does not carry out appreciable repair synthesis. In addition, we have catalogued the E. coli replication proteins that are required for plasmid DNA replication in vitro.

In vitro DNA replication systems have been useful in elucidating the role(s) of many proteins in replication, and in providing complementation assays to allow for purification of these proteins. For example, in vitro replication systems using the small circular DNAs from the phages ϕ X174, G4 and M13 have provided assays for the purification of the dnaG, dnaB and dnaC(D) proteins, and have provided information on the role of these proteins in replication. For a recent review of Escherichia coli DNA replication proteins, see Wickner (1978).

In vitro systems have also been useful for studying the DNA intermediates found during replication. Perhaps the best example of this can be seen in studies of ColE1 replication. ColE1 is a supercoiled, closed circular duplex DNA that replicates semi-conservatively and unidirectionally in Cairns-type theta structures. ColE1, like the E. coli chromosome and several of the small phages, requires RNA polymerase for the initiation of replication. It is therefore thought that RNA

serves as a primer for the initiation of DNA replication. When ColE1 DNA is replicated in vitro under conditions where only early replication intermediates are formed, molecules appear with small bubbles of about 600 b.p. located at a unique site on the DNA. These structures are comprised primarily of one single stranded and one double stranded branch, although on some molecules both branches are double stranded. When synthesis has occurred on only one strand, it is the H strand that has been copied, giving rise to a newly synthesized L strand DNA fragment. These structures are formed in both polC and dnaZ strains, indicating that E. coli polymerase III is not required for the early steps of ColE1 replication. Electron micrographic studies of ColE1 molecules replicated to different extents have shown that replication occurs by a unidirectional mechanism. The elongation stage of replication does require polymerase III. For a comprehensive review of ColE1 replication, see Staudenbauer (1978).

We have used our in vitro DNA replication system to study the replication of other Escherichia coli plasmids. RSF1030 is an ampicillin resistant plasmid that has no detectable sequence homology with ColE1, although the two have very similar replication properties. We have mapped the origin and direction of RSF1030 replication and found that, like ColE1, it replicates unidirectionally from a fixed origin.

We have also used our in vitro system to investigate pFH118, a high copy number mutant that is derived from ColE1. The increased copy number seen with this plasmid is correlated with an insertion of 20-30 base pairs into a site approximately 430 base pairs from the origin of DNA replication. The DNA sequence at this site codes for a 104 nucleotide RNA that is transcribed during in vitro DNA replication. This RNA transcript is partially homologous to the origin of

replication, and hybridizes to the origin itself. In addition, the RNA coded for by RSF1030 contains significant sequence homology to the RNA from ColE1. Revertants that have lost the insertion into the RNA-coding sequence have a wild-type copy number. These facts have led us to propose that this 104 nucleotide transcript plays a role in determining plasmid copy number, and that its synthesis may be the rifampicin sensitive step in the initiation of plasmid DNA replication.

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Chapter 3

Characterization of an Improved in vitro

DNA Replication System

for Escherichia coli Plasmids

The abbreviations used are: Form I, covalently closed circular duplex supercoiled DNA; Form I_O, covalently closed circular duplex relaxed DNA; Form II, nicked, circular duplex DNA; Form III, linear, duplex DNA; Form IV, irreversibly denatured covalently closed circular duplex DNA; DTT, dithiothreitol; PEA, phenethyl alcohol; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid; EGTA, ethylene-glycol-bis-(β -aminoethyl ether)N,N'-tetra-acetic acid.

Abstract

A modified in vitro replication system has been characterized and used to catalogue the host proteins required for the replication of plasmid RSF1030. These extracts differ from systems described previously in that endogenous DNA is removed. Replication in vitro therefore requires an exogenous RSF1030 template, even when extracts are prepared from cells carrying endogenous RSF1030. Synthesis in the in vitro system faithfully mimics in vivo replication with respect to the products synthesized, effects of specific inhibitors, and requirements for RNA polymerase and DNA polymerase I. In addition, we find that proteins encoded by dnaB, dnaC, dnaG, dnaI, dnaP and polC (DNA polymerase III) are required for in vitro plasmid synthesis. The product of dnaA is not required. Extracts prepared from E. coli mutants deficient in in vitro replication can be complemented by addition of purified proteins or of extracts carrying the wild type protein.

INTRODUCTION

Two in vitro systems have been described for the replication of plasmid DNAs, one by Sakakibara and Tomizawa (1), and another by Staudenbauer (2). These systems have been used for studying ColE1 and, more recently, RSF1030 replication (3; for summary see Refs. 4 and 5). These two plasmids have very similar replication properties. Both are present in multiple (20-30) copies per cell, and continue to replicate in the presence of chloramphenicol resulting in the accumulation of 1000-1500 copies per cell (4,6). In vivo replication of both plasmids requires DNA polymerase I and is inhibited by rifampicin (4,6,7). In the absence of DNA polymerase III, the rate of replication is decreased (7,8). In addition, both plasmids replicate unidirectionally from a unique origin (4,9). In spite of these similarities, plasmids ColE1 and RSF1030 appear to have evolved independently since they have no detectable sequence homology (6).

The two in vitro systems currently in use have several major flaws. In one (1), the rate and extent of synthesis is very low, and the extracts are unstable. In the second (2), the extent of synthesis is increased, but there is endogenous plasmid DNA present in the extracts. It is desirable to use an in vitro system in which synthesis is dependent upon the addition of exogenous template in order to be sure that initiation is occurring in vitro and that synthesis does not reflect mere elongation of intermediates formed in vivo.

In the work presented here, we have extended the system of Staudenbauer by removing endogenous DNA and by concentrating extracts to allow increased levels of synthesis. The extent of synthesis we observe in vitro is almost three times greater than previously observed, making quantitative measurements and purification of products easier. Furthermore, the system is completely dependent

upon the addition of exogenous, covalently-closed circular duplex DNA, allowing one to look at the replication of any desired added DNA or DNAs.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* W3110 thy⁻ and *E. coli* W3110 (RSF1030) were used as the standard strains for preparation of the in vitro system. Strains used to test for protein requirements are listed in Table I. RSF1030 is a relaxed plasmid (4) that carries amp^R on the transposon TN 2 (6,10).

Other Materials. Nalidixic acid, novobiocin and streptomycin sulfate were obtained from Sigma. Rifampicin was Rifampin from Calbiochem. Chloramphenicol was chloromycetin sodium succinate, Parke-Davis. [³H]dTTP (10 Ci/mmol and [α -³²P]dTTP (300 Ci/mmol) were purchased from New England Nuclear. Agarose (Seakem, ME) was from Marine Colloids, Inc.

Growth of Cells. Two liters of *E. coli* W3110 (RSF1030) were grown at 37° to O.D.₅₉₀ = 1.0 in L Broth supplemented with 0.1% glucose. Chloramphenicol (150 µg/ml) was added and the culture incubated for 4 hr with shaking at 37° (O.D.₅₉₀ = 3.6). Cells were harvested at room temperature and resuspended in 10 ml 0.025 M HEPES, pH 8.0/5 mM EGTA. The cells were frozen in liquid N₂.

Preparation of Extracts. Frozen cells were thawed by incubating at 0° for 20 min and then at 20°. The lysis procedure used was that of Staudenbauer (2). The cell suspension was brought to a final concentration of 0.15 M KCl and 0.2 mg/ml lysozyme. After incubation at 0° for 30 min, the lysate was frozen in liquid N₂ for 2 min and then thawed at 20°. The lysate was centrifuged for 30 min at 100,000 x g. The supernatant fluid, which will be referred to as Fraction I, contains about 17 mg per ml of protein and has an A₂₆₀ of 100-130.

Streptomycin Sulfate and Ammonium Sulfate Fractionation. To 2.0 ml of Fraction I, 0.2 ml of a 30% (w/v) solution of streptomycin sulfate was added. The solution was stirred for 30 min at 0°C. The precipitate was removed by centrifugation for 10 min at 20,000 x g at 4°C. To 2.0 ml of supernatant, 0.944 g solid ammonium sulfate was added over a 10 min period. The mixture was stirred for 30 min at 0° and the precipitate was then removed by centrifugation at 20,000 x g for 10 min. The pellet was dissolved in 1.0 ml 0.01 M HEPES, pH 8.0/1 mM DTT/1 mM EDTA/0.1 M KCl/10% ethylene glycol. The redissolved pellet was dialyzed against 100 ml of the same buffer for 3 hr at 0°. The dialyzed material, Fraction II, was frozen and stored in liquid N₂. Fraction II contains about 11 mg per ml of protein.

Assay for DNA Synthesis. A standard assay for DNA synthesis has been established using Fraction II. Reactions (0.05 ml) contained 40 mM HEPES, pH 8.0, 100 mM KCl, 12 mM magnesium acetate, 50 µM each dCTP, dGTP, dATP, and [³H]TTP, 2 mM ATP, 0.5 mM each rCTP, rGTP, rUTP, 1.5 nmol of RSF1030 DNA, and 20 µl extract. Incubations were carried out at 30°. Reactions were terminated by placing tubes at 0° followed by addition of 1 N HCl containing 0.1 M sodium pyrophosphate. The precipitate was collected on Whatman GF/C filters. The filters were washed, dried and radioactivity determined in a toluene-based fluid in a scintillation counter. The half-life of the DNA synthesizing activity was greater than 60 min at 30° in Fractions I and II. Fraction II has a half-life of >60 min at 40°.

Preparation of Plasmid RSF1030 DNA. *E. coli* HMS 174 recA1 (RSF1030) was grown to O.D.₅₉₀ = 1.0 in Vogel-Bonner medium (11). Chloramphenicol (250 µg/ml) was added and incubation continued for 17 hr. Cells were harvested by centrifugation at 4°, and frozen and thawed one time. Cells (25 g) were lysed

by the method of Clewell and Helinski (12), modified by the use of Triton X-100 in place of Brij 58 and sodium deoxycholate (13).

Supercoiled DNA was purified by two equilibrium bandings in CsCl ($\rho = 1.55$) EtdBr (100 $\mu\text{g/ml}$) gradients. In order to remove low molecular weight nucleic acid contaminants, the DNA was sedimented through 30 ml CsCl ($\rho = 1.3$ gm/cc) containing 100 $\mu\text{g/ml}$ EtdBr with a CsCl ($\rho = 1.7$ gm/cc) cushion. EtdBr was removed by Dowex chromatography and CsCl was removed by dialysis.

Agarose Gel Electrophoresis. Vertical slab gels (7-5/8" x 6-1/2" x 4 mm) of 0.7% agarose were formed and run in E buffer (40 mM Tris, 5 mM NaAcetate, 1 mM EDTA, pH 7.4) as described previously (9). Samples were loaded in low salt buffer plus 2.5% Ficoll, 0.02% bromophenol blue in a maximum volume of 50 μl for a ten slot gel. Neutral agarose gels were run at 40 V for 12 hrs to resolve the different forms of circular DNA. For autoradiography, gels were first dried on a Slab Gel Dryer (Hoefer Sci. Instruments). Preflashed Kodak Xr-5 film was exposed at -70°C using an intensifying screen.

RESULTS

As pointed out in the Introduction, one goal of these studies was to develop an in vitro replication system dependent on the addition of exogenous DNA. Although one approach would be to prepare extracts from cells that do not carry plasmid, such extracts would not contain products specified by plasmid genes and one could not assess their role, if any, in plasmid replication. We therefore chose to prepare extracts from cells carrying the plasmid and to subsequently remove endogenous plasmid from the extracts. Since lability of the extracts made such fractionation difficult, we first established conditions for stabilizing the replicating activity.

Sucrose and glycerol proved inhibitory (2,3). Ethylene glycol, however, proved an efficient stabilizing chemical and we have been able to develop a system (see Methods, Fraction II) that is totally dependent on addition of exogenous covalently closed, circular, duplex plasmid DNA.

In order to be sure that synthesis in Fraction II represented replication and not repair, Fraction II was compared to Fraction I with respect to the requirements for synthesis, the effect of inhibitors, and the nature of the products. Fraction I has been shown by others to represent replication (2).

Kinetics of Synthesis in Fraction I and Fraction II. The kinetics of RSF1030 DNA synthesis in Fraction I confirm the findings of Staudenbauer et al. (2). As shown in Figure 1, synthesis proceeds at a linear rate for 40 min and then plateaus at around 60 min. After an initial lag period of 10 min, the kinetics of synthesis in Fraction II, also shown in Fig. 1, are somewhat different. The rate is 30-50% higher than observed in Fraction I, with synthesis continuing at an almost linear rate for up to 90 min before a plateau is reached. The extent of synthesis obtained with Fraction II is almost three-fold that seen in Fraction I. As in Fraction I, however, a 10 min lag period is observed before the synthesis increases at a linear rate. The initial rate of synthesis in Fraction II after the 10 min lag is 180 pmols of dTMP incorporated per hour, or the equivalent of 2.6×10^{10} RSF1030 molecules per hour. Since the reaction mixture contains protein from 6×10^9 cells, this rate represents 4.5 RSF1030 molecules per hour per cell equivalent.

Requirements of the Reaction and Effects of Inhibitors. The requirements of the in vitro synthesis reaction in Fractions I and II were compared and are shown in Table II. Results with Fraction II are exactly the same as with Fraction I and are similar to those in other related in vitro systems (1,2). The most important

similarities are the requirement for ribonucleotides and the inhibition of synthesis by rifampicin. Inhibition by rifampicin indicates that RNA polymerase is required for synthesis in Fraction II, and is strong evidence in itself that Fraction II carries out replication and not repair synthesis. The kinetics of rifampicin inhibition in Fraction II (Fig. 2) are the same as those reported by others (1) and show that, in Fraction II as in Fraction I, rifampicin inhibits only initiation. Inhibition of Fraction II by novobiocin and nalidixic acid further shows its similarity to Fraction I and implicates DNA gyrase (14,15) in synthesis in Fraction II (Table II, Fig. 2b). Finally, phenethyl alcohol inhibits plasmid replication at levels equivalent to those that produce in vivo inhibition of E. coli growth.

Template Requirement in Fraction II. As shown in Fig. 3 and Table II, the amount of DNA synthesis in Fraction I in the absence of exogenous DNA is 63% of that observed in the presence of 10 $\mu\text{g/ml}$ of added Form I RSF1030 DNA. In Fraction II, however, the amount of synthesis in the absence of DNA is $< 0.3\%$ of the maximum observed when DNA is added to a final concentration of 10 $\mu\text{g/ml}$, indicating the complete dependence of synthesis in Fraction II upon exogenous DNA.

The initial rate of synthesis in Fraction II (after the 10 min lag) is proportional to the amount of DNA added to the reaction, although high concentrations are inhibitory (Fig. 3). Optimal synthesis is obtained when RSF1030 is added to a final concentration of 10 $\mu\text{g/ml}$, which represents a total of 5.3×10^{10} RSF1030 template molecules per reaction, or 9 molecules per cell equivalent of Fraction II added to the reaction. Since maximum synthesis ranges from 4.5 to 8 RSF1030 molecules produced per cell equivalent, depending on the extract used, 40-90% of the template, at most, is participating in replication. This is a more efficient use of template than in any other in vitro plasmid replication system.

The DNA requirement of Fraction II parallels the requirement for DNA observed in Fraction I extracts prepared from cells that carry no endogenous plasmid. However, the overall rate of synthesis in the latter extracts is always about three-fold less than the rate observed in extracts prepared from plasmid-carrying cells.

Sucrose Gradient Analysis. RSF1030 DNA synthesized in an in vitro reaction containing covalently closed RSF1030 as template and [α - 32 P]TTP has been analyzed by zone sedimentation through neutral and alkaline sucrose gradients (Fig. 4). Results using Fraction I are presented for comparison with Fraction II. These results clearly show that products are formed in approximately the same ratios in reactions catalyzed by either Fraction I or Fraction II. Although the proportion of newly synthesized DNA in each peak varies somewhat from experiment to experiment, the product distribution is identical to that found in Staudenbauer's Fraction I (2) and compatible with that found in detergent lysis system (1), where synthesis is known to represent replication and not repair. In summary, distribution of products suggests that up to 90% of the molecules that initiate replication in vitro complete replication, while the remainder are present as partially replicated molecules.

Gel Electrophoresis. Despite the large number of similarities between Fractions I and II already shown, we wished to demonstrate that newly synthesized DNA was not covalently attached to the template. Synthesis was carried out using an RSF1030 template and [α - 32 P]TTP (200 cpm/pmol) in the standard reaction mixture for sixty minutes. The products were phenol extracted and ethanol precipitated and analyzed on 0.7% agarose gels (Fig. 5), prior to (Lanes 2 and 4)

and after (Lanes 3 and 5) denaturing and renaturing. Lanes 2 and 3 (EtBr stained) give information about both template and product. Lanes 4 and 5 are autoradiograms of Lanes 2 and 3 and therefore only represent newly synthesized DNA.

Prior to denaturation, completely replicated molecules are seen at the position of Form I DNA (16) (Lanes 3 and 4). In addition, labeled DNA products are seen at the position of Form II and in two other bands, one migrating between Forms I and II, designated RI_{α} , and a second migrating slower than Form II indicated as RI_{β} . There is ^{32}P labeled material appearing throughout the gel from the position of Form I DNA to the position of the most slowly migrating band. The DNA has been eluted from these regions of the gel, and analysis by electron microscopy indicates that it is comprised of circular replicative intermediates that have been replicated to different extents (9).

Upon denaturation of the product (Lanes 3 and 5), all of the labeled material above the Form II position disappears, as does the diffuse material between Forms I and II. A new band, which we have not characterized, appears just below the Form II position. The amount of Form I material increases, and diffuse label now appears below the Form I position, probably representing less than full length linear molecules that have been released by denaturation from replicative intermediates. There are several very broad bands apparent in this region, indicating that replication may be prematurely terminated at several distinct sites. Thus, at least some of the newly synthesized DNA is not covalently attached to the template since it can be released by brief heating of the DNA. That the amount of Form I DNA appearing in the autoradiogram after the product is heated also increases suggests that some of the newly synthesized and therefore ^{32}P labeled DNA serves as a template for new rounds of replication in vitro.

Neutral agarose gels do not distinguish between nicked and relaxed closed circles (Form II and Form I_O, respectively). In order to do this, newly synthesized DNA was analyzed on an agarose gel containing EtdBr, a system we found readily distinguishes Forms II and I_O. Examination of the autoradiogram on the right in Fig. 6, representing newly synthesized DNA, and the photographed gel on the left, carrying a relaxed closed circular marker, indicates that the newly synthesized relaxed DNA is nicked. There is little Form I_O DNA, although there may be some, since the supercoiled band is broad and Forms I and I_O are not well resolved. We do not know whether the nicked forms are late intermediates in replication or arise due to random nicking activity in the extract or in the purification procedure used in preparing the DNA for gel analysis.

Further Characterization of Product. Because of the good correspondence between the requirements for synthesis and products of synthesis in Fractions I and II, and because the product was not all covalently attached to the template, no further product characterization by density gradients was considered necessary to prove that synthesis represented replication. This conclusion is supported below by the fact that a number of proteins known to be required for replication *in vivo*, but not thought to be repair enzymes, are absolutely required for DNA synthesis. Most importantly, however, we have shown by electron microscopy and gel electrophoresis that replication in Fraction II is initiated at a unique site and proceeds sequentially and unidirectionally (9). This result could only be due to replication and rules out the possibility that we are looking at repair synthesis, even in completely replicated molecules.

The above studies have been repeated with hosts not carrying endogenous plasmid. The results are identical, indicating that if any plasmid protein is involved in replication, a host protein can substitute for it.

Mutations Affecting DNA Polymerases. DNA polymerase I is required for plasmid replication in vivo (4) and has been shown to be required for ColE1 and RSF1030 replication in vitro in Fraction I (2,7). In addition, DNA polymerase III is implicated in plasmid replication in vivo since in polC mutants synthesis slows down, although it does not cease completely. Staudenbauer has also shown that DNA polymerase III is required for plasmid replication in vitro (7,17). Fraction II was prepared from polA or polC mutants as a further test that Fraction II was actually carrying out DNA replication.

Fraction II was prepared from E. coli DK517', which contains a thermolabile DNA polymerase I. No replication was found whether the extract was first incubated at 30° or at 40°. Evidently the activity in strain DK517' is defective in vitro even at the temperature that permits RSF1030 replication in vivo. This confirms that DNA polymerase I is required in vitro in Fraction II as well as in vivo and, as Staudenbauer has already shown, in Fraction I (17). Identical results were obtained with a polAI mutant.

Extracts of strain BT1026 polAI polC_{ts} supplemented with purified DNA polymerase I carries out synthesis at 30°, but not at 37° (17). Fraction II was prepared with E. coli dnaE486, which Livingston and Richardson (18) have shown contains no detectable DNA polymerase III activity in vitro. We found that there was no RSF1030 replication at any temperature in extracts of dnaE486 mutants, confirming that in Fraction II, as in Fraction I, DNA polymerase III is absolutely required for replication.

Host Gene Products Required for Plasmid DNA Replication in vitro. The usefulness of an in vitro replication system lies in its ability to give new insight into the mechanism of DNA replication, and a great deal has been learned about

the molecular biology of plasmid replication from in vitro replication studies. Requirements for DNA gyrase, RNA polymerase, DNA polymerase I and DNA polymerase III have recently been demonstrated (4). We have used Fraction II to investigate the additional protein requirements for plasmid replication.

A summary of the gene products required in vivo for plasmid replication is found in Ref. 4. We have prepared Fraction II from a number of mutants of E. coli defective in these required functions (dnaA, dnaB, dnaC, dnaE, and dnaG) and from several mutants defective in other functions not yet identified with plasmid replication in vivo, but that have phenotypes that suggest they might be (dnaZ, dnaP, dnaI). Extracts were prepared in most cases from strains that harbored RSF1030. A summary of the results obtained using these mutants and corresponding revertants is shown in Table III. Staudenbauer has also demonstrated requirements for dnaB, dnaC, and dnaZ (19). Extracts from strains dnaG, dnaZ, and dnaI, however, did not contain endogenous plasmid. It remains to be seen, therefore, if a plasmid gene product suppresses the requirement for any of these three activities.

Complementation Assay for DnaP Protein

In vitro replication systems are most useful if they provide a way in which to identify and isolate new components of the DNA replication apparatus. This is usually done by in vitro complementation assays where one extract provides a component missing or defective in the extract of a strain that contains a mutation in an allele known to be involved in replication. Using this in vitro replication system, we have developed a complementation assay for the purification of DnaP protein. The phenotype of dnaP mutants is that they continue replication at the

nonpermissive temperature until the ongoing round of replication is completed, but then synthesis ceases. It is therefore thought that this mutation affects initiation (or termination), and not elongation. Although extracts lacking either DnaP protein or polymerase I do not carry out DNA synthesis *in vitro*, DNA synthesis activity is restored when the extracts are mixed (Fig. 7). The reaction mixtures in the two curves contain 10 μ l of an extract missing the DnaP protein. Different amounts of an extract to be tested for complementation are then added. If more of the same dnaP extract is added (curve marked dnaP), there is no significant increase in the rate of DNA synthesis. When the complementary extract is added, however (curve marked polA dnaP⁺), there is a five-fold stimulation of activity. This stimulation is apparently proportional to the amount of extract added at low concentrations. The triple mutant E. coli polA1 polB100 polC_{ts} was chosen as a source of complementary extract since the absence of all three polymerases would reduce nonspecific stimulation of dnaP extracts.

The data in Table III and Fig. 7 indicate that there is residual DNA synthesizing activity in dnaP extracts. The nature of this activity was examined in order to define the stage of the reaction at which DnaP is required. The activity is totally abolished by the addition of 2 μ g/ml rifampicin to the *in vitro* assay, indicating that the residual synthesis requires RNA polymerase and probably represents true initiation of replication. The products synthesized in dnaP extracts were analyzed on neutral agarose gels (Fig. 8) and alkaline sucrose gradients. The newly synthesized DNA is found at the positions of Form I and Form II DNA in the autoradiogram shown in Fig. 8a. No late replicative intermediates (RI _{β}) are seen. Forty-three percent of the product sediments as an 18S species and 55% as a 5S species on alkaline sucrose gradients, while less than 0.1% is found in the 53S supercoiled peak (data not shown).

The nature of the 5S fragments was of interest since they might represent specific initiation fragments synthesized at the origin of replication that could not be elongated in dnaP extracts. We have looked at the pattern of labeling of the 6 Hinc II restriction fragments of total RSF1030 DNA synthesized in vitro. We have shown previously that the origin of an in vitro replication lies in Fragment A and the unidirectional order of labeling of the 6 fragments during replication is A, B, E, C, D, F (9). As shown in Fig. 8b, when RSF1030 DNA synthesized in the dnaP extract was subsequently degraded with Hinc II, all of the bands were labeled, as in the wild type control. Densitometer tracings indicate that the newly synthesized DNA contains approximately equimolar amounts of sequences from the whole genome and not just from the origin of replication. Therefore, the residual synthesis in dnaP mutants cannot be attributed to initiation alone, but also represents elongation. Further characterization of purified 5S fragments is required to establish if they contain sequences homologous to the entire genome or only sequences specific to the Hinc II A fragment.

dnaP mutants of E. coli were selected initially as mutants resistant to the drug PEA. The phenotype of dnaP mutants is that they grow in the presence of PEA at 30°, but do not grow in either the presence or absence of PEA at 42°. The residual replication promoting activity observed in extracts from dnaP mutants was therefore tested for sensitivity to PEA. All residual activity was sensitive to the drug, indicating that residual activity either does not require DnaP protein (data not shown) or that DnaP protein is not the target of PEA in this system.

DISCUSSION

An improved in vitro system for studying plasmid DNA replication has been developed. The extent of synthesis observed in this system is approximately

3-4 times that seen in the previous system. More importantly, synthesis is now completely dependent upon the addition of exogenous Form I DNA, although the cells from which the extracts are made contain endogenous plasmids. Evidence obtained *in vivo* using plasmids and chloramphenicol-treated cells indicates that no plasmid coded proteins are required for ColE1 replication (20,21), but does not discount the possibility that such gene products are normally involved in replication or can replace host proteins. Recently, Kahn and Helinski have shown that *in vitro* replication of the transmissible, multicopy plasmid R6K does require an R6K encoded protein (22).

Analysis of the products synthesized *in vitro* by both sucrose gradients and gel electrophoresis indicates that both fully and partially replicated molecules are formed during the reaction and that the newly synthesized DNA in the partially replicated molecules is not covalently attached to the template. Electron microscope studies of partially replicated molecules have, in fact, allowed us to map the origin and direction of RSF1030 replication (9). This system is dependent upon RNA polymerase, DNA polymerase(s) I and III and DNA gyrase, as has been reported in earlier systems. In addition, the products encoded by the genes dnaB, dnaC, dnaG, dnaP, and dnaI appear to be required for plasmid DNA replication.

The increased amounts of synthesis in this system make it ideal for studying individual steps of the replication reaction. For example, many of the dna_{ts} mutants contain low residual levels of activity, which represent enough synthesis so that the replicative intermediates can be isolated. Analysis of these DNA molecules can provide information about the stage of the reaction at which the protein is required. Staudenbauer has shown that in dnaZ extracts, the product is 7S and can be elongated into completed 26S molecules by further incubation with polymerase III

in the presence of rifampicin to prevent additional initiations (3). Although he has not analyzed this DNA, he has used it to study proteins required for its elongation. A similar approach will be helpful in analyzing residual activity in dnaI and dnaG mutants. In our hands, the levels of synthesis seen with polymerase I, dnaE, and dnaC mutants are probably too low to examine which gene products are involved at which step. However, we can isolate intermediates and add them to these mutant extracts in order to observe their fate. Future studies will be aimed at separating the reaction into stages, and looking at interactions of all the proteins involved.

In addition, the system can provide complementation assays for the purification of replication proteins. Using such an assay, we have partially purified the DnaP protein. This in vitro system will therefore be useful both for examining the role of individual proteins in replication and for purifying such proteins.

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Figure 1. Kinetics of RSF1030 DNA synthesis in Fractions I and II. Fractions I and II were prepared as described under Materials and Methods. The kinetics of synthesis were then measured using standard reaction conditions described under Materials and Methods.

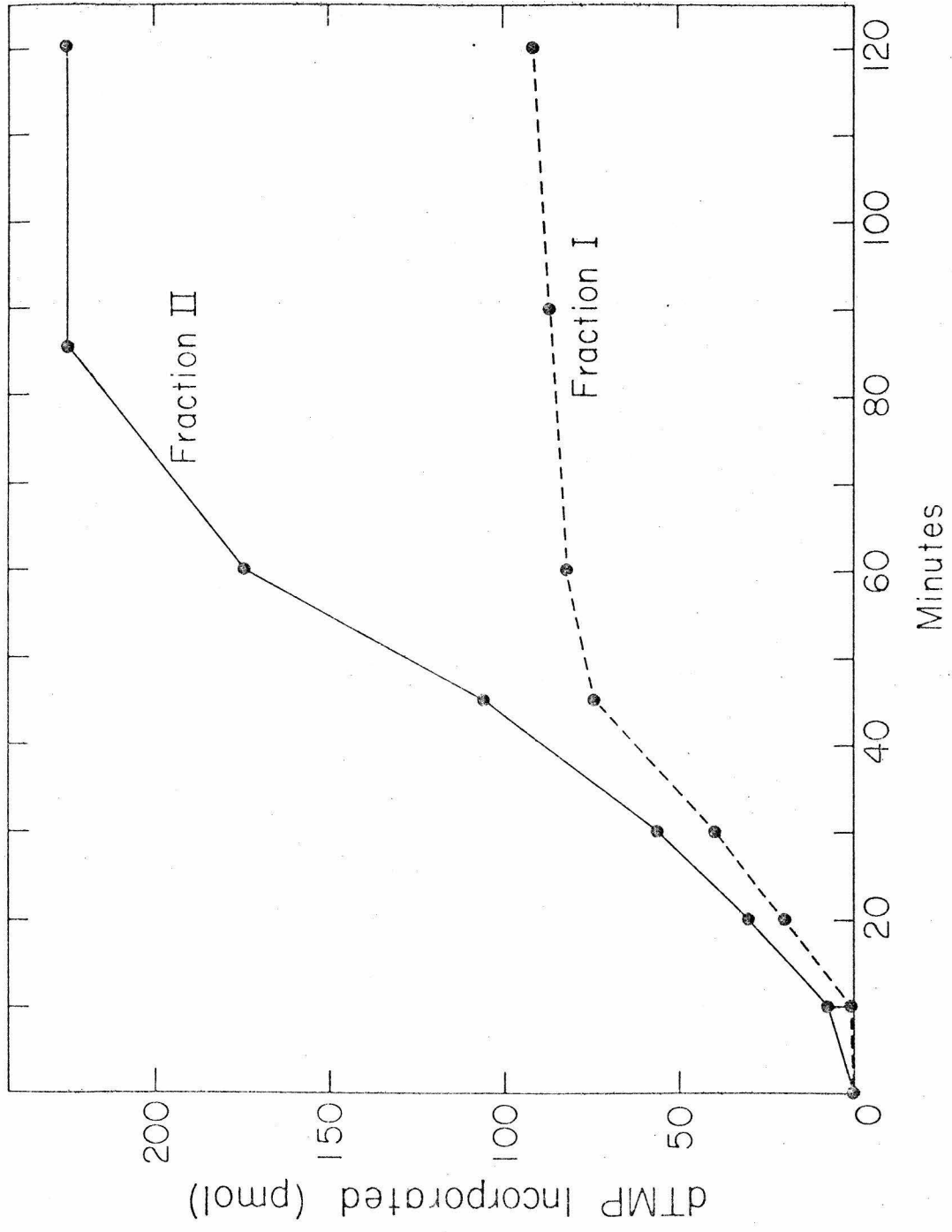


Figure 2. (a) Inhibition of synthesis in Fraction II by rifampicin. The kinetics of synthesis were measured in the standard reaction mixture. At times marked on the curves, rifampicin (10 $\mu\text{g/ml}$) was added to individual reaction mixtures and synthesis was allowed to continue in the presence of the drug. The extent of synthesis was determined at the times indicated on the abscissa. (b) Inhibition of synthesis in Fraction II by novobiocin. The kinetics of inhibition of synthesis by novobiocin (1 $\mu\text{g/ml}$) were measured as described for rifampicin.

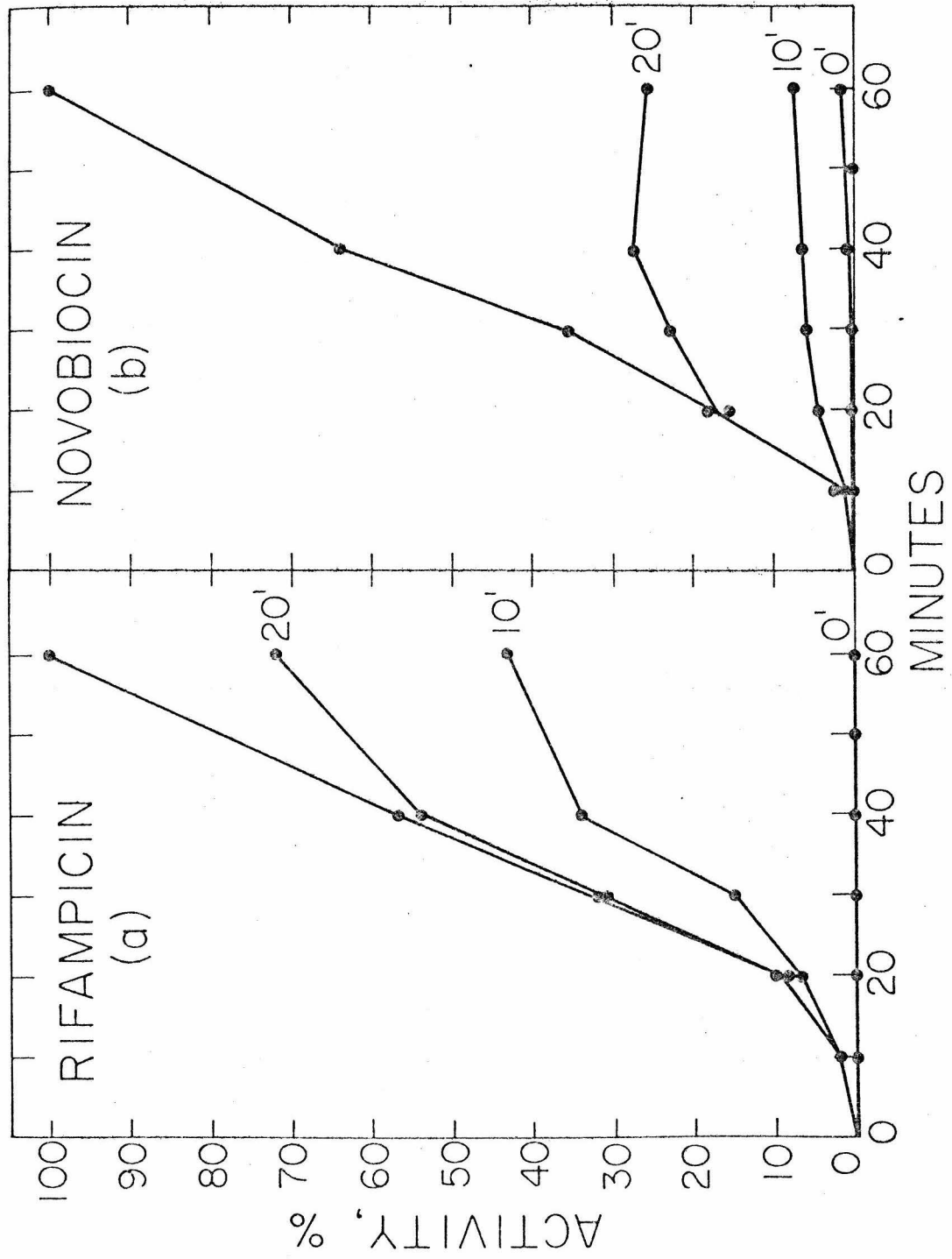


Figure 3. Effect of DNA concentration on the rate of DNA synthesis in Fractions I and II. Reactions were carried out in the standard reaction mixture except that the concentration of RSF1030 DNA was varied as shown.

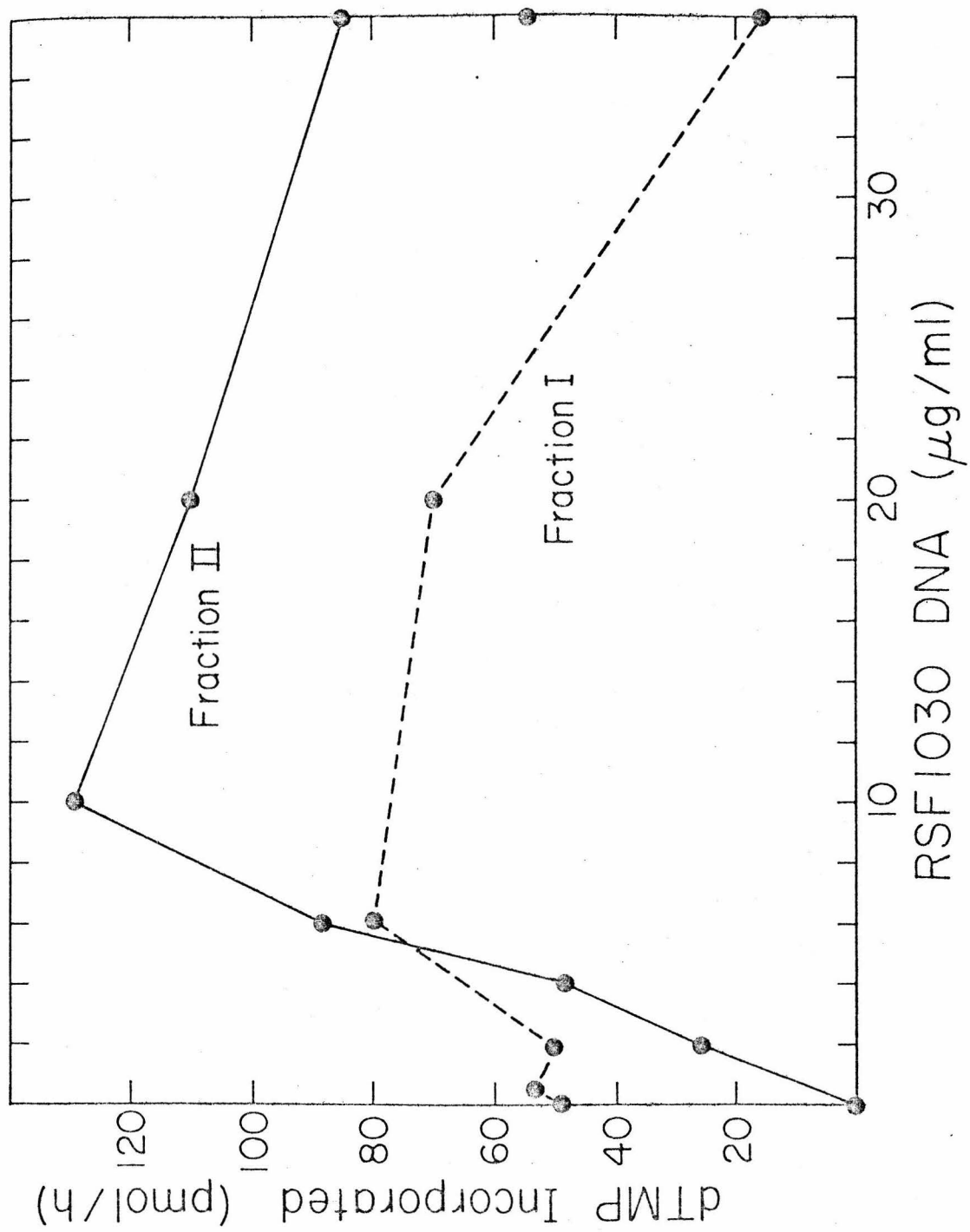


Figure 4. Sucrose gradient analysis of RSF1030 DNA synthesized in vitro. DNA was synthesized in an 0.1 ml reaction mixture containing [α - 32 P]TTP (259 cpm/pmol) at 30° for 60 min. The reaction was terminated by addition of EDTA and the reaction mixtures were extracted 1x with 0.2 ml phenol. 0.02 ml sodium acetate (10% w/v) was added and DNA precipitated by addition of 0.4 ml 95% ethanol followed by incubation at -70° for 10 min. The pellet was recovered by centrifugation, washed once with 70% ethanol (0°) and redissolved in 0.1 ml of 0.01 M HEPES-1 mM EGTA. An aliquot of each reaction was then analyzed by sedimentation through a neutral or alkaline sucrose gradient in an SW41 rotor at 35,000 rpm. A) neutral, 9 hr centrifugation; B) alkaline, 9 hr centrifugation; C) alkaline, 16 hr centrifugation. Sedimentation is from the right to left. [3 H]RSF1030 and [3 H]SV40 Form I and Form II were included in each gradient as internal markers.

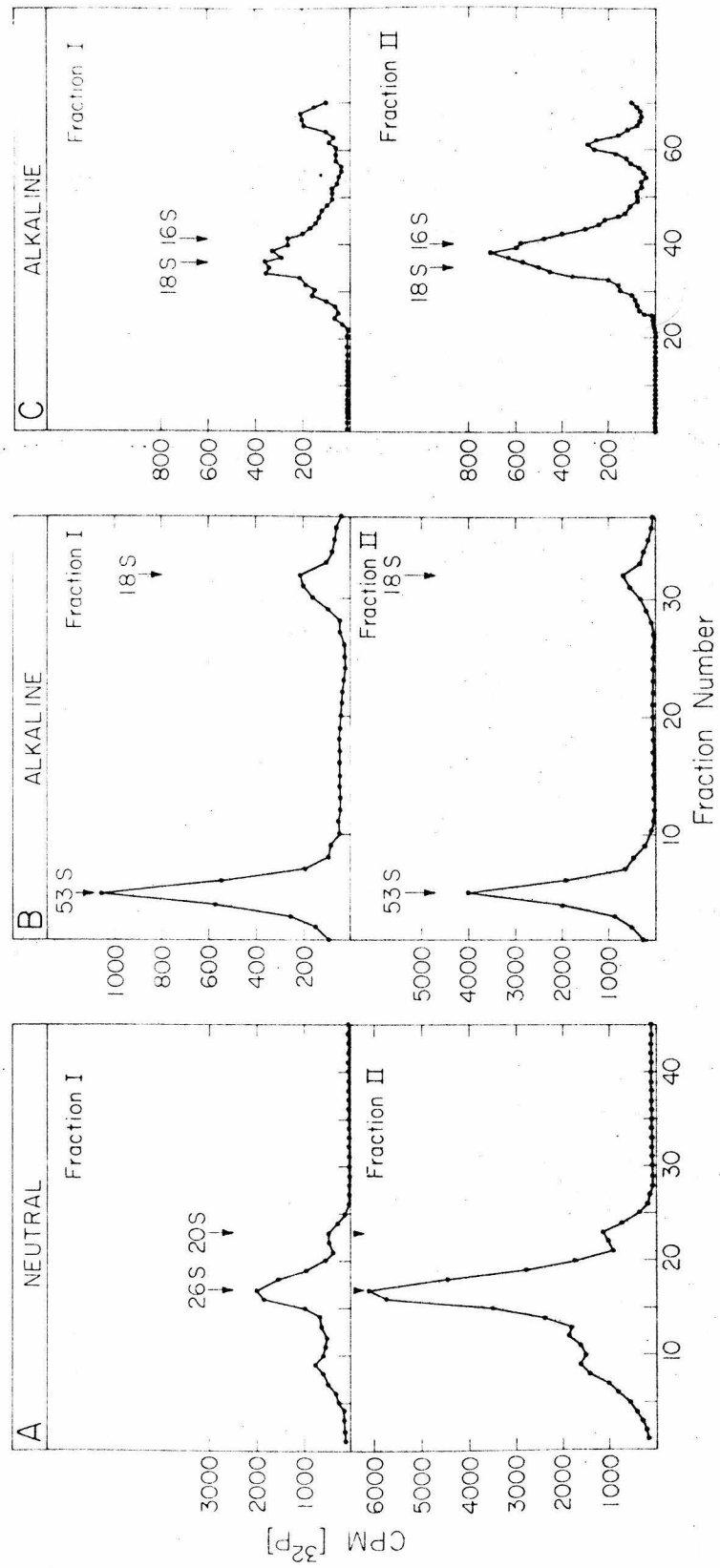


Figure 5. Agarose gel electrophoresis of RSF1030 DNA synthesized in vitro.

RSF1030 DNA was synthesized in an 0.1 ml reaction mixture for 1 hr at 30°. The reaction was terminated and DNA prepared for electrophoresis as in Fig. 4. One sample (0.05 ml) was heated at 90° for 90 sec before application to the gel. Samples were electrophoresed and gels autoradiographed as described previously (2) and in Methods. Lane 1, RSF1030 Form I and Form II markers, D + C are dimers and catenated dimers; Lane 2, products of RSF1030 synthesis stained with EtBr; Lane 3, DNA heated at 90° for 90 sec before electrophoresis; Lane 4, autoradiogram of Lane 2; Lane 5, autoradiogram of Lane 3.

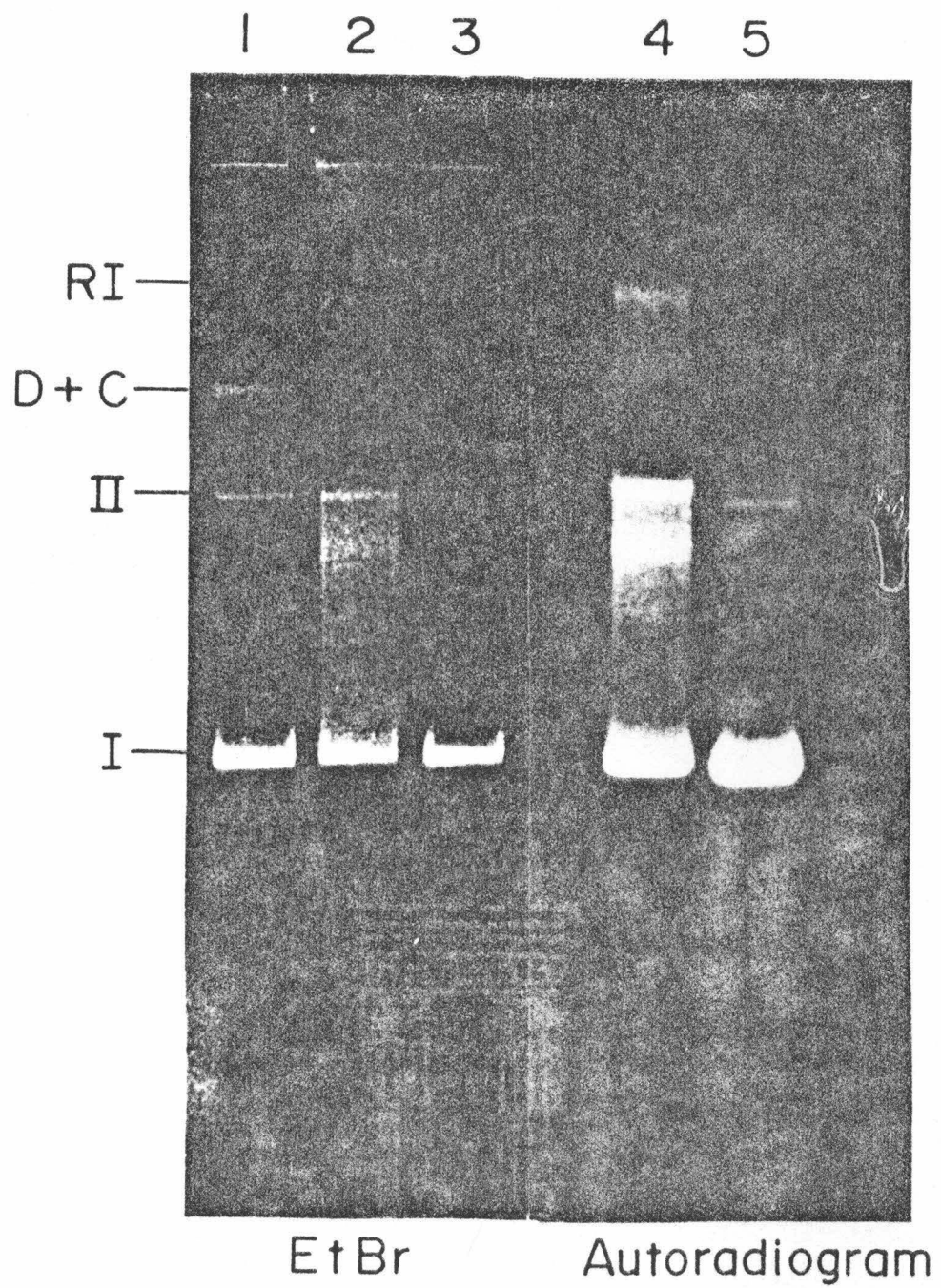


Figure 6. EtdBr-agarose gel electrophoresis of RSF1030 DNA synthesized. Samples of newly synthesized DNA were prepared for electrophoresis as in Fig. 6. The marker in Lane 1 represents RSF1030 Form I₀ which was prepared by incubating 300 pmol RSF1030 with 1 unit nicking-closing enzyme from HeLa cells for 1 hr at 37°. Lane 2, RSF1030 Form I and II as indicated; Lane 3, newly synthesized DNA stained with EtdBr; Lane 4, autoradiogram of DNA seen in Lane 3.

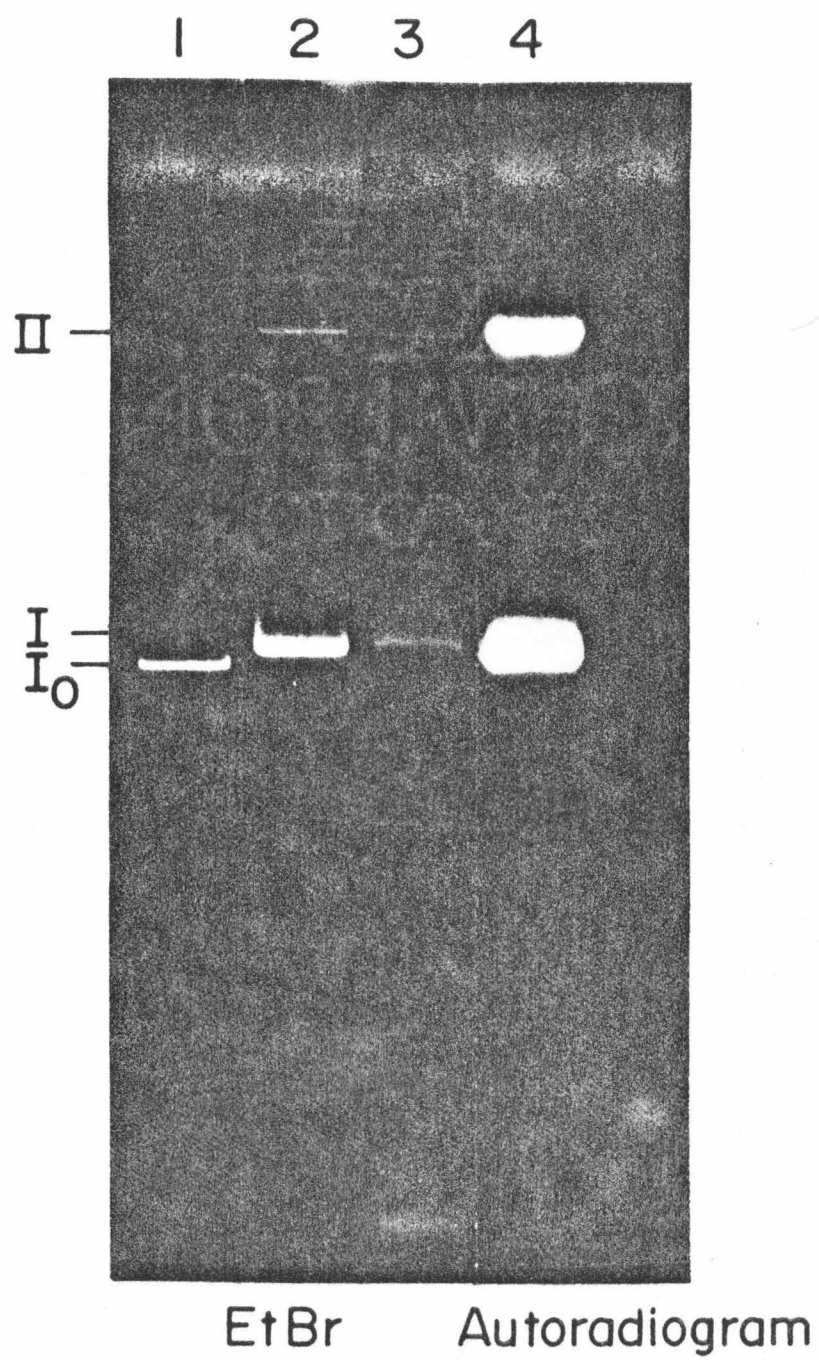


Figure 7. Complementation assay for DnaP protein. Standard reaction mixtures as described in Materials and Methods contained 10 μ l of an extract prepared from E. coli dnaP18. Increasing amounts of dnaP extract or polA extract were added as indicated and reaction mixtures incubated at 30° for 60 min.

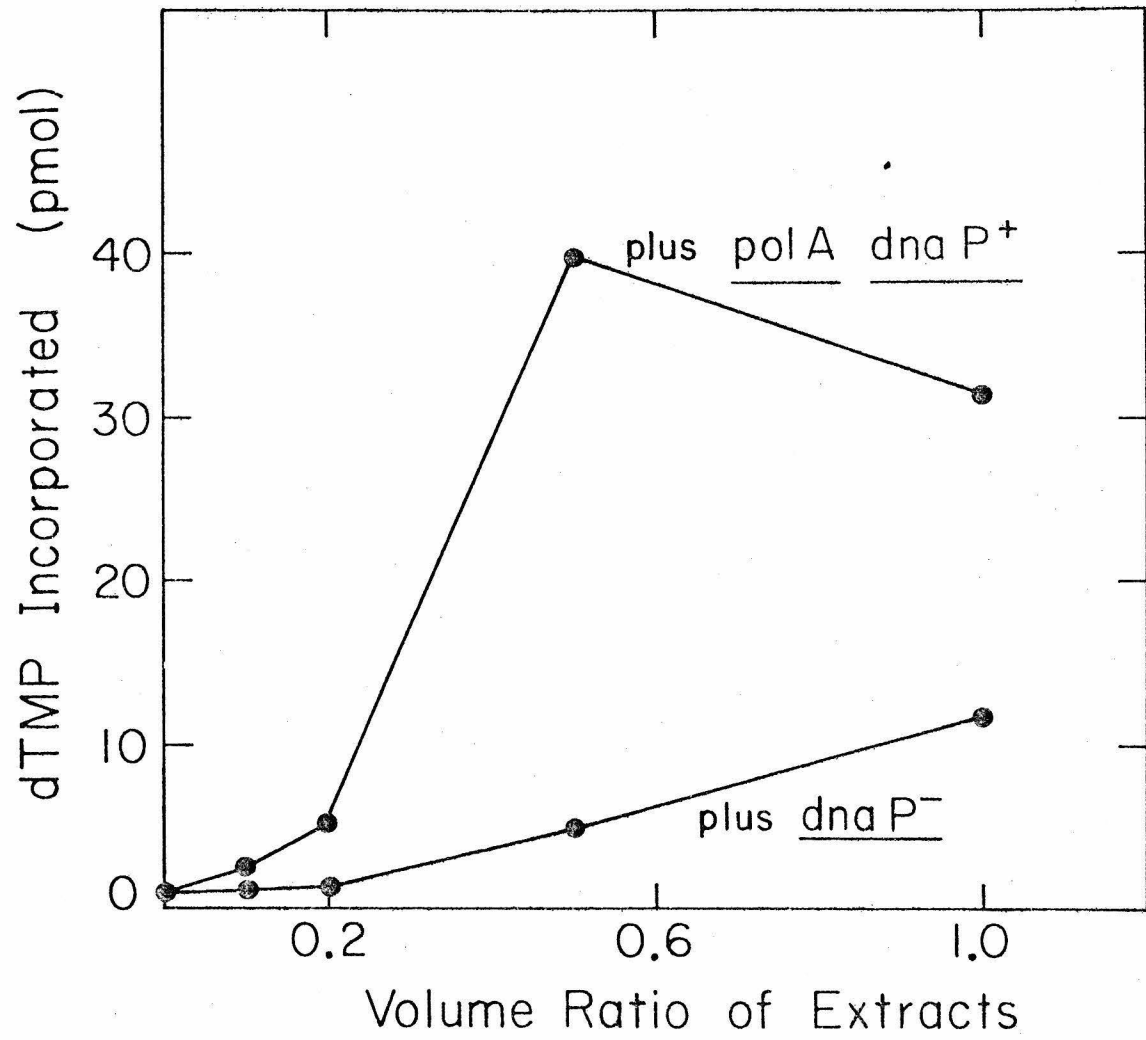
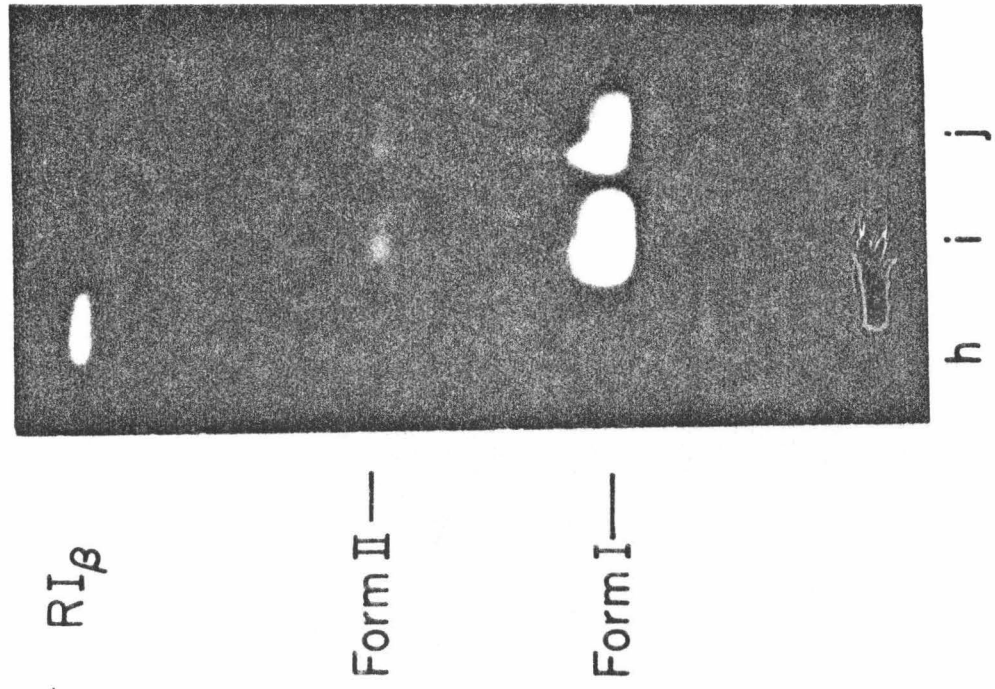


Figure 8. Agarose gel electrophoresis of RSF1030 DNA synthesized in vitro in dnaP mutants. Reaction conditions and sample preparation were as described in Fig. 5. a) Samples were electrophoresed directly and gels autoradiographed as described in Methods. b) Products were digested with restriction endonuclease Hinc II and run on a 1.4% agarose gel at 90 V for 5 hrs (22). The gel was stained and autoradiographed as described in Methods. Lanes h and k are wild type controls. Lanes i and l represent DNA synthesized in dnaP extracts using [α^{32} -P]dATP (10^9 cpm/pmol) as precursor. Lanes j and m represent DNA synthesized in dnaP extracts using [α^{32} -P]dTTP as precursor.

a)



b)

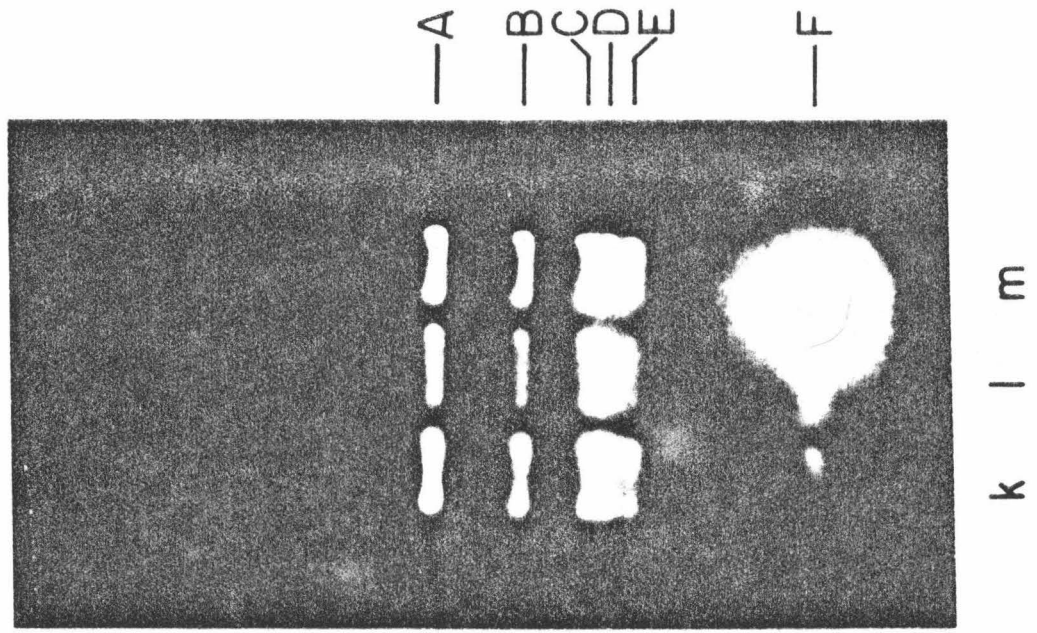


Table I
Strains Used

Strain Designation	Genotype	Source
CRT46	<u>dnaA</u> T46	Y. Hirota
PC5	<u>dnaA</u>	P. Carl
CR34/43	<u>dnaB</u>	Y. Hirota
PC1	<u>dnaC</u>	P. Carl
PC7	<u>dnaC,D</u>	P. Carl
PC2	<u>dnaC</u>	P. Carl
PC255	parent of PC1,2,5,7	P. Carl
BT1026	<u>polA1</u> <u>polC</u>	H. Bonhoeffer
E613	<u>polC</u> T486	Y. Hirota
HMS121	<u>polA1</u> <u>polB100</u> <u>polC1026</u>	J. Campbell
E101	<u>dnaF</u>	Y. Hirota
PC3	<u>dnaG</u>	Y. Hirota
WM437	<u>dnaI</u> 208	W. Messer
WM301	<u>dnaI</u> ⁺	W. Messer
KY2750	<u>dnaP</u> 18	T. Yura
KY2053	<u>dnaP</u> ⁺	T. Yura
DK517'	<u>polA</u> _{ts}	D. Kingsbury

Table II
Requirements for DNA Synthesis in vitro

Conditions	Fraction I	Fraction II
	(%)	(%)
Complete	100	100
- MgAc	< 0.3	< 0.4
- dCTP, dGTP, dATP	< 0.3	< 0.3
- dATP	N.D.	66
- dCTP or dGTP	N.D.	34
- rCTP, rGTP, rUTP	< 0.3	< 0.4
- rATP	< 0.3	< 0.3
- DNA	60-80	< 0.2
40 mM KCl	80	80
Rifampicin (5 µg/ml)	1	1
Novobiocin (1 µg/ml)	1	1
Nalidixic Acid (50 µg/ml)	43	40
Phenethyl Alcohol (0.5%)	9	8

DNA synthesis activity was measured in the standard assay as described in Materials and Methods, using either Fraction I or Fraction II prepared from *E. coli* W3110(RSF1030). In the complete system, 120 pmol and 200 pmol of [^3H]TMP represent 100% incorporation of [^3H]TMP into acid-insoluble product using Fraction I and Fraction II, respectively.

Table III

Requirements for E. coli Proteins for RSF1030 DNA Synthesis in vitro

Gene	Activity	
	Parent or Revertant	Mutant
	(pmol)	(pmol)
<u>dnaA</u>	129	131
<u>dnaB</u>	1.0	1.0
<u>dnaC</u>	70	1.5
<u>dnaE</u> (<u>polC</u>)	N.D.	1.0
<u>dnaG</u>	28	2.8
<u>dnaI</u>	128	11.7
<u>dnaP</u>	81	12.5
<u>dnaZ</u>	N.D.	3.5
<u>polA</u>	N.D.	1.0

Fractions I and II were prepared from a strain carrying the mutation indicated on the left. For dnaA, dnaC, dnaI, and dnaP, extracts were prepared from isogenic parental strains as controls. For dnaG and dnaB, revertants were prepared by plating liquid cultures in soft agar and incubating at 42°. Synthesis was measured in Fraction I and Fraction II, but only results using Fraction II are presented. In all cases, 20 µl of Fraction II were incubated in the standard reaction mixture containing 0.1 mM DTT, but without DNA, for 0, 10, 20, 30 or 40 min at 40°. RSF1030 DNA (10 µg/ml) was then added and synthesis measured for 60 min at 30°. The results with dnaA represent activity after 40 min at 40°. Values for all other strains represent synthesis after 0 min at 40°.

Chapter 4

Origin and Direction of Replication of Plasmid RSF1030

Reprint from Proc. Nat. Acad. Sci. USA 76, 736-740, 1979.

Abstract

An in vitro replication system has been used to study the origin and direction of replication of the covalently closed circular deoxyribonucleic acid of plasmid RSF1030, a non-conjugative R factor. We have enriched for replicative intermediates in these studies either by isolating them on the basis of their unique structure or by limiting the extent of synthesis in the in vitro system. Circular molecules that have replicated to varying extents migrate to characteristic positions in agarose gels, thus providing a rapid and efficient method for isolating partially replicated forms. Alternatively, replicative intermediates can be isolated directly from reaction mixtures that contain dideoxyTTP (ddTTP), a compound that limits the average extent of synthesis in vitro. Electron microscopic analysis of such intermediates linearized with either Hpa I or Bam H-I indicates that RSF1030 replicates in vitro from a unique origin located 70% from one end of Hpa I cleaved molecules and 47% from the Bam H-I site. The unidirectional mode of replication has been confirmed by the order in which the six Hinc II fragments of RSF1030 DNA are labeled in vitro when synthesis is limited to varying extents with ddTTP. Finally a physical map of RSF1030 has been constructed using the restriction endonucleases Bam H-I, Hpa I and Hinc II, and the origin and direction of replication have been defined relative to the map.

INTRODUCTION

The various known species of bacterial plasmids vary widely in their modes of replication. The plasmids Col E1 (1-3) and pSC101 (4), for example replicate unidirectionally from a unique origin. The conjugative plasmids R6K and RSF1040, however, possess two initiation and one termination sites. Replication of these plasmids usually proceeds exclusively from one or the other of the two initiation sites, although both are occasionally used simultaneously (5,6). These plasmids are unusual in that replication is asymmetrically bidirectional, that is, it begins at an origin and proceeds in one direction to the terminus before proceeding in the opposite direction from the same origin to the same termination point. Yet another plasmid, RSF1010, replicates either unidirectionally or bidirectionally with equal probability from a unique origin (7).

We have recently described an in vitro system capable of replicating the plasmid RSF1030 (unpublished data). RSF1030 is a non-conjugative plasmid of molecular weight 5.5×10^6 (8.3 kb), carrying the gene for ampicillin resistance on the transposable element Tn2 (8). In vivo replication of RSF1030 resembles that of Col E1 in several respects. Both replicate as a multicopy pool, and continue to replicate in the presence of chloramphenicol so that up to 3000 copies accumulate per cell. Replication is inhibited by rifampicin, and requires DNA polymerase I. In spite of these similarities, RSF1030 has no detectable sequence homology with Col E1 (9), so the plasmids appear to have evolved independently.

Synthesis in the in vitro system mimics in vivo replication faithfully in all respects that have been tested. Most striking are the similar protein requirements for in vivo and in vitro replication, including requirements for both RNA polymerase and DNA polymerase I. In addition, the proteins encoded by dnaB, dnaC, dnaG,

dnaP, polC (DNA polymerase III), dnaZ, dnaI, and DNA gyrase, all of which are required for E. coli DNA synthesis, are also required for plasmid replication in vitro. In this report, we show that RSF1030 replication in vitro begins at a unique origin and proceeds by an overall unidirectional mechanism. The origin of replication has been positioned on a restriction map of RSF1030 DNA, and the direction of replication has been established.

MATERIALS AND METHODS

Bacterial Strains, Media and Cell Growth

Extracts for the in vitro replication system were prepared from E. coli strain W3110 (RSF1030) grown in L Broth. Covalently closed circular RSF1030 DNA was prepared from strain HMS174 recA1 (RSF1030) grown in Vogel Bonner Medium.

Materials

Reagents and sources were as follows: Seakem Agarose (ME) from Marine Colloids, Inc.; deoxynucleoside triphosphates and ribonucleoside triphosphates from Sigma; 2',3' ddTTP from P-L Biochemicals; [α - 32 P]dTTP (300 Ci/mM) from New England Nuclear; nitrocellulose paper (0.45 μ m) from Millipore, chloramphenicol from Parke Davis; E. coli DNA polymerase I from C.C. Richardson; pancreatic DNase from Sigma; restriction endonucleases from New England Biolabs.

Preparation of DNA and in vitro Replication Extracts

The preparation of both in vitro replication extracts and the DNA used as a template in the reaction has been described previously (unpublished data).

Gel Electrophoresis and Restriction Enzyme Mapping

Agarose gels, 0.7 or 1.4%, were run in E buffer (40 mM Tris, 5 mM Na Acetate, 1 mM EDTA, pH 7.4) in slab gel apparatuses equipped with fans. Gels were stained with 0.1 μ g/ml EtBr in H₂O before photographing. DNA was removed from agarose gels by freezing a slice of the gel and then squeeze-thawing in parafilm and collecting the extruded buffer in a 50 μ l pipet. Gels that were to be autoradiographed were dried on a Peter Hoefer gel dryer. Films were preflashed and exposures were done at -70° with intensifying screens. Autoradiograms were scanned on a Joyce-Lobel densitometer.

Restriction enzyme digestions were carried out in the buffers suggested by New England Biolabs. Double-blotting was done according to the method of Sato, Hutchison and Harris (10).

Electron Microscopy

Samples were spread for electron microscopy by the formamide-Kleinschmidt technique (11). A Philips 300 electron microscope was used to take photomicrographs on 35 mm film from which molecule lengths were measured by overhead projection using a Hewlett-Packard 9280A calculator and a Hewlett-Packard digitizer.

RESULTS

Restriction Enzyme Mapping of RSF1030 DNA

A restriction enzyme map of the Bam H-I, Hpa I and Hinc II sites on RSF1030 DNA has been constructed. Both Bam H-I and Hpa I cleave RSF1030 DNA only once, giving rise to linear molecules. Hinc II cleaves the molecule into six fragments, the sizes of which are shown in Figure 1. Molecular weights were determined using Hpa I fragments of phage T7 DNA as size standards (12).

The map positions of the Hinc II fragments have been determined using the double blotting technique of Sato, Hutchison and Harris (10). A partial Hinc II digest of RSF1030 (10 μ g) was run on an agarose gel and transferred to nitrocellulose filter paper. A second gel, containing 1 μ g of a complete Hinc II digest that had been labeled with [α - 32 P]TTP in a nick translation reaction using pancreatic DNase and DNA polymerase I, was run. The DNA was transferred under hybridizing conditions from the second gel to the filter paper containing the partial digest, at a 90° angle to the first gel. By determining the combinations of 32 P-labeled fragments that

hybridize to each partial fragment, one can construct a restriction map. The sets of fragments DF, CDF, CDE, AF and BE existed in the set of partials, indicating a map order of A,B,E,C,D,F.

In order to position the Bam H-I and Hpa I cleavage sites relative to the Hinc II map, Bam H-I/Hpa I and Bam H-I/Hinc II double digests were analyzed by gel electrophoresis. The Bam H-I site is located within the Hinc II E fragment, and the Hpa I site is the same as the Hinc II site between Hinc II fragments C and D. Hpa I cleaves at a subset of Hinc II sites, due to the overlapping sequence recognitions of the two enzymes (13):



The restriction map derived from these results is shown in Figure 1.

Isolation of Replicative Intermediates

In order to characterize the mode of replication of RSF1030, replicative intermediates were studied by electron microscopy. Replicating molecules were prepared in an in vitro plasmid replication system that has been described in detail in Chapter 2. Synthesis was carried out using [α - ^{32}P]dTTP as described in the legend to Figure 2, and the products were run on a 0.7% agarose gel (Figure 2). The EtBr stained gel shown in lane b represents both template and product DNAs while the autoradiogram shown in lane c represents only product DNA. Labeled product is seen at the positions of supercoiled (Form I) and nicked circular duplex (Form II) DNAs, and in two additional bands labeled RI_{α} and RI_{β} . There is also a smear of radioactivity from the position of Form I to the position of the RI_{β} band (Figure 2).

DNA was extracted from the RI bands, as well as from intermediate positions between the Form I and RI_β bands and mounted for electron microscopy. Replicative intermediates appeared as relaxed circular molecules with replicated loops of various lengths. The extent of replication increased with decreasing distance of migration in the gel. Supercoiled intermediates were not seen, probably because the gel had been stained and photographed under conditions that nick DNA. The fact that DNA extracted from the supercoiled Form I band also appeared as relaxed circles in the electron microscope supports this contention. On the other hand, under the conditions of the formamide-Kleinschmidt method used, negative superhelical Form I DNA may be partially unwound and this could account for the open appearance of the closed molecules. This result, in addition to our previous findings, establishes that RSF1030 molecules are not replicating by a rolling circle mechanism.

Electron Microscope Studies on the Origin and Direction of RSF1030 Replication

Replicative intermediates were linearized with either Hpa I or Bam H-I for electron microscope studies of the origin and direction of replication. Replicating molecules were either extracted from agarose gels as described above or obtained by carrying out the in vitro DNA replication reaction in the presence of various amounts of ddTTP. Since ddTTP halts DNA chain growth when it is incorporated, this is an efficient way to limit the extent of DNA synthesis to varying degrees. When ddTTP was used, reaction mixtures were phenol extracted and the products digested with the appropriate restriction enzyme before being mounted for electron microscopy.

Molecules isolated from agarose gels, cleaved with Bam H-I and observed by electron microscopy are shown in Figure 3a-e in order of increasing extents of

replication. Replication begins at a point 47% from one end of the linear molecules. Molecules that are greater than about 50% replicated appear as double forks, or H forms, as seen in Figure 3d and 3e. From an analysis of 89 molecules, replication appeared to be unidirectional. It was difficult to orient the molecules, however, since the origin is located near the center of Bam H-I cleaved DNA. For this reason, replicating molecules were cleaved with Hpa I and examined. Replicative intermediates were generated with ddTTP, cleaved with Hpa I and observed by electron microscopy. Molecules containing internal loops were scored. The lengths of both unreplicated arms and the average length of the replicated loop were measured. In molecules that were < 5% replicated, the unreplicated arms were approximately 70% and 30% of the total length. The long arm was designated L_1 , and the short arm L_2 . Figure 4 is a plot of the lengths of L_1 and L_2 measured as a percentage of the total length of the molecule versus the extent of replication. The length of L_1 decreases as replication proceeds, while the length of L_2 stays constant until the molecule is 70% replicated. Thus, replication is unidirectional for at least 70% of the molecule.

These data indicate that in this in vitro system, replication of RSF1030 DNA begins at a unique origin located 70% from one end of Hpa I cleaved molecules and 47% from one end of Bam H-I cleaved molecules, and proceeds by a unidirectional mechanism for at least 70% of the molecule. We have observed only four molecules that are greater than 70% replicated. These include one Hpa I cut molecule with the termination point 9% of the molecular length away from the origin, and three Bam H-I generated double-forked molecules like the one shown in Figure 3e. The structures of these molecules suggest that the termination point is, in fact, at or near the origin of replication.

The origin of replication has been positioned on the restriction map shown in Figure 1. A unique position can be determined because the Hpa I and Bam H-I sites were physically mapped relative to the Hinc II cleavage sites. The Bam H-I site is approximately 50% and the Hpa I site approximately 70% of the molecular length of RSF1030 away from the origin in the direction of replication.

Gel Electrophoresis Mapping of the Origin and Direction of Replication

We have also studied the origin and direction of RSF1030 replication by determining the order in which the Hinc II fragments are labeled in vitro. In vitro DNA synthesis reactions were carried out in the presence of different levels of ddTTP in order to allow replication to proceed to 16, 19, 29, 53, and 76% of the maximum obtained in the absence of ddTTP. Products were digested with Hinc II, run on agarose gels, stained with EtdBr, photographed and autoradiographed. The results are shown in Figure 5. The EtdBr-stained gel (5a) shows the normal Hinc II pattern of six bands regardless of the extent of replication. In addition, at greater extents of replication a new, high molecular weight band designated A' appears (lanes k and l). The autoradiogram in Figure 5b shows a very different pattern. The first material to be labeled (lane h) appears in a diffuse band above the position of the A fragment. This material probably represents fragments containing replication bubbles. Next, (lane i), the amount of diffuse label increases and extends up to fragment A', and fragment B becomes labeled. A' and E both appear as heavily labeled bands in lane j. Densitometer tracing of the autoradiogram (lane j) indicates that the C fragment has a higher specific activity than D at this extent of replication. Thus, the order in which the first three fragments are labeled is BEC, and is consistent with the unidirectional mode of replication proposed from electron microscope studies.

According to the electron microscope mapping, however, the origin of replication lies within the A fragment, so the A fragment should be the first to be labeled. In fact, significant label does not appear in the A fragment until lane k, where 53% of maximal synthesis has occurred. The appearance of the new band A', however, accounts for this apparent discrepancy. If the origin is within the A fragment, and if replication is completely unidirectional, then the A fragment would not be completely replicated until the entire molecule was replicated. The fact that significant label does not appear in the A fragment until fragment F, the last one predicted to be replicated by a unidirectional mechanism, is also labeled (lane k), suggests that the origin of replication does lie within the A fragment and that replication is completely unidirectional. We suspected that the fragment A' was a branched molecule that had been replicated from the origin to the AB junction, but not in the direction of the AF junction. A Hinc II digest of newly replicated DNA was therefore carried out and the products run on an agarose gel with (lane m) and without (lane n) prior denaturation and subsequent renaturation (Figure 5c). If A' is in fact a branched molecule, then it should disappear upon denaturation. This does occur and a new band appears between the A and B fragments. We conclude that this is the fragment covering the region between the origin and the AB junction. Several other faint bands that have not been characterized appear between the E and F fragments. These data are consistent with the interpretation that replication begins within the A fragment and moves clockwise around the map. If there is any counterclockwise synthesis, it is limited to the region between the origin of replication and the AF junction.

DISCUSSION

The origin and direction of plasmid RSF1030 replication *in vitro* have been assigned by both traditional electron microscopic procedures and by novel applications of agarose gel electrophoresis, restriction enzymology and the DNA synthesis inhibitor, ddTTP. *In vitro* replication begins at a unique site on the DNA. The origin of replication used *in vitro* has been located on a restriction map, and replication has been shown to proceed by an overall unidirectional mechanism. The results do not, however, rule out the possibility of some synthesis in the opposite direction. In fact, among 89 Bam H-I cleaved molecules examined, two were seen with a replication loop spanning the putative origin of replication. These could have been generated either by partially bidirectional synthesis, incorrect initiation or random breakage of the DNA. In addition, six molecules were seen with a replication loop at a site other than the usual origin of replication. These also could be the products of incorrect initiation or of breakage of the DNA at sites other than the Bam H-I site during extraction from the gel. Since the Bam H-I digestion was not complete, molecules broken at random sites could still be full length, and would therefore be scored during electron microscope mapping. The majority of the evidence, however, including the structures of very late replicating intermediates, indicates that replication is unidirectional.

It is not known whether the origin and direction of RSF1030 replication *in vitro* are identical to those used *in vivo*, since the *in vivo* events have not been characterized. In the case of Col E1, replication *in vivo* (1,2) and *in vitro* (3) begins at a unique origin and proceeds unidirectionally, so that the *in vitro* events do mimic those occurring *in vivo*. We have shown previously (unpublished data) that RSF1030 replication *in vitro* corresponds to that seen *in vivo* in requiring both

DNA polymerase I and RNA polymerase (9,14). In addition, at least eight other proteins known to be required for host chromosome replication are also required for in vitro RSF1030 replication (unpublished data). We therefore anticipate that in vivo replication of this plasmid will begin at the same origin used in vitro and proceed primarily by a unidirectional mechanism.

RSF1030 resembles Col E1 in many of its replicative properties. Both are present as multicopy pools in log phase cells (9,15), and continue to replicate in the presence of chloramphenicol so that up to 3000 copies accumulate per cell. The two plasmids are also similar in their requirements for DNA polymerase I (14) and RNA polymerase (9,16). We can now add a unidirectional mode of replication to this list of similarities. Although these two plasmids appear unrelated on the basis of sequence homology, only future experiments will determine if they share some limited common sequences and/or structural features near the origin of replication.

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Figure 1. Restriction map of RSF1030 DNA. Sizes of restriction fragments were determined using a T7/Hpa I digest as standards. Molecular weight determinations were carried out on a 30 cm, 1.5% agarose gel run at 60 V for 6 hrs. Details of the mapping procedures are given in the text.

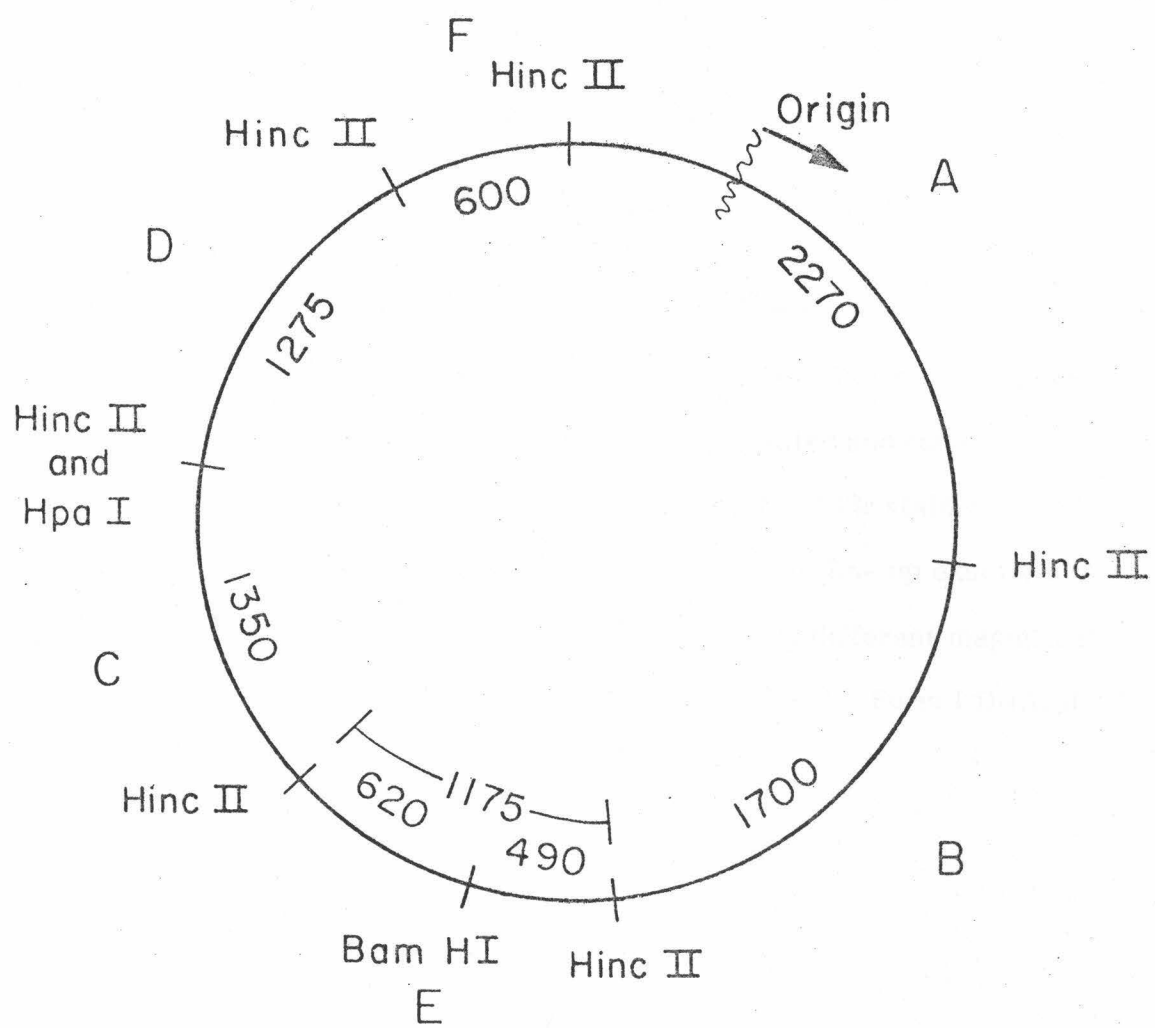


Figure 2. Agarose gel electrophoresis of RSF1030 DNA synthesized in vitro.

Extracts for in vitro DNA synthesis reactions were prepared from chloramphenicol treated cultures of *E. coli* W3110(RSF1030). Cells were lysed by lysozyme treatment followed by freezing and thawing and centrifugation to remove chromosomal DNA. FxII was prepared by precipitation of endogenous plasmid DNA with streptomycin sulfate followed by ammonium sulfate precipitation of the streptomycin supernatant. Details of the procedure will be published elsewhere. RSF1030 DNA was synthesized in a 0.05 ml reaction mixture containing 0.02 ml of W3110/RSF1030 FxII, 40 mM HEPES, pH 8.0, 100 mM KCl, 12 mM magnesium acetate, 50 μ g each dCTP, dGTP, dATP, and [α -³²P]dTTP (200 cpm/pmole), 2 mM ATP, 0.5 mM each rCTP, rGTP, rUTP, and 0.5 μ g of RSF1030 DNA. The reaction was carried out for 60 min at 30°C. Samples were phenol extracted-ethanol precipitated and run on a 0.7% agarose gel at 40 V for 12 hrs. (a) RSF1030 marker, (b) EtBr stained gel, (c) autoradiograph of (b). The bands in the autoradiograph do not line up exactly with those in the stained gel since the pictures are at slightly different magnifications. Symbols: RI_{α} and RI_{β} = Replicative Intermediate DNAs; I = Form I DNA; II = Form II DNA; D + C = Dimers plus catenanes.

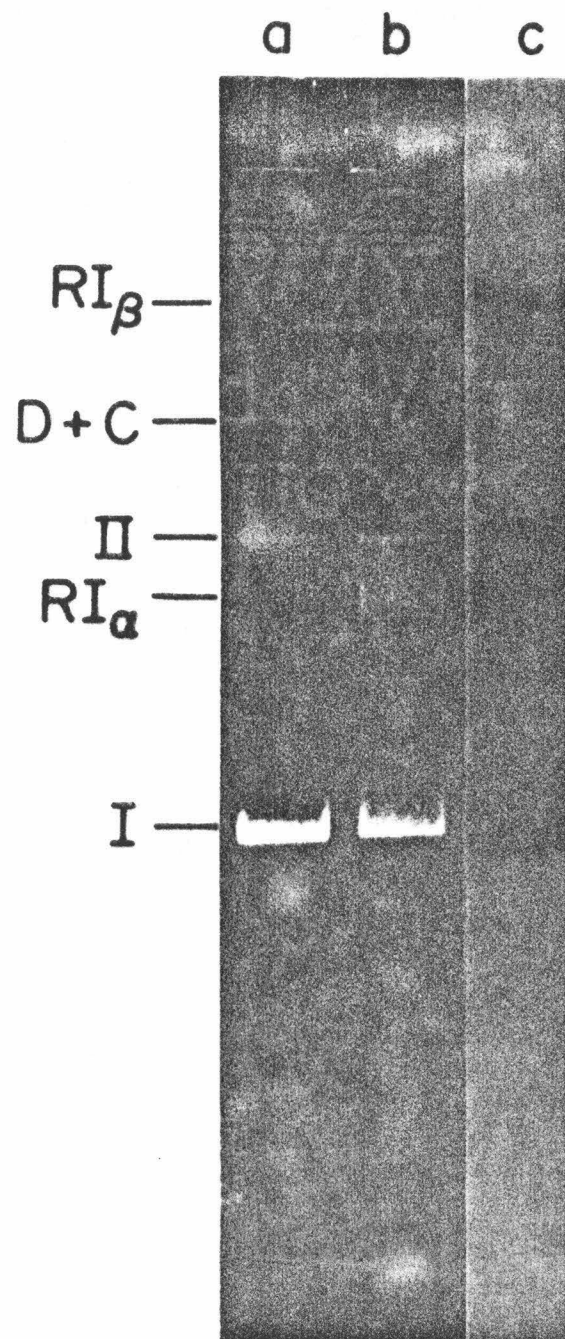


Figure 3. Electron micrographs of replicative intermediates cleaved with Bam H-I. Replicating molecules were prepared as described in the text. Molecules are arranged (a-e) in order of increasing extents of replication. Magnification is 1 cm = 0.16 μ m (a) or 1 cm = 0.311 μ m (b-e).

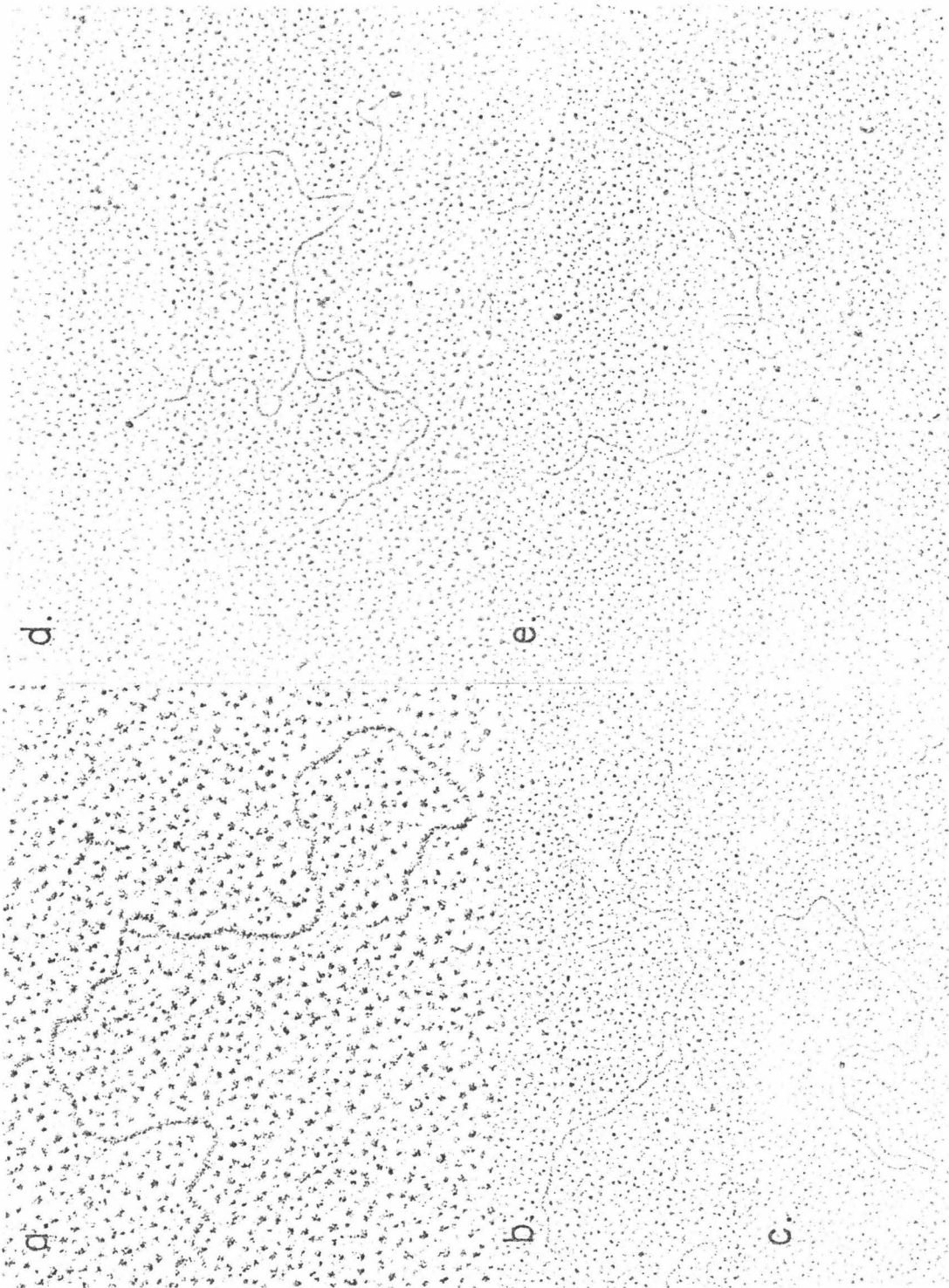


Figure 4. Analysis of the mode of replication of RSF1030. An in vitro DNA replication reaction was carried out using FxII in the presence of 25 μ M ddTTP. Hpa I cleaved replicating molecules with internal loops were photographed and measured. L_1 and L_2 are the unreplicated arms, and R_1 and R_2 are the two halves of the replicated loop. The total length of a molecule with an internal loop is then:

$$T = L_1 + L_2 + \frac{R_1 + R_2}{2}$$

The extent of replication is $\frac{R_1 + R_2}{2T}$. Symbols: (●) L_1 ; (○) L_2 .

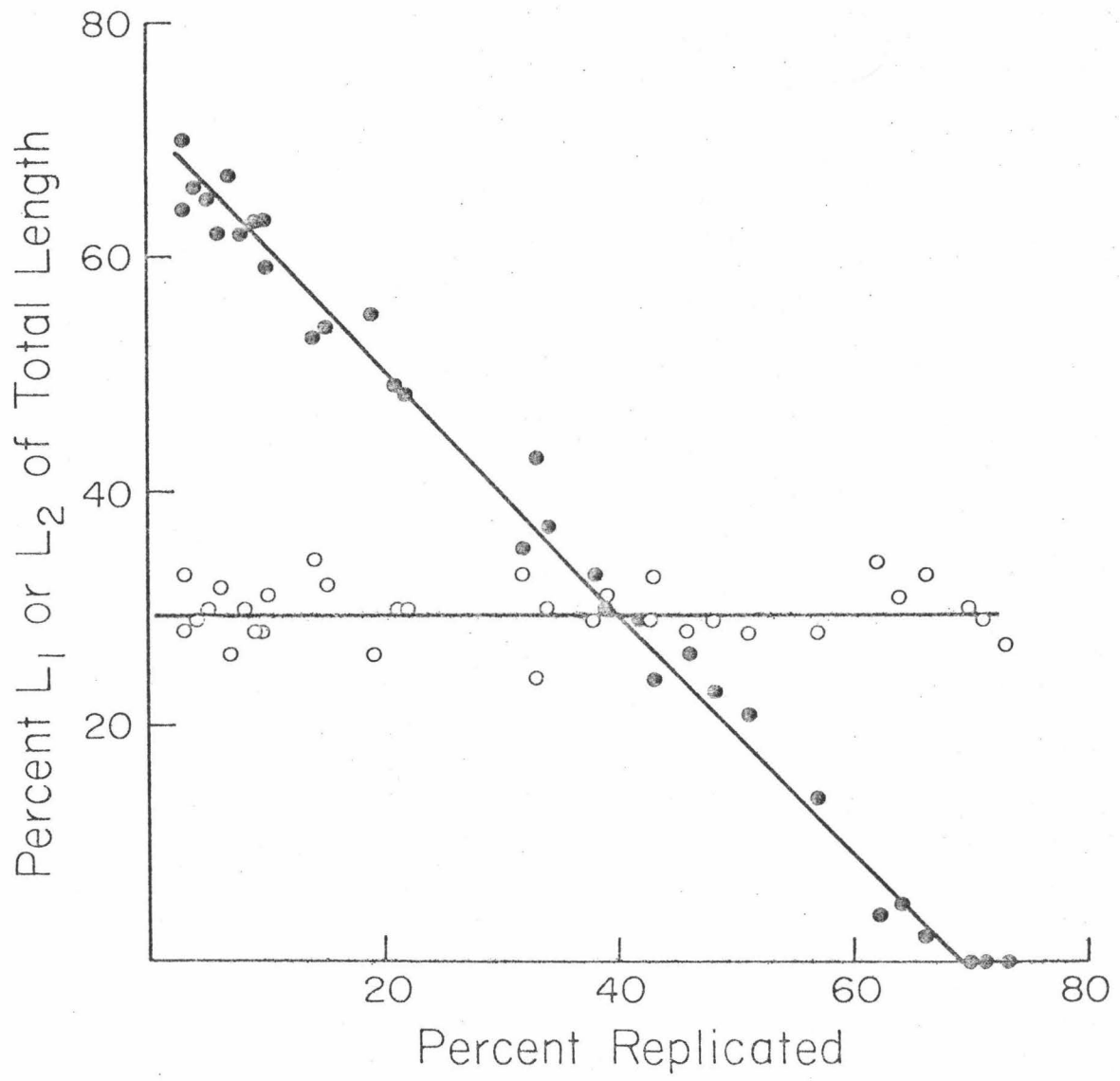
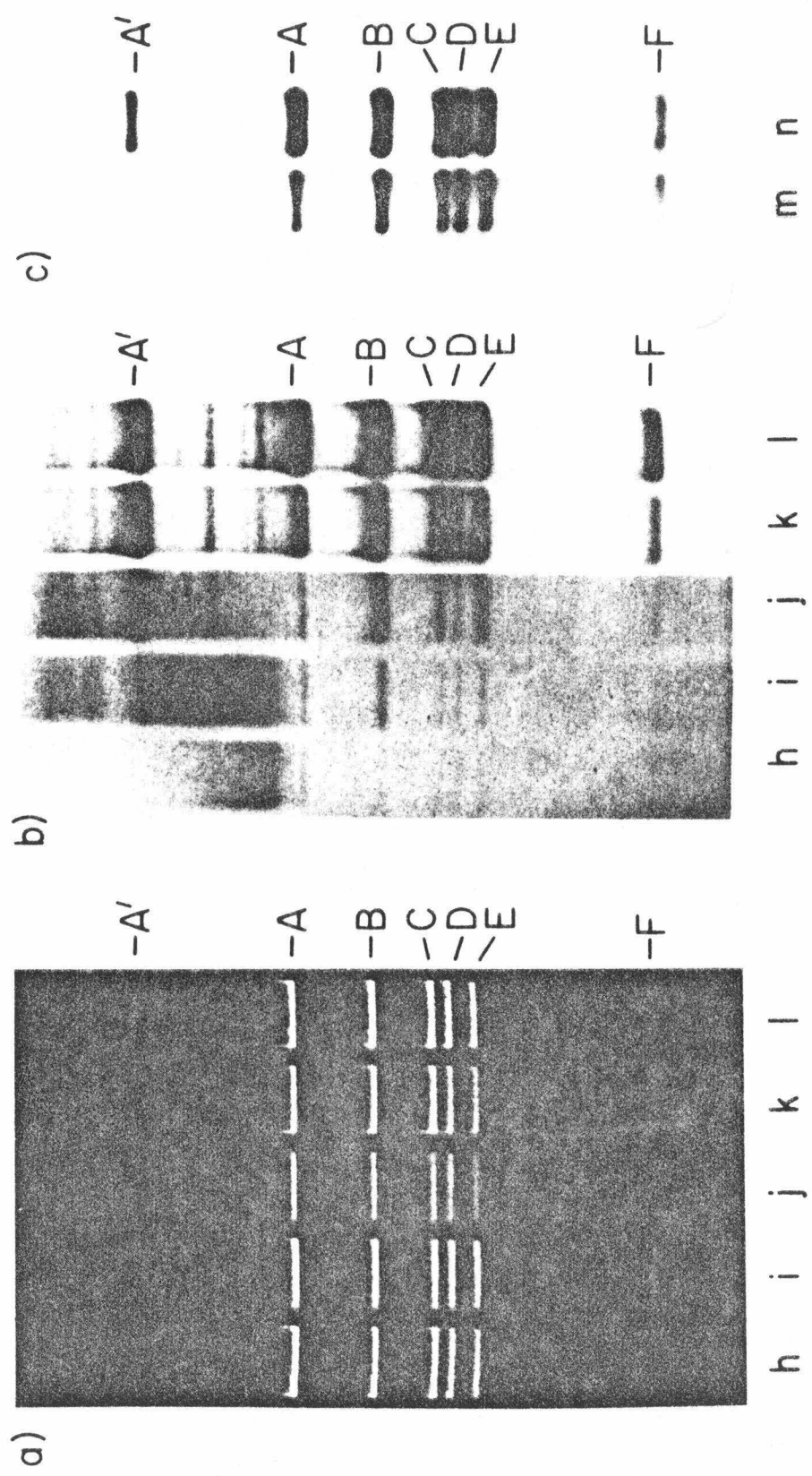


Figure 5. Analysis of replicative intermediates by gel electrophoresis. In vitro DNA synthesis was carried out in the presence of varying levels of ddTTP, as indicated, and of [α - 32 P]TTP (300 cpm/pmole). Products were digested with restriction endonuclease Hinc II and run on a 1.4% agarose gel at 90 V for 5 hrs. The gel was stained (5a) and autoradiographed (5b). Levels of ddTTP and percentages of maximal synthesis were: h = 250 μ M, 16%; i = 100 μ M, 19%; j = 50 μ M, 29%; k = 25 μ M, 53%; l = 10 μ M, 76%. (5c) shows a Hinc II digest of the products of in vitro DNA synthesis in the absence of ddTTP with (m) and without (n) prior denaturation. DNA was heated to 100°C for 3 min in gel buffer, and reannealed for 10 min at 65° before being loaded on the gel.



Chapter 5

Role of Plasmid Coded RNA and Ribonuclease III in Plasmid DNA Replication

Summary

An in vitro replication system has been used to study the control of DNA replication of the relaxed plasmids ColE1 and RSF1030. An RNA transcript approximately 100 nucleotides long is synthesized during the in vitro DNA replication reaction. This RNA is synthesized approximately 450 base pairs away from the origin of replication. A small insertion in the coding sequence for the RNA made from ColE1 DNA leads to a larger RNA species and simultaneously to an increase in plasmid copy number. Revertants missing the specific insertion show shorter RNA transcripts and wild type copy number. Although plasmids ColE1 and RSF1030 have no extensive sequence homology, the RNA synthesized during RSF1030 replication has almost the same mobility as the ColE1 RNA on polyacrylamide gels and hybridizes to the ColE1 origin region. Extracts prepared from mutants of Escherichia coli deficient in ribonuclease III do not replicate RSF1030 or ColE1 plasmids in vitro. When supplemented with homogenous RNase III, such extracts do support DNA replication on these templates, indicating that RNase III is required for DNA replication. We propose that the 100 nucleotide RNA species is involved in regulating the initiation of DNA replication of these plasmids, and that RNase III may be involved in processing this RNA.

Introduction

A unique feature of bacterial plasmids is their ability to replicate autonomously and to be distributed in an orderly fashion to daughter cells during cell division. The number of copies of plasmid per cell is carefully controlled and characteristic of each plasmid type. Both ColE1 (Helinski, 1976) and RSF1030 (Crosa, Luttrup, and Falkow, 1975) are small, non-transmissible plasmids that replicate under relaxed control, meaning that they are present in multicopy pools of 10-30 copies per chromosome. They also continue to replicate in the presence of chloramphenicol so that several thousand copies accumulate per cell. The biochemical mechanism of this type of control is the subject of studies to be presented here.

Two independent groups have recently described high copy number mutants of this class of plasmid that are maintained at 100-200 copies per chromosome in log phase cells (Gelfand, Shepard, O'Farrell, and Polisky, 1978; Heffron, So and McCarthy, 1978). Heffron et al. (1978) have constructed a high copy number mutant of plasmid RSF1050, an ampicillin resistant derivative of ColE1 that contains a single Eco RI site. The mutant, pFH118, was generated by a technique of in vitro mutagenesis that employs synthetic oligonucleotide Eco RI sites as mutagens. Circular plasmid DNA is linearized, synthetic Eco RI sites are ligated onto the ends, and the DNA is recircularized. The resulting DNAs thus contain an additional Eco RI site at the position of the mutation. This has allowed the copy number mutation to be localized to a region several hundred base pairs upstream (in the 5' direction to the direction of replication) from the origin of replication, defined as the site at which DNA synthesis begins. Levine and Rupp (1978, unpublished results) have recently identified and determined a partial nucleotide sequence

of a 100 nucleotide RNA that is transcribed from the general region containing the copy number mutation in pFH118. Backman, Betlach, Boyer and Yanofsky (1978) have isolated a 580 base pair fragment that is both necessary for and sufficient to determine ColE1 type replication. This fragment extends from a site 13 base pairs downstream from the origin of DNA replication to a site approximately 30 base pairs past the coding sequence for the 100 nucleotide RNA transcript. Large insertions within the 580 base pair fragment, including ones in the RNA coding sequence, interfere with ColE1 type replication in ColE1-pSC201 recombinant plasmids, although small insertions can be tolerated. In this communication we have used an in vitro DNA replication system to demonstrate that the mutation in the copy number mutant pFH118 is due to an insertion within the coding sequence for the small RNA transcript. Reversion to a low copy number phenotype occurs by deletion of this specific insertion, confirming that the RNA transcript does play a role in determining plasmid copy number.

We have also shown that ribonuclease III is required for plasmid DNA replication. RNase III cleaves double stranded RNA preferentially, and its action on single stranded RNA indicates that it cleaves regions of secondary structure (Dunn, 1976). Ribonuclease III is known to act as a processing enzyme in several systems. It is required in *E. coli* for the processing of 30S ribosomal RNAs (Dunn and Studier, 1973; Nikolaev, Silengo, and Schlessinger, 1973) and has been shown to cleave the polycistronic transcript of phage T7 early genes to yield five specific messages (Dunn and Studier, 1975; Dunn, 1976). The requirement shown here for RNase III in in vitro plasmid DNA replication suggests that RNA processing might be significant in DNA replication.

Results

Detection of a 100 Nucleotide Transcript Synthesized During DNA Replication *in vitro*

Using an *in vitro* replication system that carries out extensive, DNA-dependent replication of plasmids RSF1030 and ColE1 (Conrad, Wold and Campbell, 1979), we looked for production of the 100 base pair RNA previously described by others (Levine and Rupp, 1978). The replication properties of RSF1030 are very similar to those of ColE1 in that it is present in multicopy pools in the cell, continues to replicate in the presence of chloramphenicol (Crosa et al., 1975), requires DNA polymerases I and III (Falkow, 1975) and RNA polymerase (Crosa et al., 1975), and replicates unidirectionally from a unique origin (Conrad et al., 1979). However, RSF1030 and ColE1 have been thought to have no sequence homology (Crosa et al., 1975; see below).

An *in vitro* replication reaction was carried out in a reaction mixture containing [α - 32 P]UTP and either ColE1, RSF1050, or RSF1030 DNA as described in Experimental Procedures. Extensive RNA synthesis is observed. In a typical 0.05 ml reaction mixture containing 0.02 ml extract, 1-2 nmols of total ribonucleotides are incorporated into acid insoluble material in a 10 minute incubation at 30°. If plasmid DNA is omitted, less than 100 pmol is incorporated, indicating that RNA synthesis requires a plasmid DNA template. The RNA products were analyzed by electrophoresis on 10% polyacrylamide gels after phenol extraction and ethanol precipitation of reaction mixtures. An autoradiogram of such a gel is shown in Figure 1. The position of a non-radioactive *E. coli* 5S RNA marker run on the same gel is indicated. A prominent band is seen in each lane just below

the position of the 5S marker. As shown below, this is the 100 nucleotide transcript described in previous studies.

Mapping of the RNA Transcripts of ColE1 and RSF1030

We have located the coding region for the 100 nucleotide transcript in each of the plasmids we are studying using the blot-hybridization technique of Southern (1975). ^{32}P -labeled RNA was synthesized in the in vitro replication system using ColE1 DNA as template. The reaction mixture was phenol extracted and ethanol precipitated and the total RNA synthesized was hybridized to a Hae II digest of ColE1 DNA run on an agarose gel. Hae II cleaves ColE1 DNA into six fragments. A restriction map of the origin region of ColE1 DNA is shown in Figure 2. The RNA hybridizes most strongly to the A fragment, but also hybridizes to the C and E fragments (Figures 3a and b). Fragment A contains the region coding for the 100 nucleotide transcript described by others, and Fragment E contains the origin of replication of ColE1. To determine if this hybridization was due to the 100 nucleotide RNA transcript, the RNA was purified by extracting it from a 10% acrylamide gel (see Figure 1). The purified RNA also hybridizes to the Hae II-A fragment and, very slightly, to the E fragment (Figure 3c).

The sites of hybridization of the purified 100 nucleotide transcript have been mapped more precisely using additional restriction enzymes. ColE1 DNA was digested with the restriction endonuclease Hae II and run on a 1.4% Seaplaque agarose gel. Seaplaque agarose melts at 60°, and remains in a liquid state at 37° (Parker and Seed, 1979). Bands can therefore be cut out of gels, melted, and a second digestion carried out in the agarose. The ColE1 Hae II-A fragment was redigested with Hae III, giving rise to the four fragments diagrammed in Figure 2.

Band C' is the same as fragment F of a Hae III digest of ColE1, and according to the map positions and the sequencing data of Backman et al. (1978) should contain the sequence homologous to the 100 nucleotide transcript. Purified 100 nucleotide transcript hybridizes only to the C' fragment in a Hae III digest of the Hae II-A fragment (Figure 4a), indicating that our prominent RNA is indeed the 100 nucleotide RNA described by others.

The RNA transcribed from RSF1030 DNA is approximately the same size as that transcribed from ColE1 and hybridizes to the restriction fragment containing the origin of RSF1030 replication (Conrad, Wold and Campbell, 1979, and Figure 5a). We therefore looked for homology in the RNAs from ColE1 and RSF1030, although it has been reported that there is no detectable sequence homology between the two plasmids (Crosa et al., 1975). Total RNA transcribed from RSF1030 DNA was hybridized to a Hae II digest of ColE1 DNA (Figure 3d). The RNA hybridizes to both the A and E fragments. The purified 100 nucleotide RNA transcribed from RSF1030 (Figure 5b,c) hybridizes to the origin region of RSF1030 replication and to the origin region of ColE1. There is therefore significant sequence homology in the origin regions of these two plasmids.

Copy Number Mutant pFH118 Encodes an Altered RNA Transcript

The position of the insertion mutation in pFH118 has been determined. The parental plasmid RSF1050 has a single Eco RI site located approximately 1080 base pairs upstream (i.e., opposite to the direction of replication) from the origin of replication defined as the site at which the first deoxyribonucleotide is incorporated (Tomizawa, Ohmori, and Bird, 1977; Bolivar, Betlach, Heynecker, Shine, Rodriguez, and Boyer, 1977). As described in the Introduction, the mutant

pFH118 was constructed by the insertion of an additional Eco RI site into RSF1050 (Heffron et al., 1978). The approximate location of this insertion in pFH118 DNA has been determined by digesting pFH118 DNA with Eco RI, which produced one nearly full length fragment and one fragment of 650 base pairs. The mutation in pFH118 therefore lies 430 base pairs upstream from the origin of replication, a region that should be contained in the Hae II-A fragment of RSF1050, and that is in the area of the coding sequence for the 100 nucleotide RNA transcript. Indeed, the 100 nucleotide RNA hybridizes to the Hae II-A fragment of pFH118 and RSF1050 DNAs. In addition, the RNA hybridizes to the Hae III subfragment of Hae II-A corresponding to the ColE1 C' fragment (Figure 2). This Hae III subfragment of Hae II-A is cleaved by Eco RI when prepared from pFH118, but is resistant to Eco RI when prepared from RSF1050. Thus, the 100 nucleotide RNA is hybridizing to the restriction fragment containing the inserted Eco RI site, suggesting that the pFH118 mutation lies adjacent to or within the coding sequence for the RNA.

Because of the similarity in their map positions, we have investigated the relationship between the copy number mutation in pFH118 and the 100 nucleotide transcript. As shown in Figure 6a, the prominent RNA transcribed in the in vitro DNA replication system using RSF1050 as template migrates faster than the 5S RNA marker. If the copy number mutant pFH118 is used as template, however, the position of this RNA has shifted and is above the position of the 5S marker (Figure 6b). This shift in the size of the RNA confirms that the inserted Eco RI site in pFH118 is within the transcribed region, as was suggested by the blotting and hybridization data. Because these are non-denaturing gels, it was impossible to use them to measure the size of the insertion in the RNA accurately.

The size of the insertion can, however, be determined using restriction enzyme-gel electrophoresis analysis of the DNA. When RSF1050 and pFH118 DNAs were digested with the restriction endonuclease Hae III and run on a 4% acrylamide gel, nineteen bands were detected (Figure 7). The sixth largest fragment, F, differed in size between the two plasmids by about 30 b.p. In Hae III/Eco RI double digests of pFH118, fragment F disappears, and two new lower molecular weight bands appear. Fragment F is unchanged in Hae III/Eco RI double digests of RSF1050. This confirms that the insertion containing an Eco RI site in pFH118 DNA is within the F fragment.

The Altered RNA Transcript Mediates the High Copy Number Phenotype

The results presented above indicate that the 100 nucleotide RNA in the copy number mutant pFH118 is altered by the insertion of approximately 30 nucleotides. The following experiment is a genetic reversion study that shows that this 30 nucleotide insertion causes the high copy number phenotype. In our hands, pFH118 DNA is unstable in many genetic backgrounds. Specifically, attempts to prepare DNA from cultures of E. coli W3110(pFH118) consistently gave rise to DNA that had lost one Eco RI site. In order to investigate the effect of this change on the copy number phenotype, we have developed the following rapid screening procedure for the copy number phenotype. E. coli W3110 and E. coli HB101 were transformed with both RSF1050 and pFH118 DNAs. Three transformants of each were colony purified, and then grown overnight in medium containing ampicillin. Subcultures (5 ml) were prepared from each overnight culture, chloramphenicol treated, and the cells lysed as described in Experimental Procedures. Cleared lysates from chloramphenicol treated cells were divided in half, and one

half digested with Eco RI. DNA in the cleared lysates was then run on 1.0% agarose gels. Cultures of E. coli W3110 (pFH118) showed no increase in the amount of DNA over E. coli W3110 (RSF1050). Two out of the three cultures of HB101 (pFH118), however, gave an increased amount of plasmid DNA over HB101 (RSF1050) controls (Figure 8). When Eco RI digests of these DNAs were analyzed, the two isolates of HB101(pFH118) that gave increased amounts of DNA on gels still contained the second Eco RI site, whereas DNA from the remaining HB101 (pFH118) culture and the three W3110 (pFH118) cultures contained only one Eco RI site (data not shown). Large cultures (50 ml) of the six strains carrying pFH118 were grown and DNA replication extracts (Fraction I) prepared as described in Experimental Procedures. In vitro DNA replication reactions were carried out using [α -³²P]UTP at 30° for 15 min, and the products were run on 10% acrylamide gels (Figure 9). Two of the cultures of HB101 (pFH118) gave rise to an RNA the size of the pFH118 control. These were the same two cultures that still contained the added Eco RI site, and that had an increased copy number. The remaining culture of HB101 (pFH118) and all three cultures of W3110 (pFH118) gave rise to RNAs that were smaller than pFH118 controls, although they were not identical in size to the RNA from RSF1050.

In summary, these results indicate a functional correlation between the insertion of a 30 b.p. fragment into the 100 nucleotide RNA and the high copy number phenotype. When the insert, or most of it, has been removed, the copy number phenotype as detected by the amount of plasmid DNA in cleared lysates has also been reduced.

Requirement for Ribonuclease III for in vitro Replication

We have thus far described only the prominent RNA transcript in the region

of the 5S RNA markers. A second, faster migrating band labeled β is also seen reproducibly on these gels (see Figure 1). Since the minor band migrates faster, we considered that it might be a processed product of the major low molecular weight RNA. The β band was eluted from the gel and found to hybridize to the same Hae III subfragment of the Hae II-A band of ColE1 as the prominent transcript (Figure 4b). As discussed in the Introduction, a good candidate for a processing enzyme is RNase III. We first tested whether RNase III mutants supported replication of RSF1030. Strain BL107 rnc-105 (Dunn and Studier, 1975) was transformed at normal efficiency by RSF1030 DNA. Thus, in vivo, BL107 rnc-105 supports the growth of RSF1030, perhaps due to residual levels of RNase III in the cells. We have therefore used our in vitro replication assay to test for a requirement for RNase III in plasmid DNA replication in vitro. Extracts were prepared from a mutant missing RNase III (Kindler, Keil, and Hofschneider, 1973; Dunn and Studier, 1975) and assayed to see if they supported RSF1030 replication in vitro. As shown in Table 1, the rnc mutant incorporated less than 6 pmol of dTMP under conditions where the parental strain BL15 incorporated 133 pmol of dTMP. No inhibition of synthesis was observed by addition of rnc⁻ extract to extract from wild type cells. Addition of an extract prepared from E. coli polApolB100polC_{ts}, which donates RNase III but which does not support replication itself, resulted in partial restoration of activity (Table 1) in a standard complementation assay.

These results are open to the objection that something other than RNase III is being donated by the complementing extract, and that it is not the enzyme itself that is involved in replication. Since NH₄Cl and glycerol inhibit in vitro replication (unpublished result) and since the standard purification procedure yields homogeneous RNase III in a buffer containing these two compounds, it was first necessary to

find conditions where the enzyme was stable but not stored in an inhibitory buffer. This was accomplished by filtration of homogeneous RNase III obtained from J. J. Dunn over a Sephadex G-100 column equilibrated with 0.01 M HEPES - 0.15 M KCl - 10% ethylene glycol - 1 mM EDTA (Dunn, 1976). These reagents do not inhibit in vitro replication (unpublished result). This enzyme preparation was then added to an in vitro replication extract and synthesis measured. As shown in Figure 10, the purified RNase III complemented the rnc⁻ extract. Activity was proportional to the amount of enzyme added. Thus, RNase III is required for plasmid DNA replication in vitro.

Discussion

We have made use of an in vitro DNA replication system and a high copy number mutant to show that a region of ColE1 DNA encoding a nominally 100 nucleotide RNA transcript has a role in determining plasmid copy number. Three lines of evidence support this statement. First, the position of the mutation in the high copy number mutant pFH118 falls in a restriction enzyme fragment that contains the coding sequence for the RNA transcript. Second, the insertion of approximately 30 base pairs found in the high copy number mutant pFH118 DNA gives rise to a larger RNA species. Third, in revertants that have lost the high copy number phenotype, all or most of the inserted DNA has been removed and the RNA is smaller.

There is little or no direct evidence specifying the mechanism by which the RNA coding region exerts its effect on plasmid copy number. One possibility is a model of negative control. The insertion mutation in pFH118 could be inactivating a repressor of plasmid replication. The revertants we have observed that have

lost the insertion could again make an active repressor. Gelfand et al. (1978) have isolated an independent high copy number mutant derived from ColE1. The mutant plasmid, designated pOP1 (11×10^6 MW), accounts for approximately 30% of the total intracellular DNA in log phase cells. A smaller high copy number plasmid, designated pOP1 Δ 6 (4.6×10^6 MW), has been derived from pOP1 by deletion of a region that does not contain the copy number mutation or the origin of replication. This plasmid also represents 30% of the total cellular DNA, resulting in a higher copy number for the smaller plasmid. This result could also be explained by a negative control mechanism. High copy number mutations would inactivate the repressor, allowing the plasmid replicons to compete with the host chromosome for replication enzymes. The total amount of plasmid DNA present in the cell would then be limited by the level of replication enzymes in the cell, taking into account the fact that some percentage of these enzymes are necessary for chromosome replication.

Alternatively, the RNA region may act as a positive effector of DNA replication. Both this work and that of Backman et al. (1978) indicate that small insertions can be tolerated in the RNA-coding region. Backman et al. (1978), however, have shown that insertion of the tetracycline resistant plasmid pSC201 at any one of several sites in the 580 base pair fragment abolishes ColE1 type replication. In the scenario of positive control, they propose that the RNA could act as a positive effector of DNA synthesis by serving as the primer at the origin of replication. Thus, the primer for DNA replication would not be synthesized by RNA polymerase at the origin, but would be a preformed molecule synthesized at a second site on the DNA (Backman et al., 1978). This model is supported by the circumstantial evidence that the RNA contains a sequence of 12 nucleotides,

10 of which are the same as the sequence where the first deoxyribonucleotide is incorporated during replication (Tomizawa et al., 1977; Bolivar et al., 1977; and RNA sequence quoted in Backman et al., 1978). In addition, we have shown here that the total RNA synthesized from both ColE1 and RSF1030 templates in vitro hybridizes both to the site from which the 100 nucleotide RNA is transcribed and to the origin of replication of ColE1 itself. The purified 100 nucleotide RNA from ColE1 also hybridizes to the origin, although weakly. This discrepancy in the strength of hybridization might be explained by a second RNA in the total RNA preparation that is causing most of the hybridization at the origin of replication. However, this second RNA would have to be present in RSF1030 as well. An example of a preformed RNA serving as a primer for DNA synthesis exists in the case of the RNA tumor viruses, where reverse transcription of the 70S RNA is primed by a tryptophan tRNA that is found within the virion (Verma et al., 1971). There are, of course, other ways in which the RNA could act as a positive effector of DNA synthesis. It might, for instance, aid in denaturing the DNA at the origin of replication by hybridizing to its homologous sequence, stimulate a protein involved in replication, or be required for efficient elongation.

The fact that the RNA coding region is preserved in both ColE1 and the otherwise unrelated plasmid RSF1030 (Figures 3 and 5) suggests that the region is an important determinant of relaxed plasmid replication. Whether it is the DNA sequence itself, the RNA, or a polypeptide translated from the RNA (the least likely possibility from a review of the sequence) that is active is not known. The replicon model of Jacob, Brenner, and Cuzin (1963) proposes that any autonomous replicon must consist of both a gene coding for a specific "initiator" molecule and a sequence upon which the "initiator" acts. Recent studies on the transmissible

plasmid R6K have described a trans-acting protein required for the initiation of DNA replication (Inuzuka and Helinski, 1978; Kolter, Inuzuka, and Helinski, 1978). Studies on ColE1 type plasmids, however, have demonstrated that no plasmid encoded protein is required for replication (for review, see Staudenbauer, 1978; Donohue and Sharp, 1978; Kahn and Helinski, 1978), although mutant plasmids with a temperature-sensitive phenotype have been described (Kingsbury and Helinski, 1973; Kingsbury, Sieckmann, and Helinski, 1973). It will now be of interest to investigate these mutants carefully and to look for altered, temperature-sensitive RNAs.

The requirement for RNase III, an enzyme known to act only on RNA, is indicative that an RNA species is involved in plasmid replication. The fact that RNase III mutants support *in vivo* but not *in vitro* replication of plasmids may be due to back-up systems that function *in vivo* but not *in vitro*, or may be due to residual RNase III activity present *in vivo* but inactivated by preparation of *in vitro* replication extracts. RNase III is probably a processing enzyme (see Introduction), and its involvement in replication suggests that RNA processing may be significant in DNA replication. Although we have presented no evidence for RNA processing here, we have demonstrated (Figures 1 and 8) the presence of an RNA species migrating ahead of the 100 nucleotide species that hybridizes to the coding sequence for the larger RNA. Present studies in this laboratory are aimed at elucidation of both the mechanism of action of the 100 nucleotide transcript and the role of RNase III in DNA replication.

Experimental Procedures

Materials

Reagents and sources were as described previously (Conrad et al., 1979). In addition, SeaPlaque Agarose (ME) was from Marine Colloids, Rockland, ME. [α - 32 P]UTP was from New England Nuclear. RNase III (Fraction V) was the generous gift of J. J. Dunn.

Bacterial Strains and Plasmids

E. coli W3110 thy⁻ and E. coli HB101 pro⁻gal⁻r⁻m⁻recA1 have been described previously (Conrad et al., 1979) and were used as plasmid hosts in these studies. E. coli BL107 rna⁻rnc⁻ and the parental strain E. coli BL15 rna⁻ were obtained from F. W. Studier (Dunn and Studier, 1975). Standard plasmid strains used are RSF1030 and ColE1. Construction of the ampicillin resistant derivatives of ColE1, RSF1050, and pFH118 (M.W. 5.0×10^6) are described in Heffron et al. (1977). The copy number mutant 118 was constructed by a new method for in vitro mutagenesis of a circular DNA molecule (Heffron et al., 1978). This method employs synthetic oligonucleotide restriction sites to generate insertion mutations in randomly cleaved circular DNA.

Preparation of in vitro Replication Extracts

Fraction I (FxI) is prepared by lysozyme treatment and freeze-thaw lysis of cells harboring endogenous plasmid essentially according to Staudenbauer (1976). Synthesis in Fraction I is not dependent on addition of covalently closed circular exogenous DNA. Fraction II (FxII) was therefore prepared as follows: To 2.0 ml of Fraction I, 0.2 ml of a 30% (w/v) solution of streptomycin sulfate was added.

The solution was stirred for 30 min at 0°C. The precipitate was removed by centrifugation for 10 min at 20,000 x g at 4°C. To 2.0 ml of supernatant, 0.944 g solid ammonium sulfate was added over a 10 min period. The mixture was stirred for 30 min at 0° and the precipitate was then removed by centrifugation at 20,000 x g for 10 min. The pellet was dissolved in 1.0 ml 0.01 M HEPES, pH 8.0/1 mM DTT/1 mM EDTA/0.1 M KCl/10% ethylene glycol. The redissolved pellet was dialyzed against 100 ml of the same buffer for 3 hr at 0°. The dialyzed material, Fraction II, was frozen and stored in liquid N₂. Fraction II contains about 11 mg per ml of protein. Replication is entirely dependent on addition of exogenous DNA and represents true replication (Conrad et al., 1979 and manuscript submitted).

Assay for DNA Synthesis

A standard assay for DNA synthesis has been established using Fraction II. Reactions (0.05 ml) contained 40 mM HEPES, pH 8.0, 100 mM KCl, 12 mM magnesium acetate, 50 µM each dCTP, dGTP, dATP, and [³H]TTP, 2 mM ATP, 0.5 mM each rCTP, rGTP, rUTP, 1.5 nmol of RSF1030 DNA, and 20 µl extract. Incubations were carried out at 30°. Reactions were terminated by placing tubes at 0° followed by addition of 1 N HCl containing 0.1 M sodium pyrophosphate. The precipitate was collected on Whatman GF/C filters. The filters were washed, dried and radioactivity determined in a toluene-based fluid in a scintillation counter.

Preparation of Plasmid DNA

A modification of the method of Clewell and Helinski (1969) was used to prepare plasmid DNAs.

Agarose and Polyacrylamide Gel Electrophoresis

Agarose gels were run as described previously (Conrad et al., 1979).

Acrylamide gels were run in the same buffers and apparatuses as agarose gels.

A bis:acrylamide ratio of 1:30 was used unless otherwise noted. RNA was extracted from gels by the method of Maxam and Gilbert (1977) in 0.05 M Tris, pH 8.0, 0.2 M NaCl-1 mM EDTA-0.1% SDS. After 12-17 hr at room temperature, acrylamide was removed by centrifugation and the RNA was either purified further by passage over a DEAE 52 column, or was precipitated with ethanol and used directly. Yeast tRNA (100 µg/ml) was added to the extraction as carrier.

Restriction Enzyme Mapping and Blotting

Restriction enzyme digestions were carried out in the buffers suggested by New England Biolabs. Double digests of specific restriction fragments were accomplished by digestion with one enzyme followed by fractionation on SeaPlaque agarose (Parker and Seed 1979). Regions of the gel containing the desired fragment were cut out, melted at 65°, equilibrated at 37° and the second restriction enzyme and buffer added. The products were then fractionated on Seakem agarose or acrylamide gels as necessary.

Blotting onto nitrocellulose filter paper and hybridization of RNA to restriction enzyme digests was done according to the method of Southern (1975). Hybridizations were carried out at 43° in 50% formamide, 0.025 M HEPES, pH 8.0, 0.3 M NaCl, 1 mM EDTA, 0.1% SDS.

Preparation of Cleared Lysates for Screening of Plasmids

5 ml cultures of strains containing plasmids were grown with or without

chloramphenicol treatment. The cells were spun down and resuspended in 0.1 ml of 10 mM Tris, 2 mM EDTA, pH 8.0. Cells were lysed at 60° by adding 10X lysis buffer so that the final concentrations were: 0.5% SDS, 2.5% glycerol, 0.01 M EDTA, pH 8.0. The lysed cells were incubated at 0° for 30-60 min, and the cell debris and chromosomal DNA removed by centrifugation at 15,000 rpm for one hour in an SS34 rotor. The supernatant was treated with RNase (10 µg/ml RNase A, 50 units/ml T₁) and was then either loaded directly onto a gel to visualize plasmid DNA, or was extracted with phenol and precipitated with ethanol before being digested with a restriction endonuclease.

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Figure 1. Analysis of RNA Transcribed during in vitro DNA Replication by Gel Electrophoresis.

An in vitro DNA replication reaction was carried out using W3110(FxII) extract and [α - 32 P]UTP for 15 min at 30°. Products were phenol extracted, ethanol precipitated, run on a 10% acrylamide gel at 80 V for 10 hr and autoradiographed as described in Experimental Procedures. DNA templates used in the reactions were: a) ColE1; b) RSF1050; c) RSF1030.

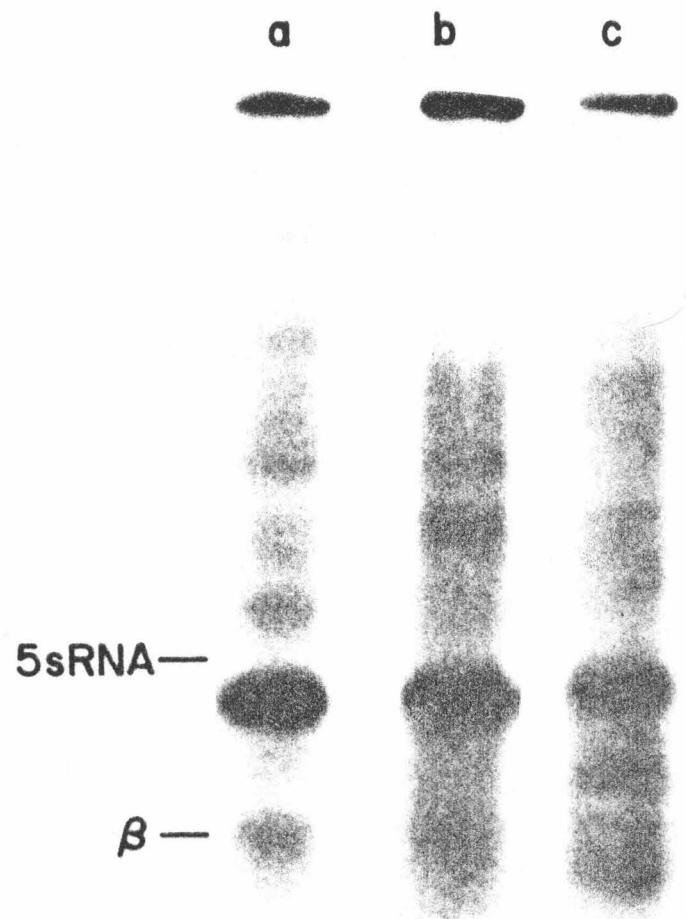


Figure 2. Restriction Map of the Origin Region of ColE1 DNA.

Replication proceeds from the origin towards the right (Tomizawa et al., 1977; Oka and Takanami, 1976).

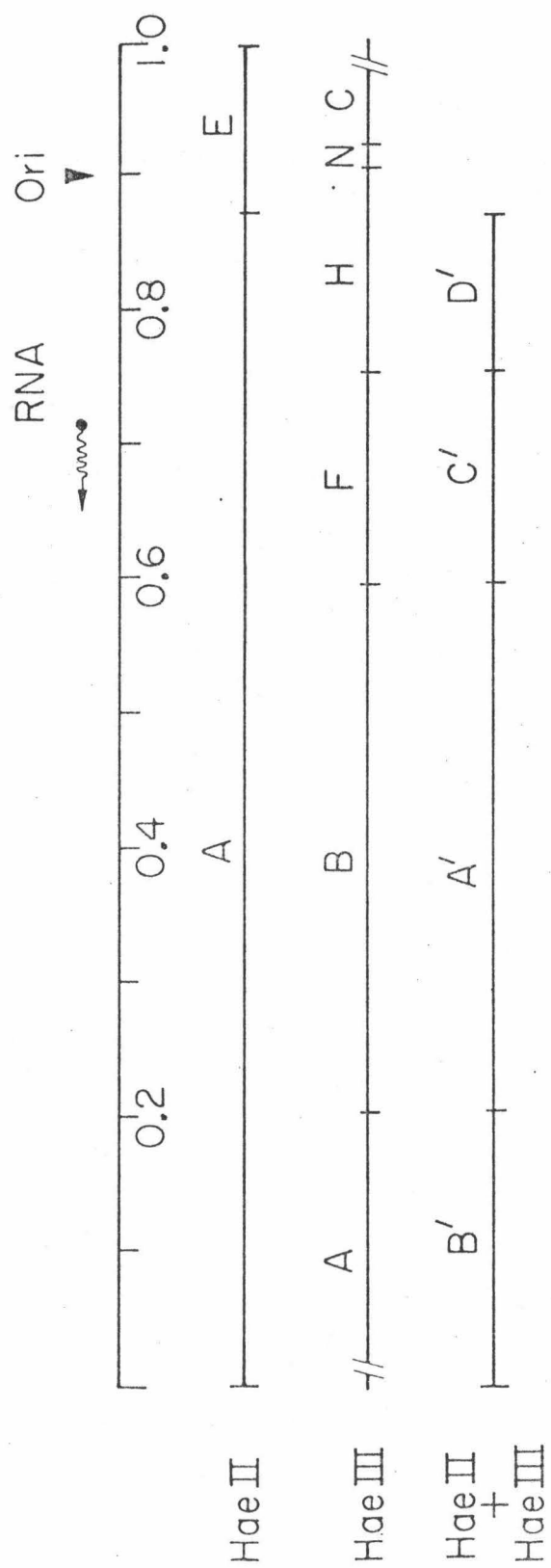


Figure 3. Hybridization of Transcribed RNA to a Hae II Digest of ColE1 DNA.

32 P-labeled RNA probes were synthesized and purified as described in Experimental Procedures. Hae II digests of ColE1 DNA were run on a 1.4% agarose gel at 60 V for 7 hr. The DNA was then transferred to millipore paper (0.22 μ), and hybridized to [α - 32 P]RNA as described in Experimental Procedures. If the DNA was transferred to 0.45 μ paper, no hybridization was seen to the E fragment. RNA probes used were: a and b) total RNA transcribed from ColE1 DNA; c) purified 100 nucleotide transcript from ColE1; d) total RNA transcribed from RSF1030 DNA; e) EtdBr stained gel.

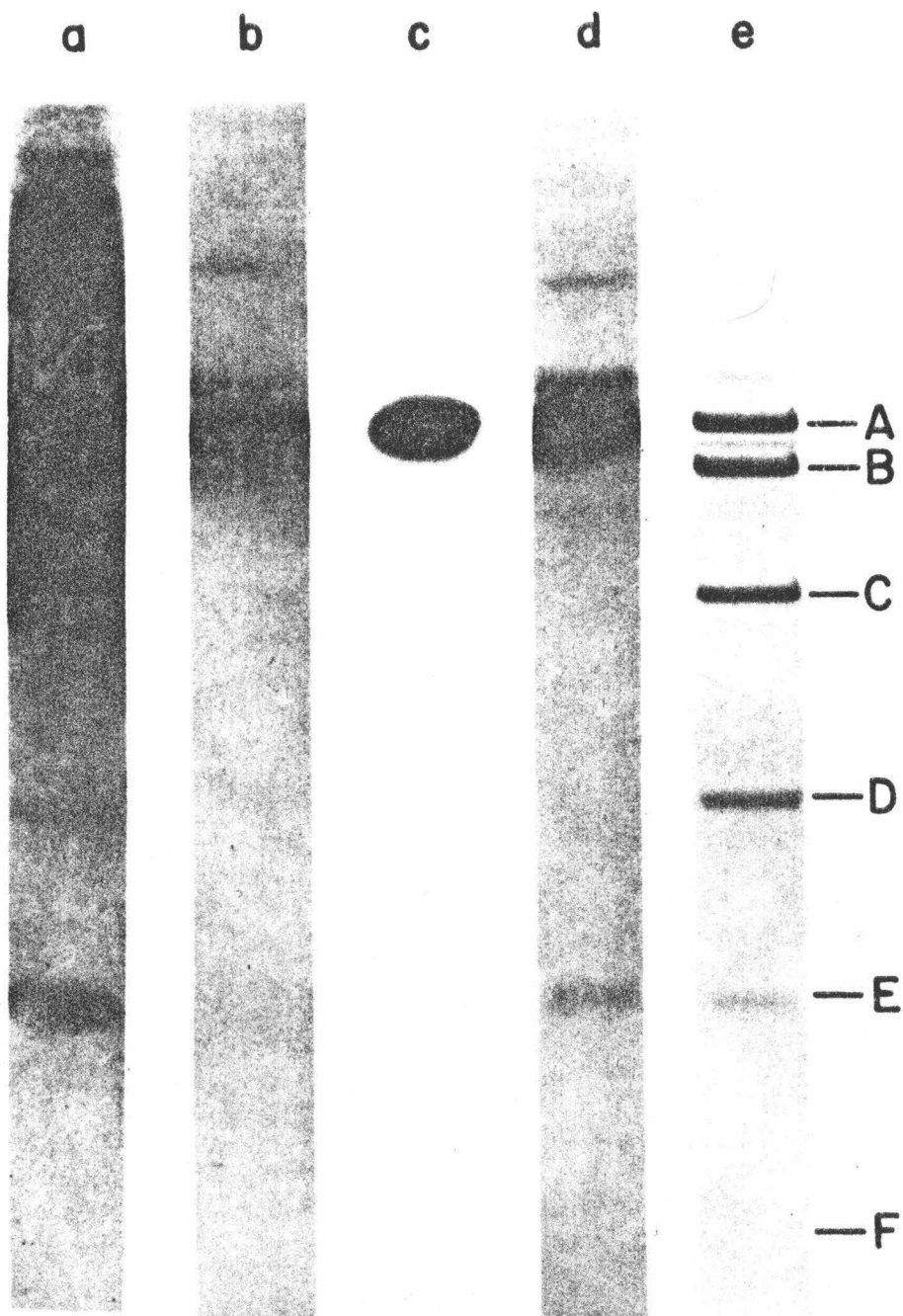
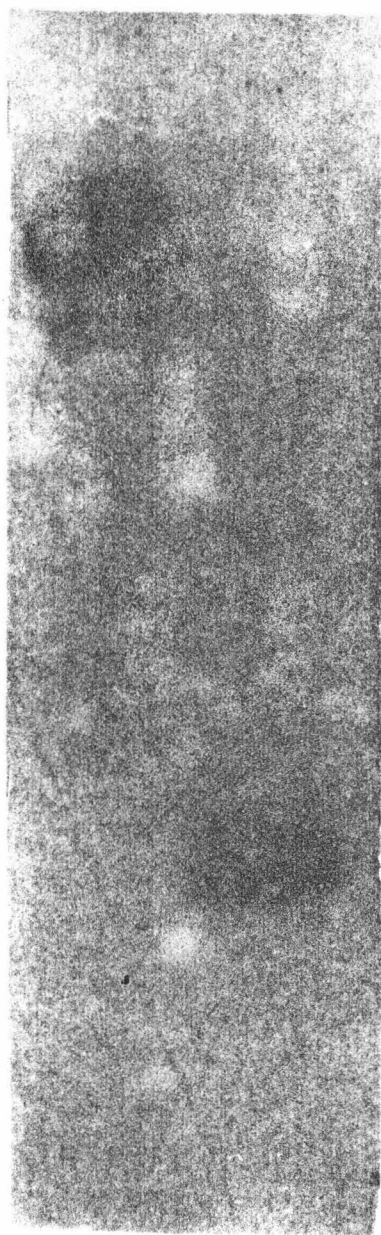


Figure 4. Hybridization of Purified RNAs to the Origin Region of ColE1.

The HaeII-A fragment of ColE1 DNA was isolated from SeaPlaque agarose (1.4%), redigested with Hae III and run on a 1.5% Seakem agarose gel at 50 V for 7 hr.

The DNA was transferred to 0.45 μ millipore paper and hybridized to the indicated RNA as described in Experimental Procedures. The first lane on these gels shows hybridization to the Hae II-A fragment run on the same gel. RNA probes were isolated from a 10% acrylamide gel (see Figure 1), and were as follows: a) purified 100 nucleotide transcript from ColE1; b) purified β band from ColE1.

a**b**

— A' —

— B' —

— C' —

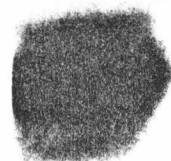


Figure 5. Hybridization of RSF1030 [α - 32 P]RNA to RSF1030 and pBR322 DNAs.

RNA was transcribed in the in vitro replication system using RSF1030 DNA as a template. a) The Hinc II-A fragment of RSF1030, which contains the origin of replication, was purified on SeaPlaque agarose and redigested with Hae III. The Hae III fragments A, B and C were separated by electrophoresis and transferred to nitrocellulose filters by the method of Southern. Total 32 P labeled RNA transcribed from RSF1030 was used as a probe. Lane 1: EtBr stained gel; Lane 2: Autoradiogram of hybrids formed. Hybridization is to fragment A and to the partial digestion product A + B. b) The 100 nucleotide RNA transcript of RSF1030 was purified as described in Experimental Procedures, and hybridized to a Hae II digest of pBR322 (lane 3). pBR322 contains the ColE1 origin region, and the Hae II-A fragment contains the coding sequence for the 100 nucleotide transcript (Backman et al., 1978). The purified transcript was also hybridized to the RSF1030 Hinc II-A fragment (lane 4).

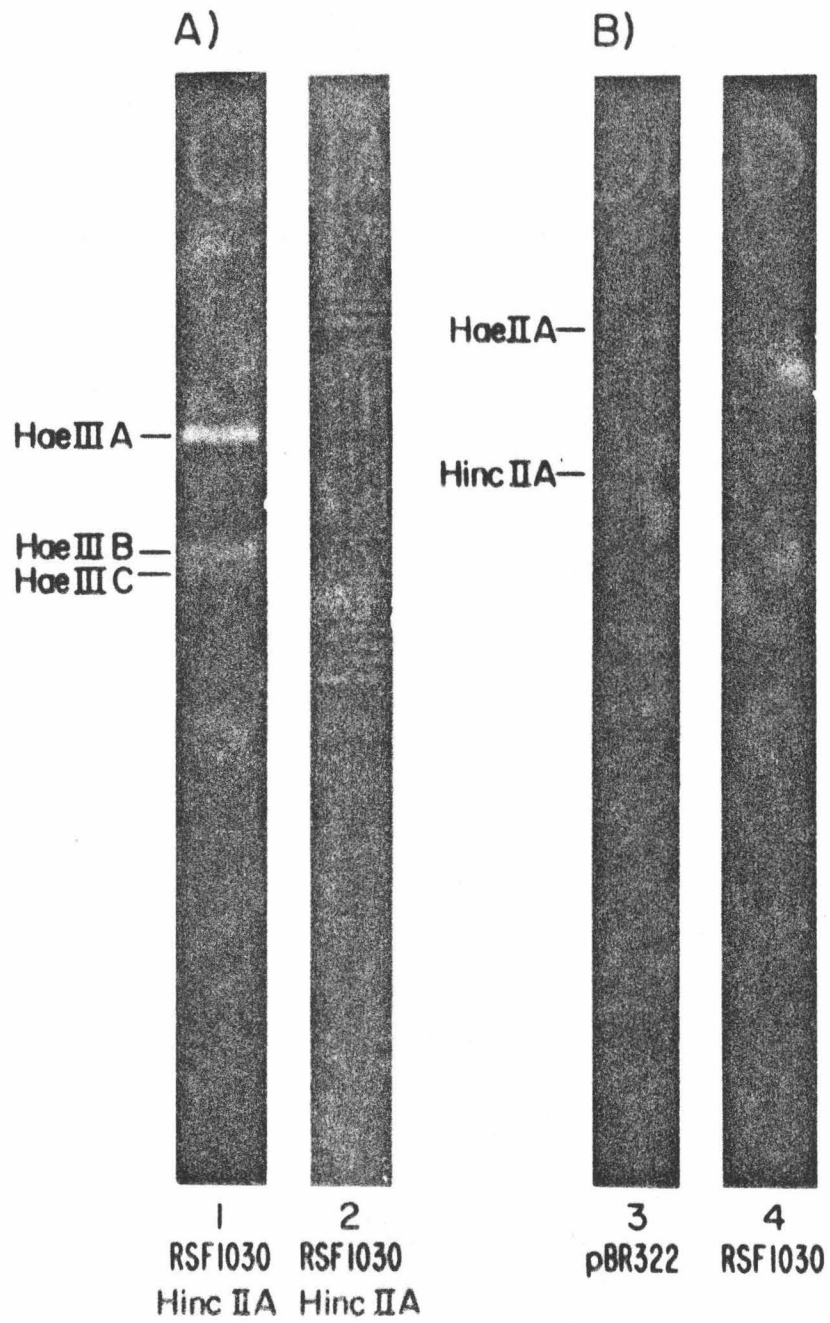


Figure 6. RNA Transcribed from Wild Type and Copy Number Mutant DNA.

^{32}P -RNAs transcribed during in vitro DNA replication reactions were phenol extracted, ethanol precipitated and run on a 10% acrylamide gel at 80 V for 10 hr. The position of a non-radioactive 5S RNA marker run on the same gel is indicated. DNAs used as templates were: a) RSF1050; b) pFH118.

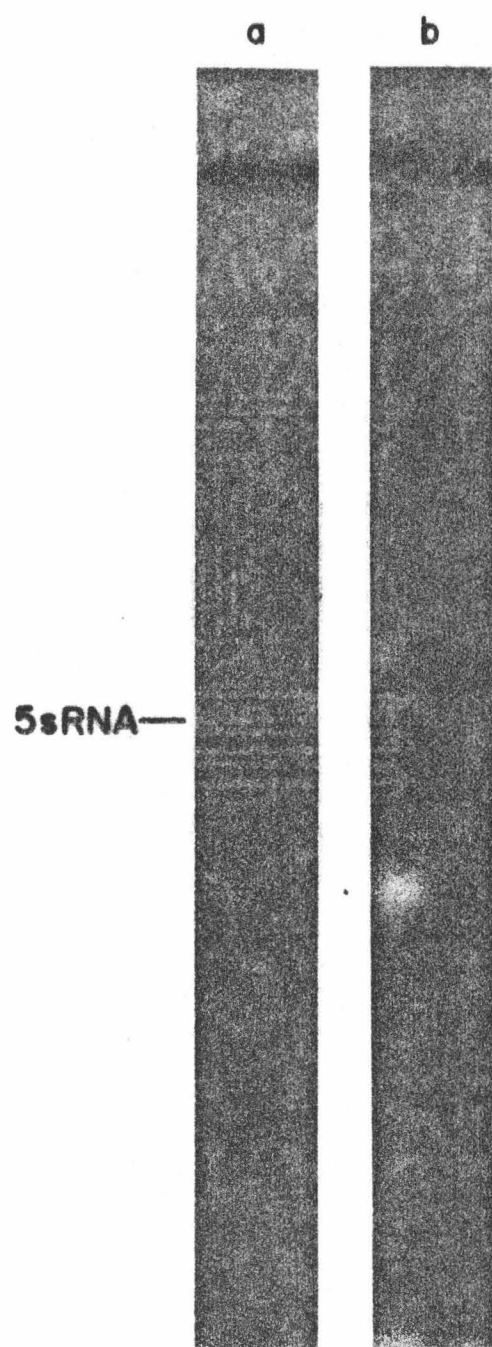
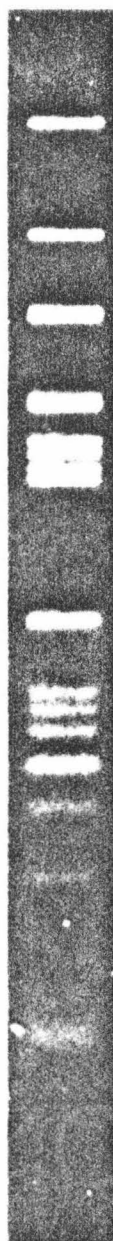


Figure 7. Restriction Enzyme Analyses of Wild Type and Copy Number Mutant DNAs.

Both Hae III and Hae III/Eco RI double digests of RSF1050 and pFH118 DNAs are shown. The products were run on a 4% acrylamide gel (1:20, bis:acrylamide ratio). Molecular weights were determined using both Hae II and Hae III digests of pBR322 DNA as size standards. a) pFH118/Hae III; b) RSF1050/Hae III; c) pFH118/Hae III + Eco RI; d) RSF1050/Hae III + Eco RI. Sizes of the fragments of interest are: Hae III F fragment of pFH118, 455 b.p.; Hae III F fragment of RSF1050, 425 b.p.; new bands in Hae III/Eco RI double digest of pFH118, 330 and 110 b.p.

a



b



c



d



Figure 8. Analysis of Plasmid DNA in Cleared Lysates of Cells Containing Wild Type and Copy Number Mutant DNA.

Small (5 ml) cultures of strains containing plasmids were grown with overnight chloramphenical treatment. Cells were harvested and DNA prepared as described in Experimental Procedures. The DNAs were run on 1% agarose gels at 50 V for 12 hr. Sources of DNA were as follows: a-c) HB101(pFH118); d) HB101(RSF1050); e-g) W3110(pFH118); h) W3110(RSF1050).

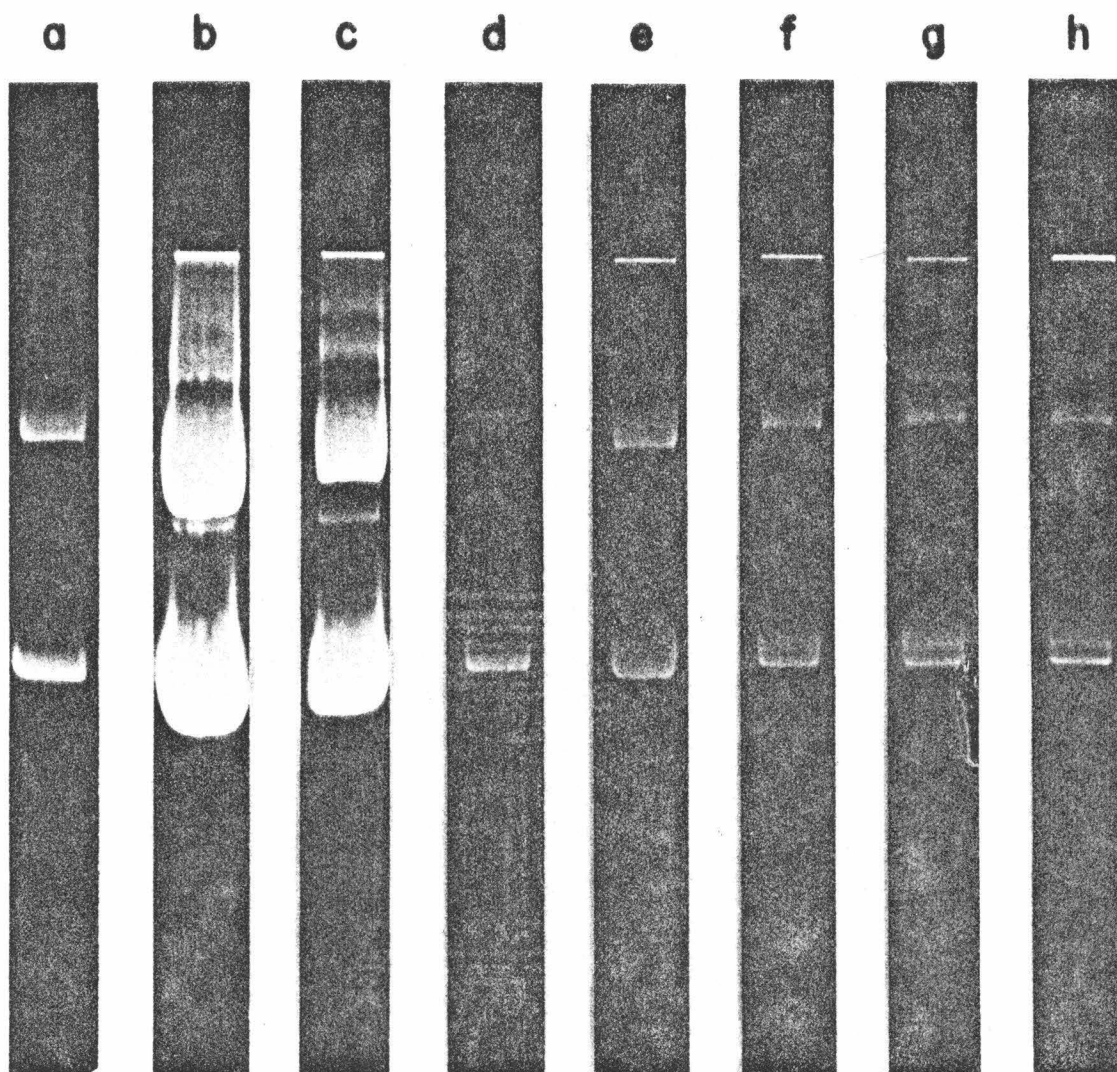


Figure 9. Analysis of RNA Transcribed from both Copy Number Mutants and Revertants.

FxI in vitro DNA replication extracts were made from 50 ml cultures of strains containing plasmid DNA. Reactions were carried out for 15 min at 30° with [α -³²P]UTP label using only the endogenous plasmid DNA as template. Products were run on a 10% acrylamide gel at 80 V for 10 hr. Extracts used for RNA synthesis were as follows: a) HB101(RSF1050); b-d) HB101(pFH118); e-g) W3110(pFH118); h) W3110 FxII, RSF1050 DNA as template; i) W3110 FxII, pFH118 DNA as template.

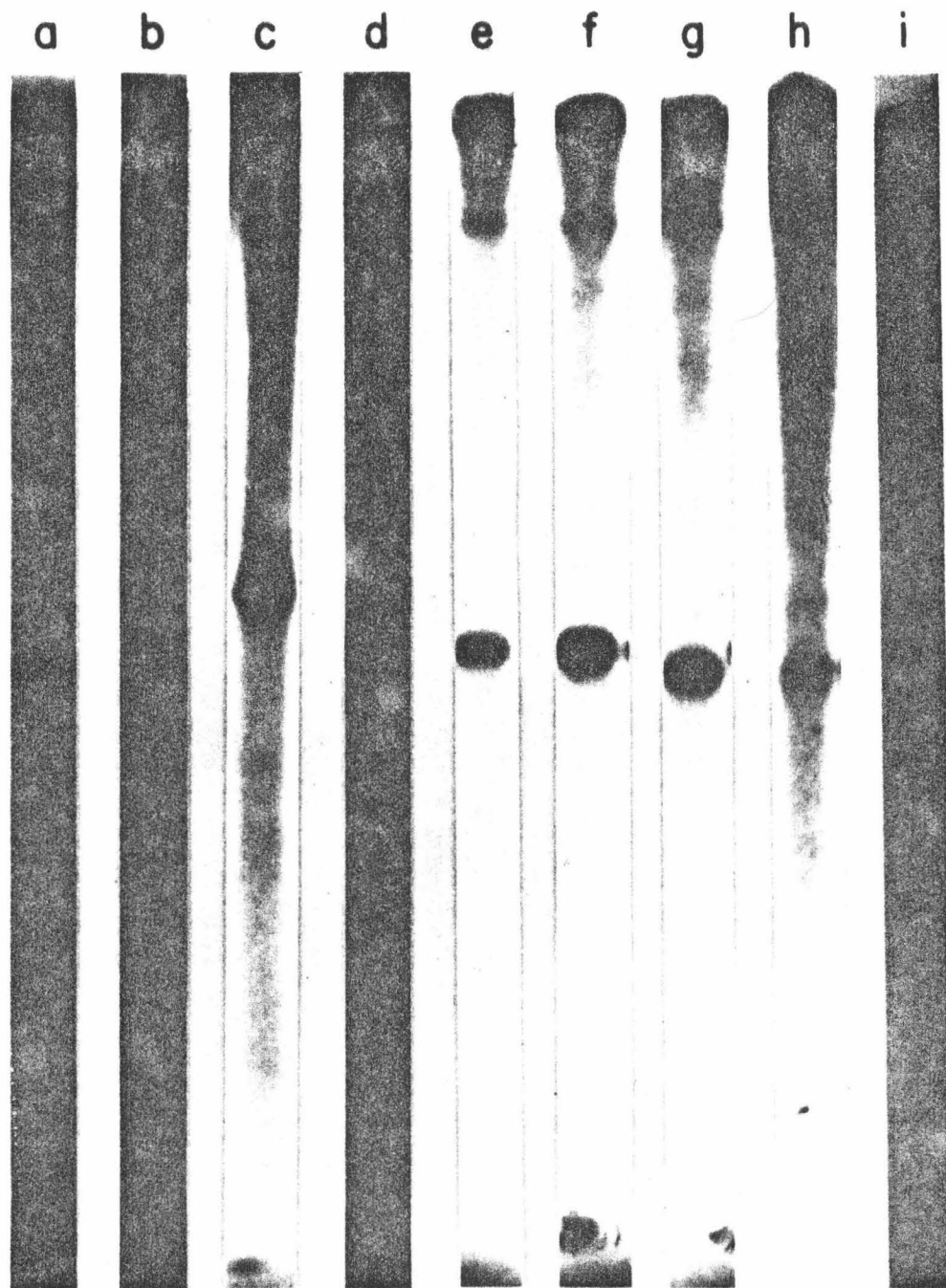


Figure 10. Complementation of the rnc⁻ Extract with Purified Ribonuclease III.

Ribonuclease III was prepared by J. Dunn and further purified as described in the text (Dunn, 1975). Fraction II was prepared from E. coli BL107 rnc⁻ and reactions carried out for 60 min at 30° as described in Experimental Procedures, using the Sephadex G-100 fraction of RNase III. Reactions contained 20 µl of extract.

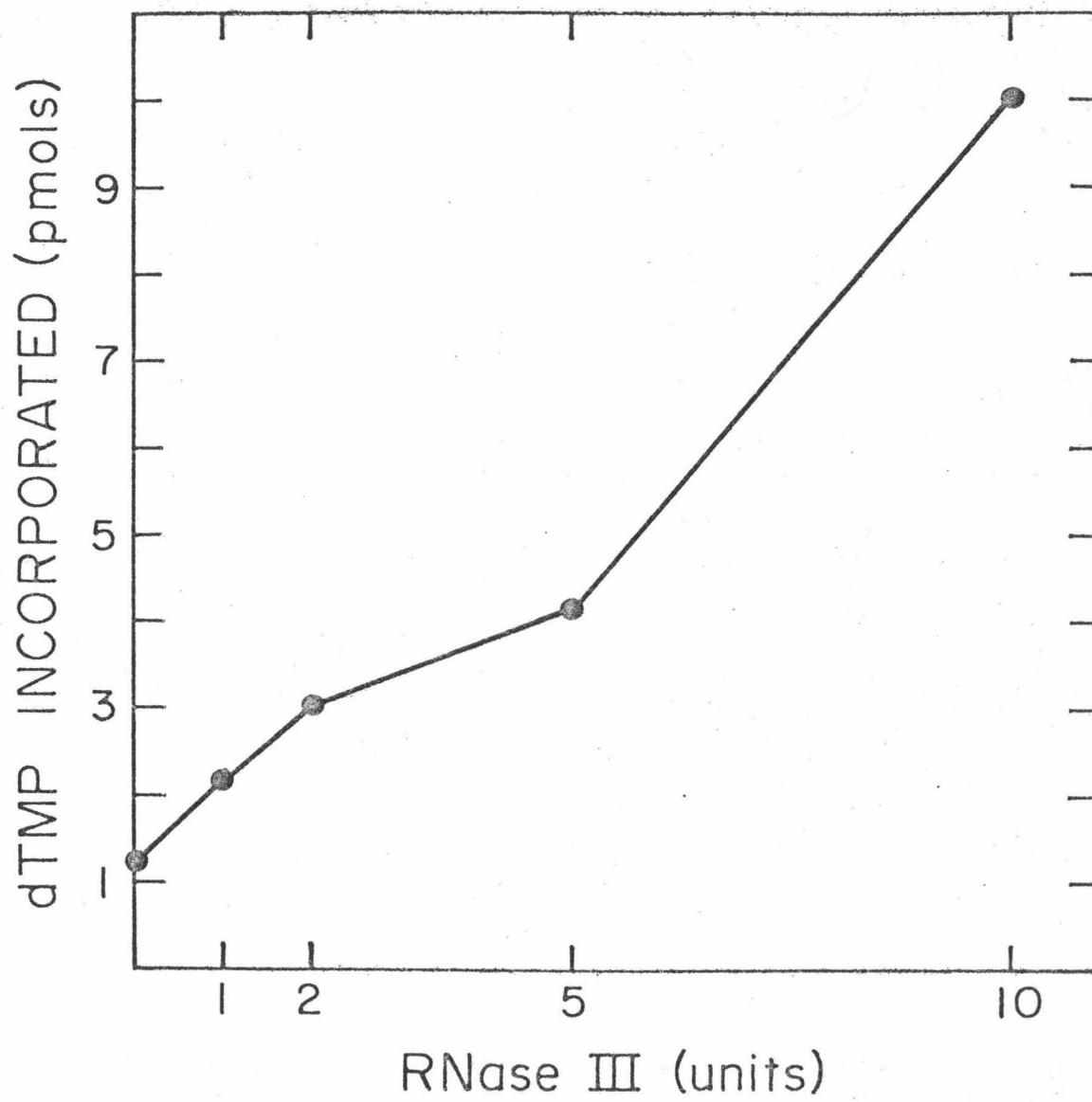


Table 1. Requirement for RNase III for DNA Synthesis in vitro

Extract	dTMP incorporated
	pmol
<u>rnc</u> (20 μ l)	4.0
<u>rnc</u> ⁺ (20 μ l)	200
<u>rnc</u> + <u>rnc</u> ⁺ (20 μ l + 20 μ l)	197
<u>rnc</u> + <u>polApolBpolC</u> _{ts} (20 μ l + 2 μ l)	30

Synthesis was measured in the standard reaction mixture.

Strain rnc was E. coli BL107 and strain rnc⁺ was E. coli BL15 as described in Experimental Procedures.