

RESTRICTION MAP, PARTIAL CLONING AND LOCALIZATION OF 9S AND 12S KINETOPLAST RNA GENES ON THE MAXICIRCLE COMPONENT OF THE KINETOPLAST DNA OF *Leishmania tarentolae*

(Restriction endonucleases *EcoRI*, *BamHI*, *HaeIII*, *SalI*, *BglII*, *HindIII*; hemoflagellate protozoa; pBR322 plasmid; *E. coli*)

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SUMMARY

We have constructed a restriction map of the maxicircle component of the kinetoplast DNA of *Leishmania tarentolae* for the enzymes *EcoRI*, *BamHI*, *HaeIII*, *HpaII*, *SalI*, *BglII* and *HindIII*. The 9 and 12S kinetoplast RNAs were localized on this map. Two fragments of this maxicircle molecule were cloned in the bacterial plasmid, pBR322, including a $4.4 \cdot 10^6$ dalton *EcoRI/BamHI* fragment which contains the 9 and 12S RNA genes.

INTRODUCTION

The kinetoplast DNA (KDNA) of the hemoflagellate protozoa consists of a high molecular weight network composed of thousands of catenated minicircles and a smaller number of catenated maxicircles (Simpson, 1972; 1979; Borst and Fairlamb, 1976; Borst et al., 1976; 1977; Steinert et al., 1976 a and b; Simpson and Da Silva, 1971; Simpson and Berliner, 1974; Simpson and Simpson, 1974; 1978; Wesley and Simpson, 1973a-c; Kleisen et al., 1975, 1976a; 1976b; Kleisen and Borst, 1975; Riou and Yot, 1970, 1977; Cheng and Simpson, 1978; Steinert and Van Assel, 1975; Weislogel et al., 1977; Fairlamb et al., 1978; Simpson and Hyman, 1976; Simpson and Braly, 1970; Englund, 1978; Renger and Wolstenholme, 1972; Fouts et al., 1975). We have shown previously (Simpson and Simpson, 1978) that the maxicircle KDNA of

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L. tarentolae codes for the two major stable RNA species found in the kinetoplast, and we have reported a method to isolate the maxicircle species from the network after cleavage with a single hit restriction endonuclease such as *EcoRI* or *BamHI* (Simpson, 1979). This method involves the buoyant separation of the maxicircle DNA in CsCl in the presence of the At-binding dye, Hoechst 33258. We have also reported that the maxicircle DNA was relatively high in %AT (Simpson and Hyman, 1976) and that there was an intramolecular base composition heterogeneity (Simpson, 1979). In this report we have used this isolation method to obtain large quantities of pure *EcoRI*-linearized maxicircle DNA ("RI Maxi DNA") from *L. tarentolae* KDNA for the purposes of restriction enzyme mapping and cloning in the bacterial plasmid, pBR322.

MATERIALS AND METHODS

Cells

L. tarentolae clonal strain c-1 was grown in Difco Brain Heart Infusion medium as described previously (Simpson and Braly, 1970). Large quantities (12–15 l) were grown in a fermentor at 27°C for 4 days to a stationary phase density of $300 \cdot 10^6$ cells/ml.

Isolation of KDNA and linearized maxicircle DNA

Large quantities of KDNA (4–6 mg per 12–15 l culture) were obtained as described previously (Simpson, 1979). The RI Maxi and Bam Maxi linearized maxicircle DNAs were isolated by the Hoechst 33258-CsCl method described previously (Simpson, 1979).

Gel electrophoresis

Horizontal agarose gel electrophoresis, vertical acrylamide gel electrophoresis, and EthBr staining and photography were performed as described previously (Brunk and Simpson, 1977; Cheng and Simpson, 1978; Simpson, 1979). The agarose gel dimensions were 18 × 13 × 0.2 cm. Molecular weights were calculated by use of the least squares curve fitting program of Parker et al. (1977) using reference molecular weight values from Murray and Murray (1975) and Sanger et al. (1977). Sizes of the larger maxicircle fragments were obtained in 0.8% agarose and sizes of smaller fragments were obtained in 1%, 1.2%, 1.5%, 2.0% agarose or in 10% acrylamide.

Restriction enzyme digestion

All enzymes were purchased from New England Biolabs. Digestions were performed in 200- μ l volumes at 37°C for 1 h in the recommended buffers. Double digestions were performed either sequentially or simultaneously. The *HindIII* buffer was used for all simultaneous digestions except those involving *EcoRI*. After digestion the DNA was ethanol precipitated and taken up in sample buffer for electrophoresis (Cheng and Simpson, 1978).

In vitro labeling of DNA and RNA

RNA was labeled with ^{32}P by polynucleotide kinase after mild alkali hydrolysis as described previously (Simpson and Simpson, 1978). DNA was labeled with $[\alpha\text{-}^{32}\text{P}]\text{TTP}$ by nick translation (Maniatis et al., 1975) using DNase I and *E. coli* DNA polymerase (Böhringer). The reaction was carried out for 1 h at 14°C , after which the solution was phenol-chloroform and ether extracted and the labeled DNA was separated on a Sephadex G75 column. The DNA was precipitated with ethanol and resuspended in hybridization buffer.

Southern transfers

This was performed as described previously (Simpson and Simpson, 1978) except that entire slab gels were used instead of single slots. A Schleicher and Schuell BA85 membrane filter was used for the transfer. Hybridization of labeled RNA was performed in $2\times$ SSC at 47°C for 48 h. The wet filters were wrapped in Saran Wrap and sealed inside plastic bags to prevent desiccation. For DNA-DNA hybridization the DNA filters were presoaked in Denhardt's medium (Denhardt, 1966) at 65°C for 6 h and then in Denhardt's medium plus $10\ \mu\text{g}/\mu\text{l}$ sonicated denatured *E. coli* DNA for 3 h at 65°C . Hybridization was performed in 50% formamide—0.6 M NaCl—0.2 M Tris·HCl (pH 8)—0.02 M EDTA (Dawid, 1977) at 37°C for at least 24 h. Filters were washed extensively in hybridization medium at 25°C and then in $2\times$ SSC. Autoradiography was performed at -70°C with a Dupont Cronex Quanta II Intensifier screen.

Cloning: restriction, ligation, transformation

The plasmid, pBR322 (Bolivar et al., 1977), was prepared after chloramphenicol amplification (Clewell, 1972) by EthBr CsCl centrifugation of the cleared SDS lysate (Guerry et al., 1973). Plasmid DNA was digested with either *Hind*III, *Bam*HI, or *Eco*RI plus *Bam*HI plus *Hind*III, and was then treated with bacterial alkaline phosphatase (Worthington BAPF, pretreated for 10 min at 95°C in 10 mM Tris·HCl (pH 7.4)—10 mM MgCl_2) to reduce self-annealing (Ulrich et al., 1977). The appropriate, digested plasmid was then mixed with either *Hind*III or *Bam*HI digested total KDNA or *Bam*HI digested RI Maxi DNA in *Eco*RI buffer, and then an equal volume of ligase buffer was added (0.12 M Tris·HCl (pH 7.5)—10 mM dithiothreitol—0.8 mM ATP) together with several units of T_4 ligase (New England Biolabs). The ligation was carried out for 2 days at 14°C . Transformation of *E. coli* RRI cells was performed as described by Kaplan et al. (1978). Transformants were selected by plating on TYE agar containing ampicillin (Amp) ($100\ \mu\text{g}/\text{ml}$) and transformants containing inserts in the tetracycline resistance gene were selected by replica plating onto TYE-Amp plates and TYE-Tet plates ($15\ \mu\text{g}/\text{ml}$). Clones containing maxicircle or minicircle inserts were selected by colony hybridization using nick-translated ^{32}P -labeled RI Maxi DNA or ^{32}P -labeled total monomeric minicircle DNA (Wesley and Simpson, 1973b) as probes. Colonies on filters were subjected

to chloramphenicol (Cap) amplification of plasmid DNA prior to hybridization. After overnight growth on TYE-Amp plates, the filters were transferred to TYE-Amp + Cap (170 $\mu\text{g}/\mu\text{l}$) plates and incubated 12–18 h at 37°C. Hybridization with ^{32}P -DNA probes was performed in 0.6 M NaCl–0.2 M Tris·HCl (pH 8.0)–0.02 M EDTA–50% formamide for 24 h at 37°C (Dawid, 1977). Final identification of the insert was performed by agarose gel electrophoresis of the restricted recombinant plasmid. All recombinant DNA experiments were performed under P2 + EK1 containment conditions, as described in the NIH Guidelines.

Isolation of recombinant plasmids

Clones were grown to an A_{540} of 1.5 in TYE medium and then chloramphenicol was added to 170 $\mu\text{g}/\text{ml}$, and the cultures incubated an additional 12–18 h at 37°C. Closed circular plasmid DNA was isolated by EthBr CsCl centrifugation of the cleared SDS lysate. The dye was removed by isopropanol extraction. The maxicircle inserts were isolated by Hoechst 33258 dye CsCl equilibrium centrifugation (Simpson, 1979) of the digested plasmid DNA.

Mapping

Mapping was performed by comparisons of double digests of the RI Maxi and single digests of total KDNA as described in RESULTS. All molecular weight values used for mapping were adjusted to an assumed total maxicircle molecular weight of $19.47 \cdot 10^6$. The actual mean molecular weights obtained for the various fragments in several gels together with the standard deviations of the values are given in Table I. The “adjusted” molecular weight values are given in Table I and in the figure diagrams; the values are merely to be considered relative and will probably have to be adjusted in an absolute sense later on.

RESULTS

Isolation of the EcoRI linearized maxicircle and the BamHI linearized maxicircle

Total closed network DNA was digested with *EcoRI* or with *BamHI* and the single cleaved maxicircle linears (“RI Maxi DNA” or “Bam Maxi DNA”) were separated from the minicircles and undigested catenanes by buoyant centrifugation in CsCl in the presence of Hoechst 33258. The purified RI Maxi DNA and the Bam Maxi DNA ran as single bands in agarose with a molecular weight of approx. $18\text{--}20 \cdot 10^6$. Most of the mapping was performed using the RI Maxi DNA. The Bam Maxi DNA was isolated for confirmatory digestions and to check on the possibility of maxicircle heterogeneity.

Restriction mapping of the RI maxicircle

(a) *Localization of sites for BamHI, BglII and HhaI.* The purified RI Maxi DNA was digested with *BamHI*, *BglII*, *HhaI*, *HpaII*, *HindIII*, *HaeIII* and *SallI*, and the fragments separated by electrophoresis in agarose (Fig. 1A, Table I).

It is evident that there is a single site for *Bam*HI and there are two sites for *Bgl*II and *Hha*I, and four sites for *Hae*III, *Hpa*II and *Sal*I. There are five sites for *Hind*III, although the $0.16 \cdot 10^6$ dalton fragment, is only visualized in acrylamide (not shown).

TABLE I

MOLECULAR WEIGHTS (M.W.) OF MAXICIRCLE FRAGMENTS RESULTING FROM SINGLE DIGESTIONS OF RI MAXI

Enzyme	$\bar{X} \pm \text{S.D. (N)}^c (\cdot 10^6)$	Total M.W. ($\cdot 10^6$)	Adjusted M.W. ($\cdot 10^6$) ^a
<i>Eco</i> RI/ <i>Bam</i> HI	(A) 14.48 \pm 0.83(4)	19.04	15.07
	(B) 4.56 \pm 0.40(4)		4.40
<i>Eco</i> RI/ <i>Bgl</i> II	(A) 8.78 \pm 1.06(5)	20.14	8.69
	(B) 8.78 \pm 1.06(5)		8.26
	(C) 2.58 \pm 0.29(5)		2.52
<i>Eco</i> RI/ <i>Hha</i> I	(A) 11.64 \pm 1.15(4)	20.19	11.49
	(B) 6.81 \pm 0.78(4)		6.18
	(C) 1.74 \pm 0.14(4)		1.80
<i>Eco</i> RI/ <i>Hpa</i> II	(A) 10.74 \pm 1.35(6)	21.13	9.59
	(B) 5.56 \pm 0.68(6)		5.00
	(C) 2.85 \pm 0.24(6)		2.97
	(D) 1.32 \pm 0.08(4)		1.26
	(E) 0.66 \pm 0.03(4)		0.65
<i>Eco</i> RI/ <i>Hae</i> III	(A) 8.16 \pm 1.27(6)	19.98	7.70
	(B) 6.07 \pm 0.88(6)		5.80
	(C) 3.36 \pm 0.37(6)		3.28
	(D) 1.40 \pm 0.44(5)		1.69
	(E) 0.99 \pm 0.05(4)		1.00
<i>Eco</i> RI/ <i>Sal</i> I	(A) 13.03 \pm 0.50(4)	19.26	12.80
	(B) 2.62 \pm 0.22(4)		2.85
	(C) 1.50 \pm 0.07(4)		1.65
	(D) 1.17 \pm 0.04(5)		1.17
	(E) 1.01 \pm 0.05(5)		1.00
<i>Eco</i> RI/ <i>Hind</i> III	(A) 12.25 \pm 1.09(5)	18.91	12.69
	(B) 2.86 \pm 0.38(5)		2.80
	(C) 2.00 \pm 0.13(5)		2.05
	(D) 1.19 \pm 0.08(4)		1.18
	(E) 0.61 \pm 0.06(4)		0.59
	(F) 0.16 ^b		0.16
Average 19.81 \pm 0.79			

^a Values adjusted to total M.W. of $19.47 \cdot 10^6$ in accordance with results of double and triple digestions.

^b Measured by acrylamide electrophoresis. All other values measured by agarose electrophoresis.

^c Mean \pm standard deviation (number).

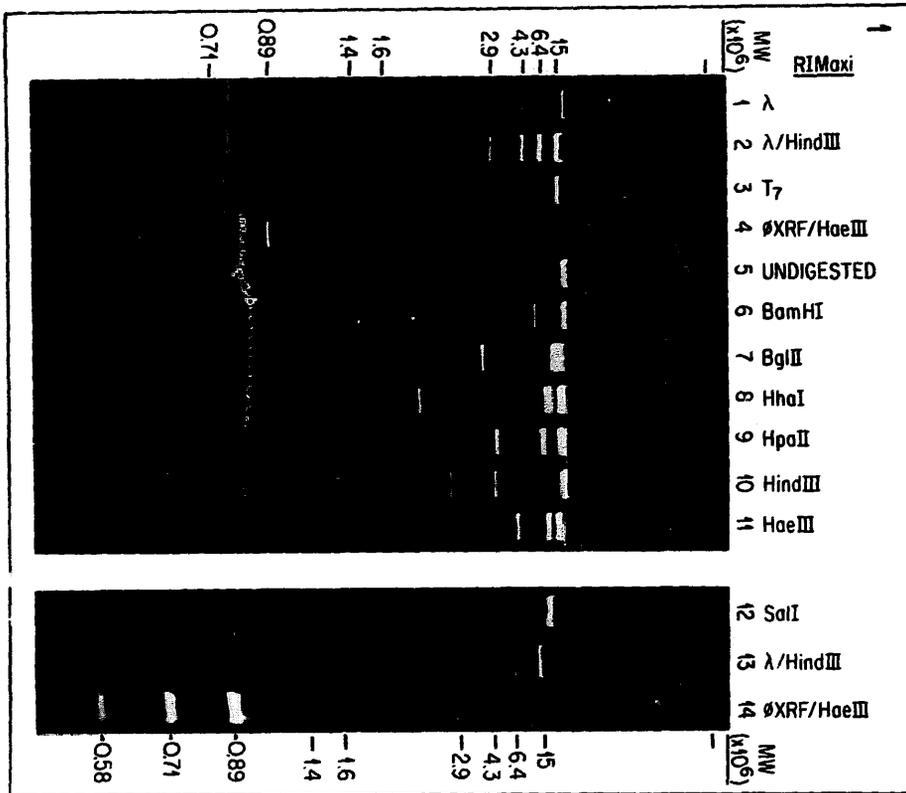


Fig. 1. Agarose gels of RI Maxi DNA digested with 7 restriction enzymes. Slots 1–11, 1.2% agarose; slots 12–14, 1% agarose.

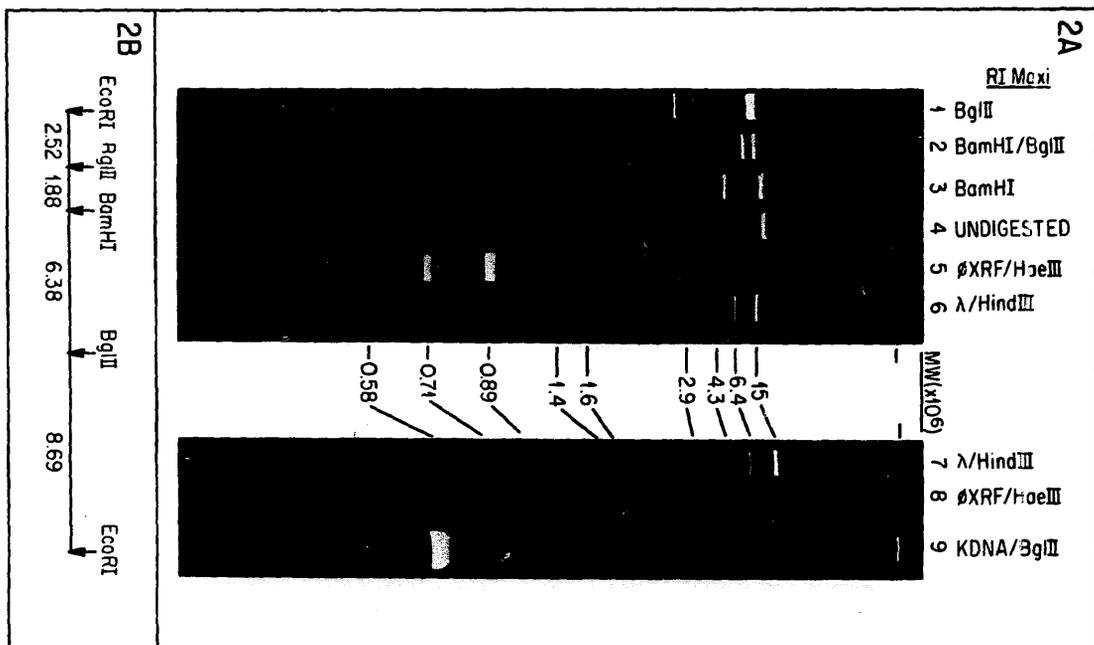


Fig. 2. Agarose gels of double digests of RI Maxi DNA, and a *Bgl*II digest of KDNA. (A) 1% agarose. (B) Diagram of sites for *Bam*HI and *Bgl*II. Fragment molecular weights given in daltons ($\cdot 10^6$).

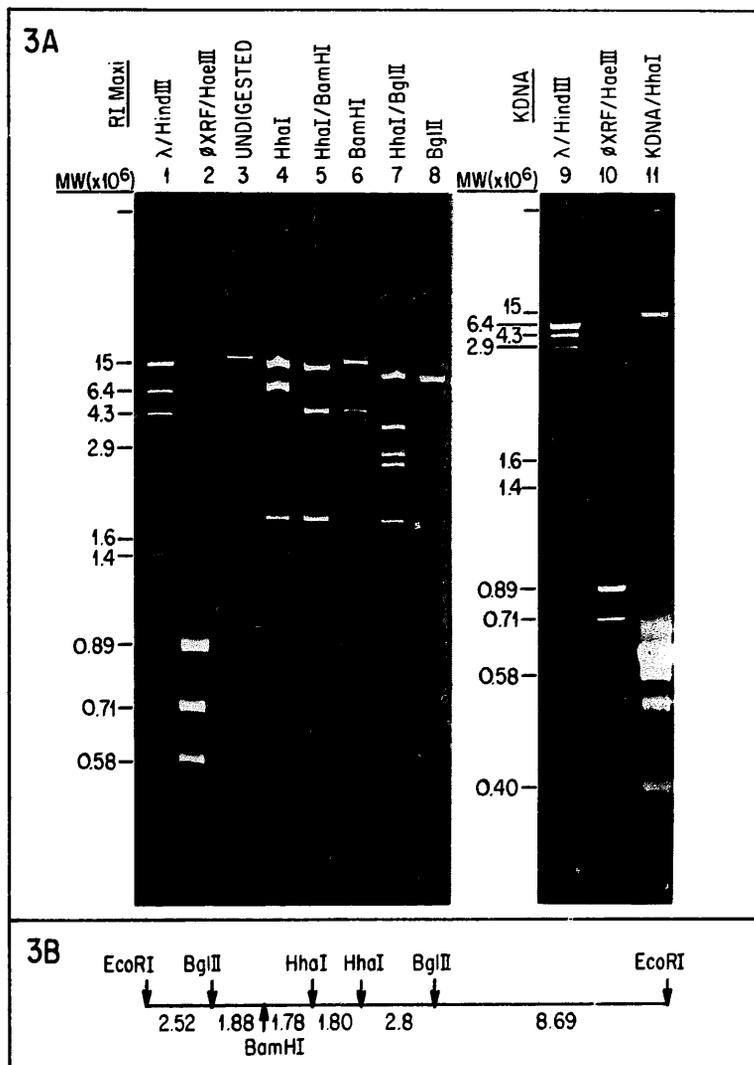
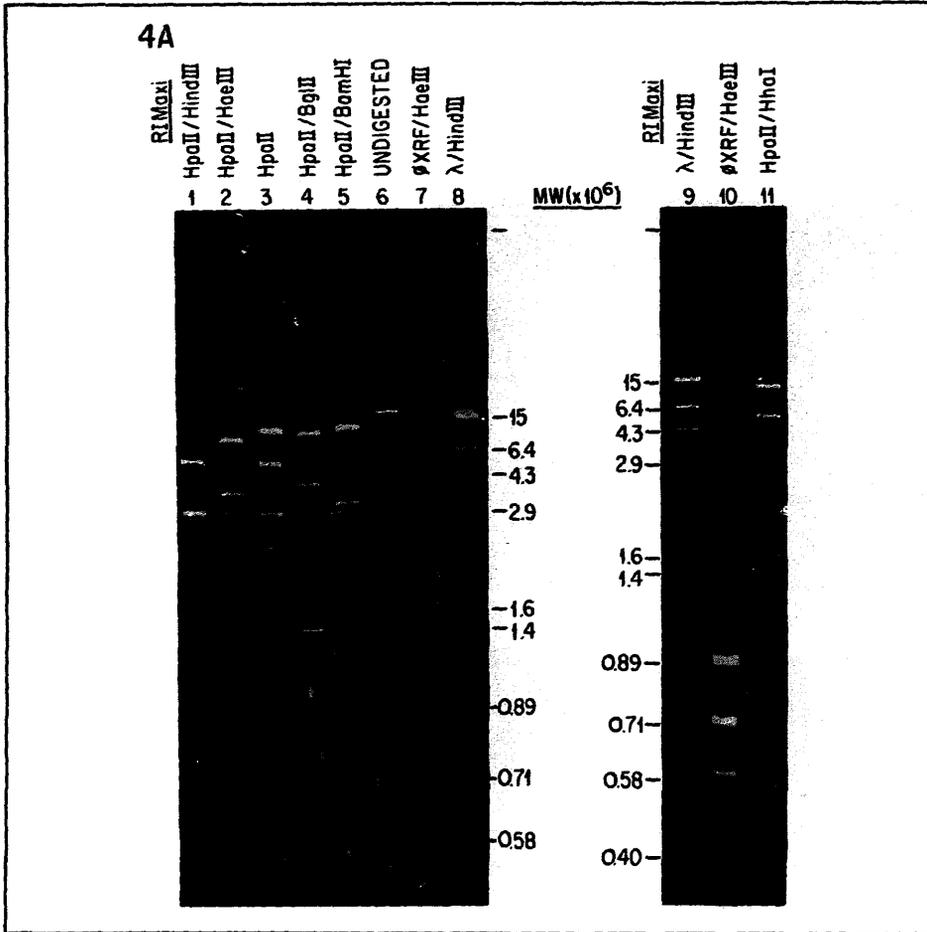


Fig. 3. Agarose gels of double digests of RI Maxi DNA and a *HhaI* digest of KDNA. (A) 1% agarose. (B) Diagram of sites for *BamHI*, *HhaI* and *BglII*.

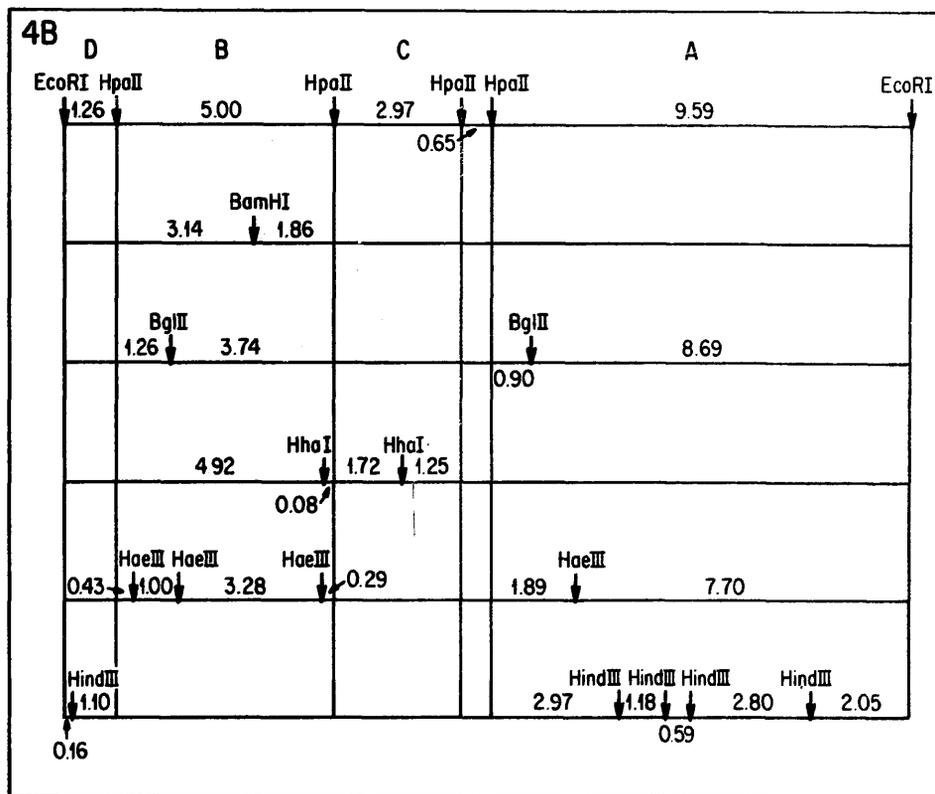
The orientation of the various fragments relative to the single *EcoRI* site was established by comparing single digestions of the intact maxicircle within the KDNA network with single and double digestions of the purified RI Maxi linear. We will first describe the localization of the 1- and 2-site enzymes — *BamHI*, *BglII* and *HhaI*.

The single *BamHI* site is located $4.4 \cdot 10^6$ daltons from the *EcoRI* site (Fig. 1, slot 6). The existence of two *BglII* sites can be seen clearly in the gel in Fig. 2A by a comparison of the *BglII* digest of the RI Maxi with the *BglII*/*BamHI* double digest of the RI Maxi. *BglII* fragments A and B comigrate in the



gel (Fig. 2A, slot 1) but fragment B contains a single *Bam*HI site and gives rise to two smaller fragments after *Bam*HI digestion (Fig. 2A, slot 2). Digestion of total KDNA networks with *Bgl*II gives rise to two bands containing the maxicircle fragments B and A + C, to a minicircle band, and to undigested minicircle catenanes remaining at the origin (Fig. 2A, slot 9). The relative orientation of these sites is diagrammed in Fig. 2B.

The localization of the two *Hha*I sites was determined by the same procedure. Double digestion of the RI Maxi with *Hha*I and *Bam*HI (Fig. 3A, slot 5) shows the single *Bam* site to be within the *Hha*I B fragment, with one product of this digestion comigrating with the RI/*Hha* C fragment. Furthermore, the release of one fragment identical in size to the *Bam* B fragment (Fig. 3A, slot 6) localizes the RI/*Hha* B fragment on the left side of the RI Maxi as shown in Fig. 3B. The relative localization of the RI/*Hha* A and C fragments was established by digestion of intact maxicircle DNA with *Hha*I (Fig. 3A, slot 11) in which two maxicircle fragments are released, corresponding to the RI/*Hha* A + B and C fragments.



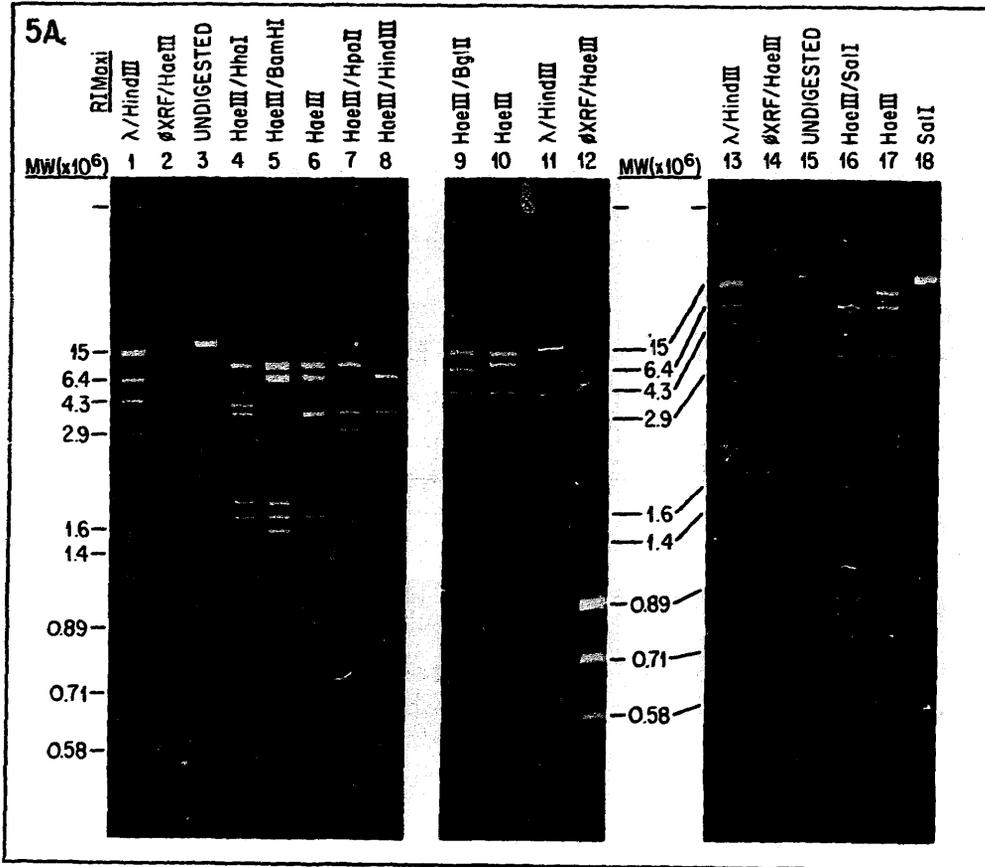
HpaII - DOUBLE DIGESTIONS OF RI MAXI

Fig. 4. Agarose gels of double digests of RI Maxi DNA. (A) 1% agarose. (B) Diagram with separate localizations of sites for *Bam*HI, *Bgl*II, *Hha*I, *Hae*III and *Hind*III within the 4 *Hpa*II fragments. The *Hpa*II fragments are labeled A–D in decreasing molecular weight sequence.

The *Bgl*II/*Hha* double digestion of the RI Maxi (Fig. 3A, slot 7) yields the five fragments predicted by the map in Fig. 3B.

(b) *Localization of sites for HpaII.* The localization of the 4- and 5-site enzymes — *Hpa*II, *Hae*III, *Sal*I and *Hind*III — will be established by first presenting the final restriction map and then showing all possible confirmatory double digests predicted by this map. This method of presentation is simpler than attempting to explain the step-by-step reasoning involved in the derivation of this map.

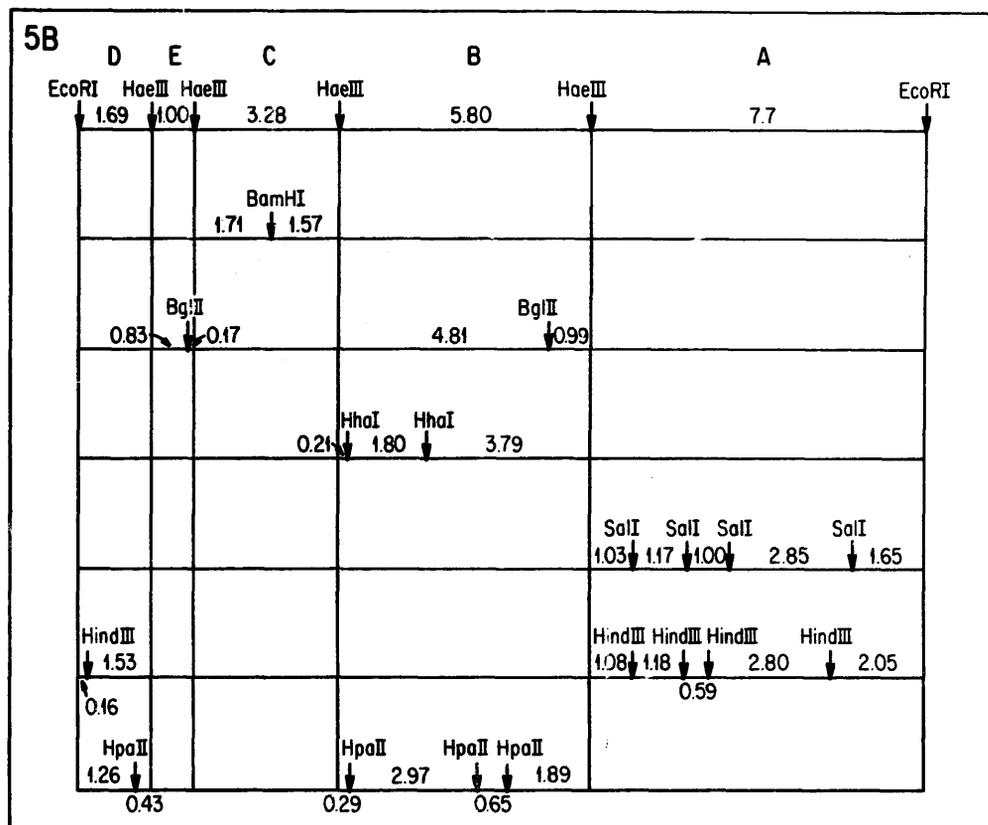
The *Hpa*II map is presented in Fig. 4B together with individual maps of double digests of the RI Maxi with *Hpa*II plus *Bam*HI, *Bgl*II, *Hha*I, *Hae*III and *Hind*III. The actual results of these double digests are shown in the agarose gels in Fig. 4A. In each case the predicted fragments of the double digests are seen in the gel, except for several small fragments which were not visualized in this gel system and which were sized in 1.5% agarose or 10% acrylamide. For example, two of the four products of the digestions of the *Hpa*II B fragment with *Hae*III were not visualized in this gel, and neither was



the $0.16 \cdot 10^6$ dalton fragment produced by *Hind*III digestion of the *Hpa*II D fragment.

(c) *Localization of sites for Hae*III. The *Hae*III map is presented in Fig. 5B together with individual maps of double digestions of the RI Maxi with *Hae*III plus *Bam*HI, *Bgl*II, *Hha*I, *Sal*I, *Hind*III and *Hpa*II. The actual results of these double digestions are shown in the agarose gels in Fig. 5A. In each case the predicted fragments are seen in the gels except for small fragments not visualized in this gel system, such as the $0.21 \cdot 10^6$ dalton fragment released from the *Hae*III fragment B by *Hha*I, the $0.17 \cdot 10^6$ dalton fragment released from *Hae*III fragment E by *Bgl*II, and the 0.29 and $0.65 \cdot 10^6$ dalton fragments released from *Hae*III fragment B by *Hpa*II. The four *Sal*I sites are all located within the *Hae*III fragment A. One of the *Hha*I/*Sal*I fragments comigrated with the *Hae*III fragment E, producing a band with greater than normal intensity.

(d) *Localization of sites for Sal*I. The *Sal*I map is presented in Fig. 6B together with individual maps of double digestions of the RI Maxi with *Sal*I plus *Bam*HI, *Bgl*II, *Hha*I, *Hpa*II, and *Hind*III. The actual results of these digestions are shown in the agarose gels in Fig. 6A. The map predictions were verified in each case in terms of the fragments released by each double digestion of the RI Maxi. In the case of the *Sal*I/*Hind*III double digestion (Fig. 6A, slot 15), however, several small fragments were released which could only be



HaeIII - DOUBLE DIGESTIONS OF RI MAXI

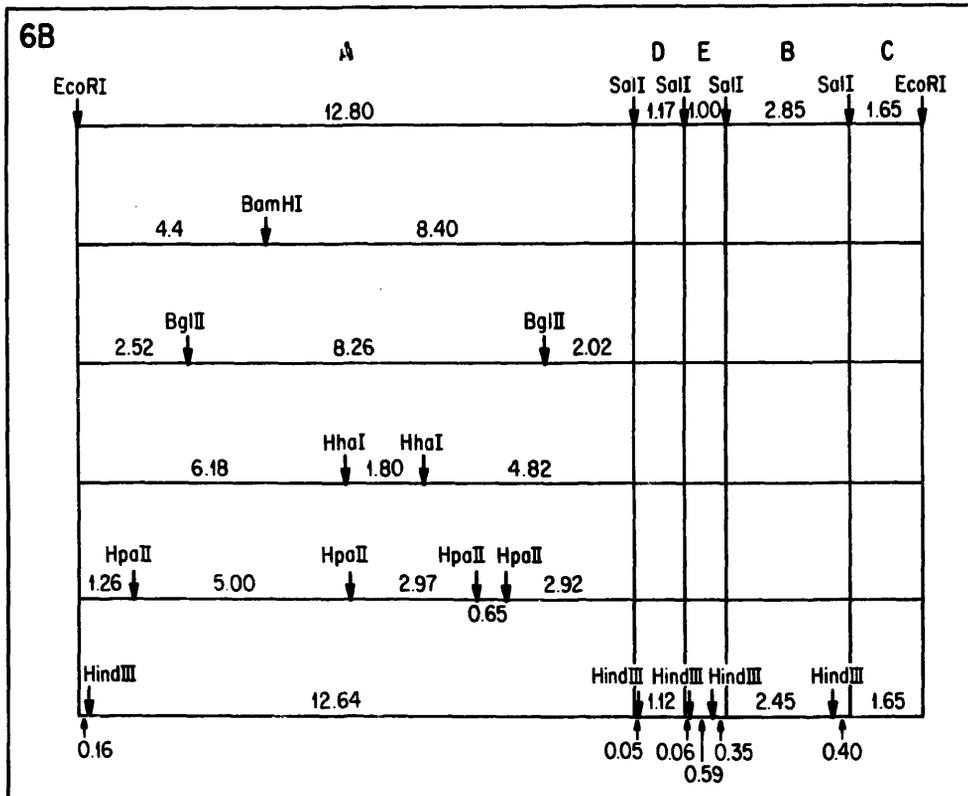
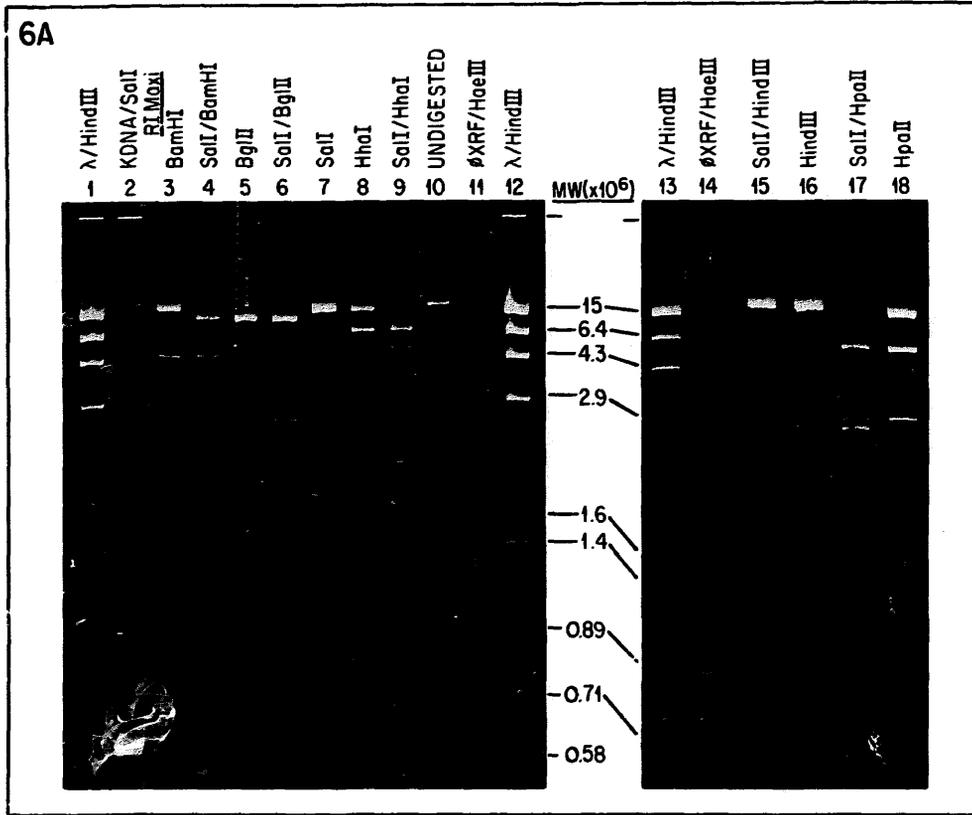
Fig. 5. Agarose gels of double digests of RI Maxi DNA. (A) 1% agarose. (B) Diagram.

visualized in acrylamide gels (not shown). There is some uncertainty about the exact sizes of the small *SalI*/*HindIII* fragments.

The juxtaposition of *RI/Sal* fragments A and C was established by the *Sal* digestion of intact maxicircle DNA (shown in Fig. 6A, slot 2) in which the *RI/Sal* fragment C disappears.

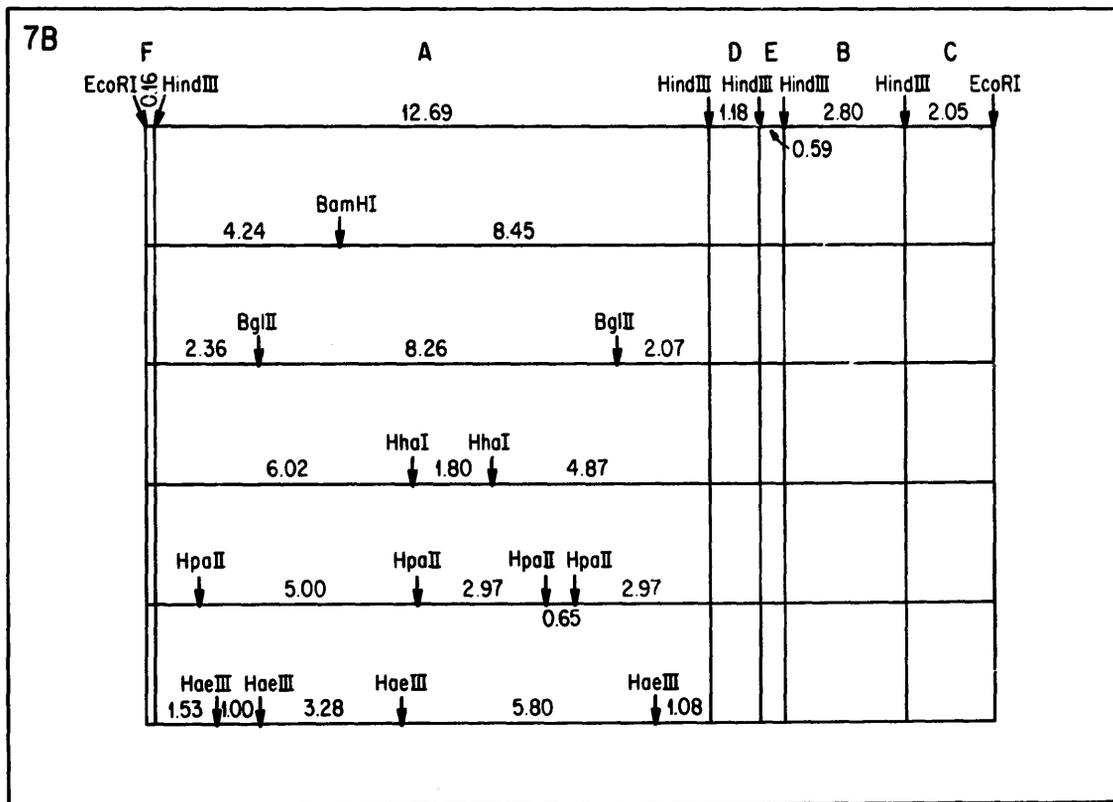
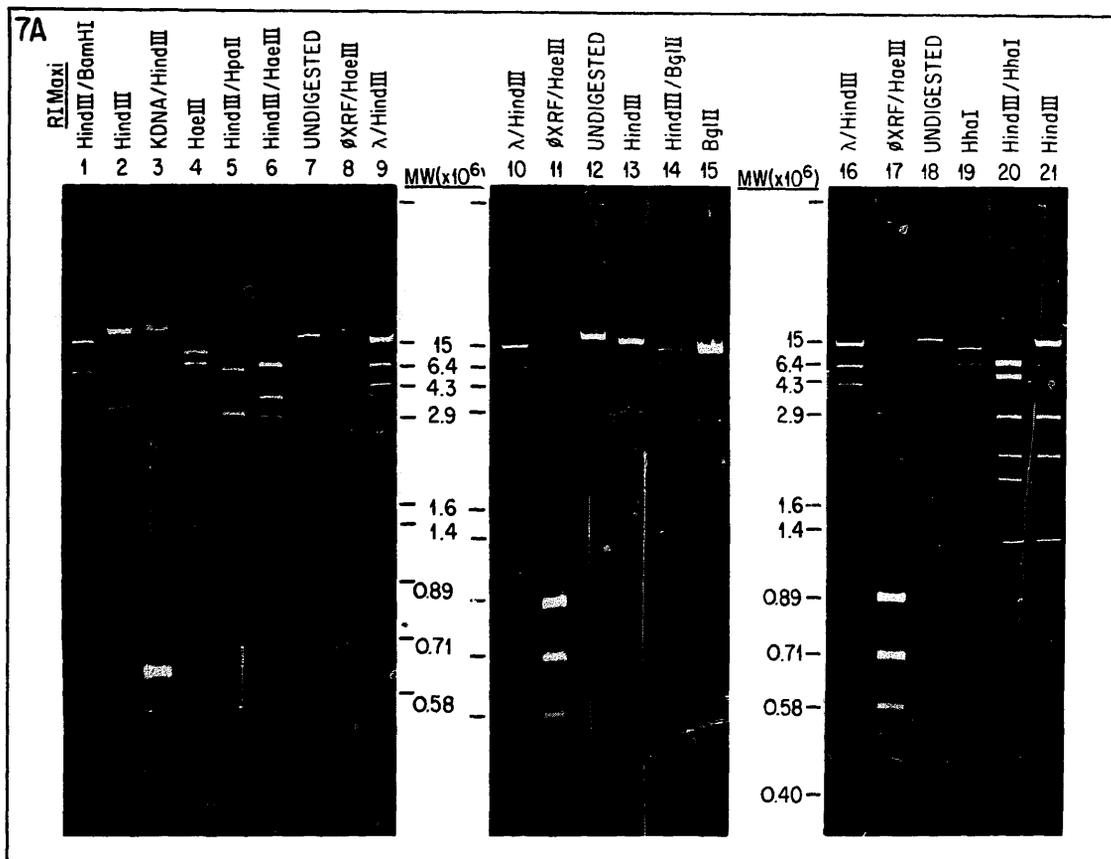
It is of some interest that all of the enzymes tested except for *HindIII* have sites only in the *SalI* A fragment.

(e) *Localization of sites for HindIII.* The *HindIII* map is presented in Fig. 7B together with individual maps of double digestions of the RI Maxi with *HindIII* plus *BamHI*, *BglII*, *HhaI*, *HpaII* and *HaeIII*. The actual confirmatory results of these digestions are presented in the agarose gels in Fig. 7A. The map predictions were verified in each case. There is one small discrepancy with respect to the size of one fragment released from *RI/HindIII* fragment A by digestion with *BglII*. The map predicts a size of $2.07 \cdot 10^6$ daltons, which should comigrate with the $2.05 \cdot 10^6$ dalton *RI/HindIII* C fragment. The gel (Fig. 7A, slot 14) shows that the fragment in question clearly migrates faster than the $2.05 \cdot 10^6$ dalton *RI/HindIII* C fragment and is approx. $1.8 \cdot 10^6$ daltons instead of $2.07 \cdot 10^6$ daltons. This small discrepancy is probably a result of inaccuracies in the size measurements of the large *RI/BglII* fragments A and B.



SalI - DOUBLE DIGESTIONS OF RI MAXI

Fig. 6. Agarose gels of double digests of RI Maxi DNA and a *SalI* digest of KDNA. (A) 1% agarose. (B) Diagram.



HindIII - DOUBLE DIGESTIONS OF RI MAXI

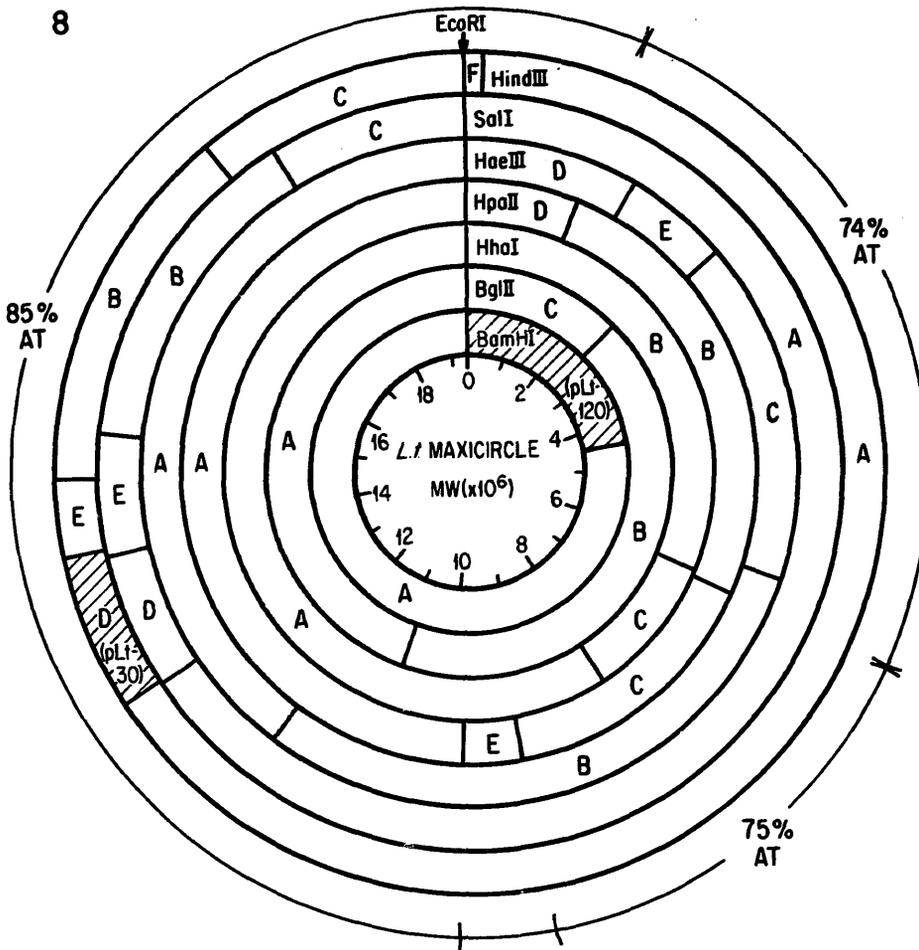
Fig. 7. Agarose gels of double digests of RI Maxi DNA. (A) 1% agarose. (B) Diagram.

The juxtaposition of *RI/HindIII* fragments F and C is established by a comparison of the digestion of the *RI Maxi* with *HindIII* with the digestion of intact maxicircle DNA with *HindIII* (Fig. 7A, slots 2 and 3). The disappearance of fragment C and the appearance of a slightly larger fragment in its place confirms this localization.

(f) *Composite circular restriction map of the RI maxicircle DNA.* A composite circular map of all the above restriction sites is presented in Fig. 8. The AT contents of three portions of the molecule are also indicated, as obtained previously by buoyant *CsCl* analysis of the three largest maxicircle *HpaII* bands (Simpson, 1979).

Restriction mapping of the BamHI linearized maxicircle

The above map was constructed with the purified *RI Maxi* DNA, although in each case a digestion of total *KDNA* was performed to confirm the relative



RESTRICTION MAP OF *L. tarentolae* *KDNA* MAXICIRCLE

Fig. 8. Composite circular restriction map of maxicircle. AT% of 3 *HpaII* fragments is indicated. Cloned fragments indicated by shading.

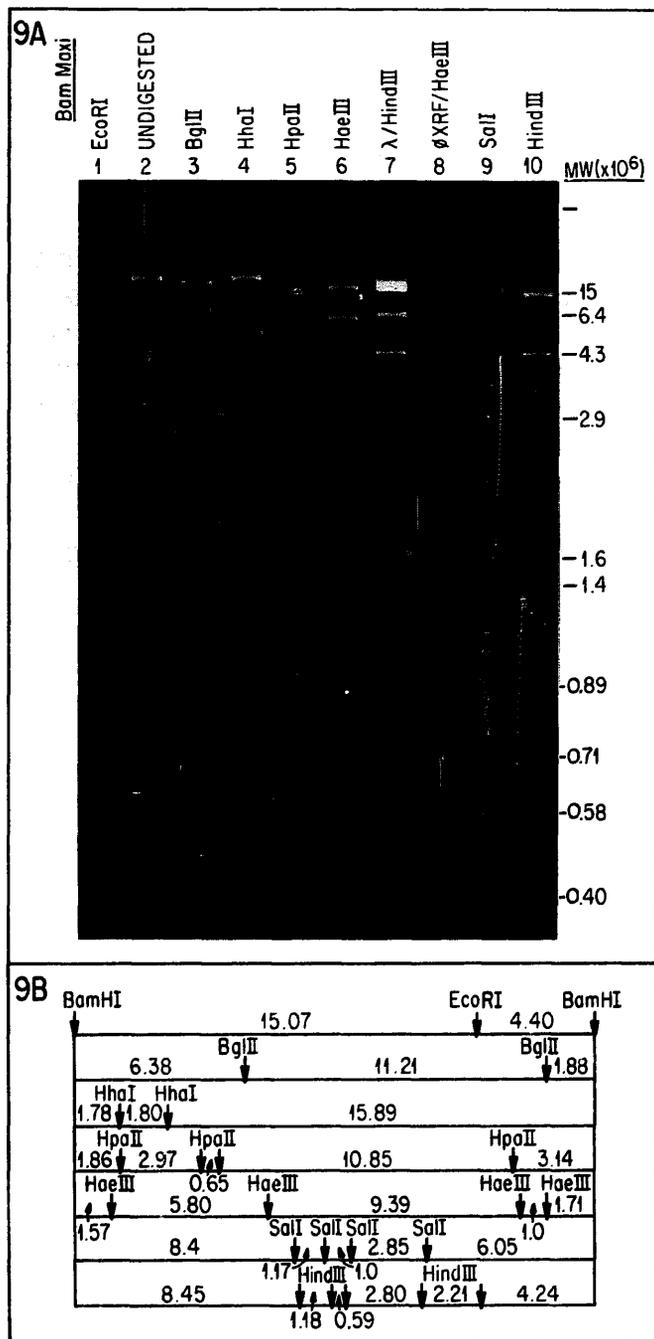


Fig. 9. Agarose gel of Bam Maxi DNA digested with 7 restriction enzymes. (A) 1% agarose. (B) Diagram.

localizations of the terminal fragments. It could be argued that we selected a homogeneous class of maxicircles by this method, i.e. those with a single *EcoRI* site, and that we have not proved that the entire maxicircle population is homogeneous. Therefore, we isolated linearized maxicircle DNA cleaved at the single *BamHI* locus and performed confirmatory digestions. As shown in Fig. 9, the predicted fragments were released in each case. This experiment

makes it unlikely that there is a different major sequence class of maxicircles lacking an *EcoRI* site but possessing a *Bam*HI site.

Cloning maxicircle fragments in pBR322

Two fragments of the maxicircle have been cloned in the bacterial plasmid pBR322. In the first cloning protocol, total KDNA was digested with *Hind*III or *Bam*HI and the released fragments, both minicircle-derived and maxicircle-derived, were cloned into the *Hind*III or *Bam*HI locus of the plasmid. Most tetracycline-sensitive transformants contained unit length $0.6 \cdot 10^6$ dalton minicircle inserts (e.g. pLt-19 in Fig. 10, slot 3), which will be discussed in a future report, but one maxicircle sequence was also obtained in several clones (e.g. pLt-30 in Fig. 10, slot 1). The maxicircle-derived insert of pLt-30 apparently corresponded to the $1.18 \cdot 10^6$ dalton *Hind*III D fragment (Fig. 10, slot 2).

In the second cloning protocol, purified RI Maxi DNA was digested with

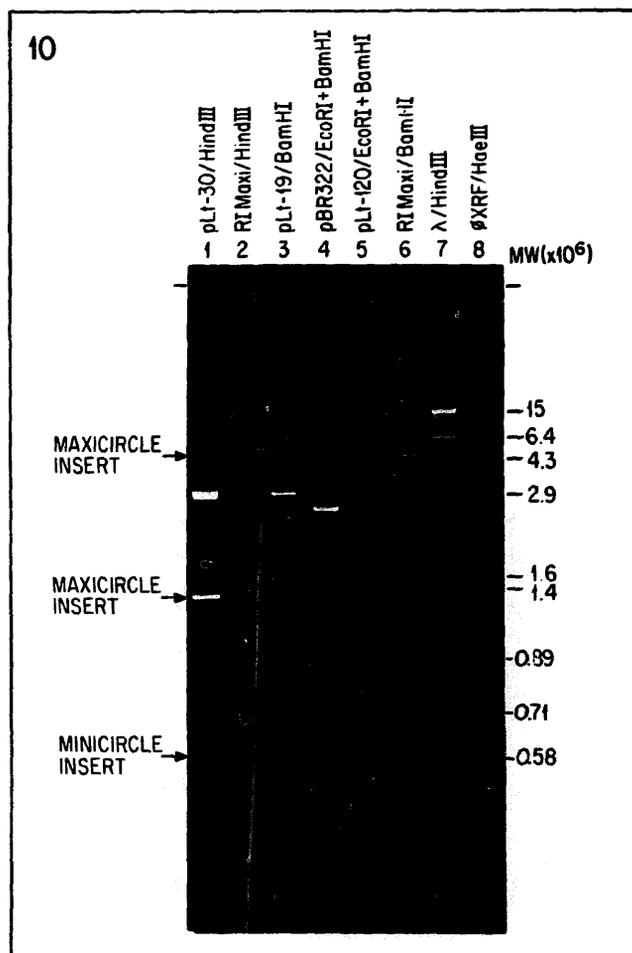


Fig. 10. Agarose gel (0.8%) of the recombinant plasmids pLt-30, pLt-19 and pLt-120. The plasmids were digested to release the inserts. *Hind*III or *Bam*HI digested RI Maxi DNA was co-run to identify the cloned inserts.

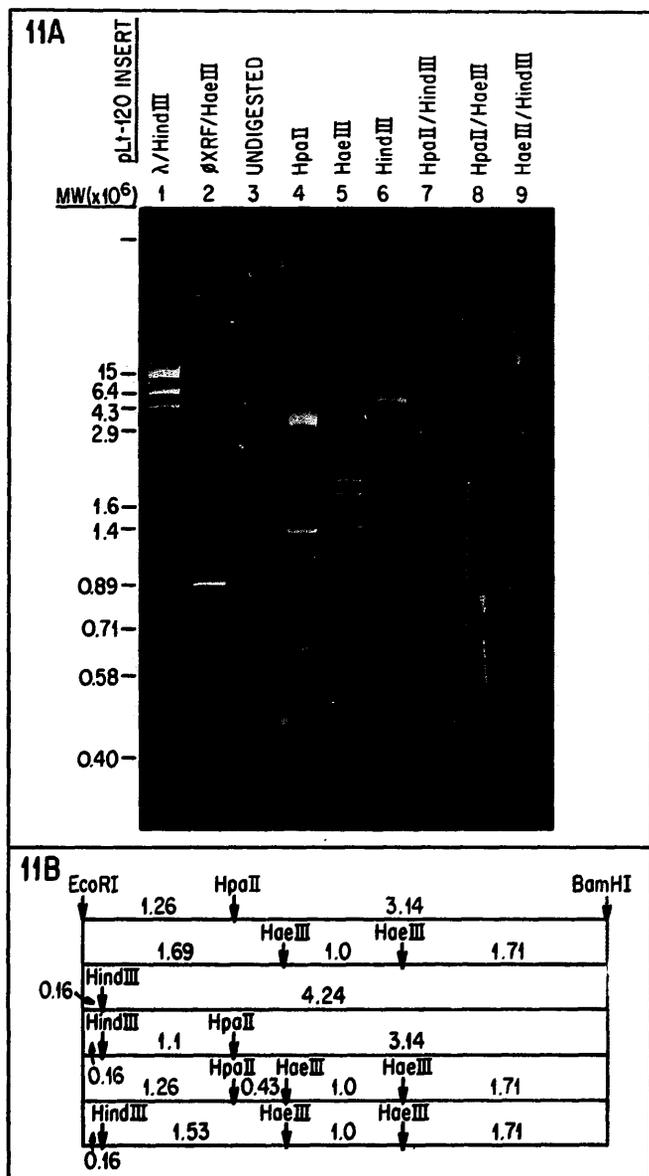


Fig. 11. Agarose gel of purified pLt-120 insert DNA digested with several enzymes. (A). 1.2% agarose. (B) Diagram showing expected localizations of sites for each enzyme.

*Bam*HI and the resulting *RI/Bam* maxicircle fragments were cloned into the *RI/Bam*-digested plasmid. Two transformants (pLt-120 and pLt-133) were selected which contained inserts which corresponded to the *RI/Bam* maxicircle fragment B (Fig. 10, slots 5 and 6). The localization of the cloned fragments is indicated on the maxicircle map in Fig. 8.

Both cloned maxicircle inserts were isolated from the host plasmid by appropriate digestion and buoyant separation in CsCl in the presence of the AT-binding dye, Hoechst 33258. The separation of the pLt-30 insert from the plasmid was larger than the separation of the pLt-120 insert, implying that the pLt-30 insert has a higher AT content. This is consistent with the previous

evidence that this portion of the maxicircle molecule has an AT content of 85% vs. a 74% AT content of the remainder of the molecule (Fig. 8). Digestion of the purified pLt-120 insert with several enzymes as shown in Fig. 11 produced fragments consistent with the restriction map derived from uncloned maxicircle DNA (Fig. 8).

The purified pLt-120 insert was labeled with ^{32}P by nick-translation and this probe was used to confirm the restriction map by detection of these sequences within other fragments by Southern hybridizations. The hybridization of the labeled pLt-120 insert probe to Southern transfers of RI Maxi DNA digested with several enzymes is shown in Fig. 12. The labeled bands in the autoradiograph demonstrate the presence of the *RI/Bam* B fragment sequence in each case within the fragments as predicted by the map.

Localization of the genes for the kinetoplast 9 and 12S RNAs

The kinetoplast 9 and 12S RNAs were isolated by electrophoresis in 5% acrylamide and were labeled with ^{32}P by polynucleotide kinase after mild alkali hydrolysis. The labeled RNA probes were then hybridized to Southern transfers of agarose gel profiles of purified RI Maxi DNA, total KDNA, and the cloned *RI/Bam* B fragment, each digested with several enzymes, and the

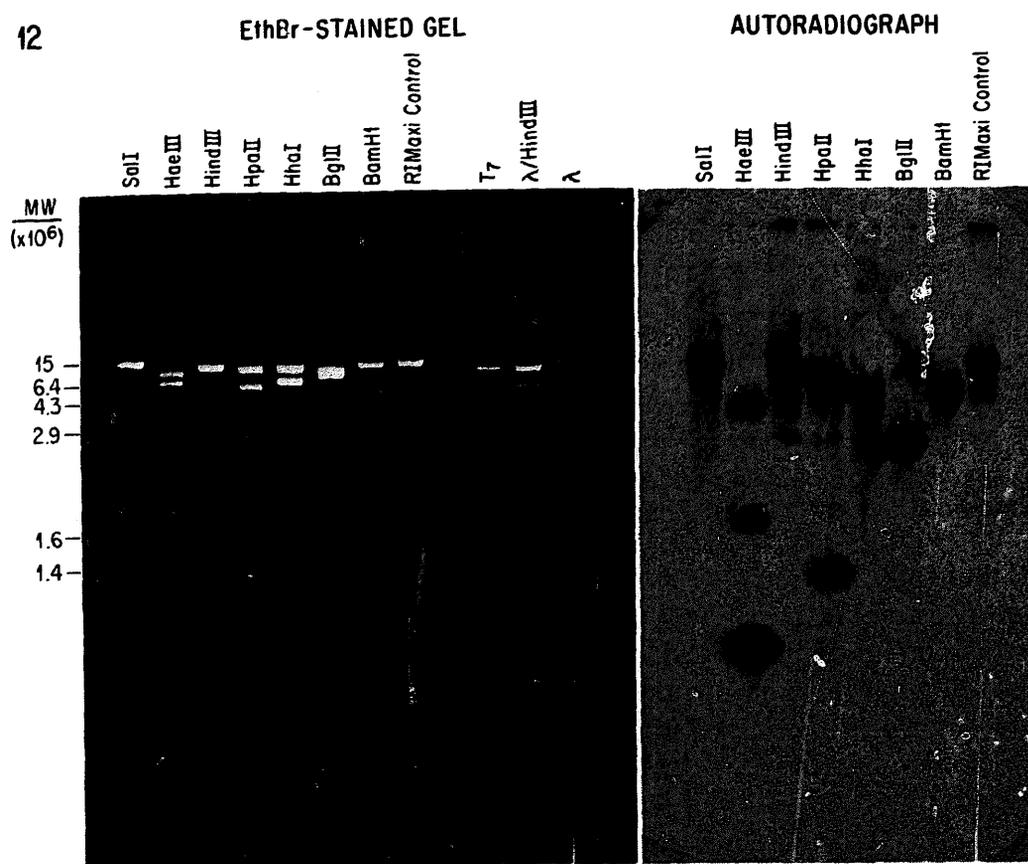


Fig. 12. Southern transfer of agarose gel of RI Maxi DNA digested with several enzymes. Hybridization probe was ^{32}P -pLt-120 insert DNA.

labeled bands were detected by autoradiography as shown in Fig. 13. In the case of the RI Maxi RNA and the cloned *RI/Bam* B fragment, both the 9 and 12S RNAs hybridized mainly to a *Hind*III subfragment of the *Hpa*II fragment D. No hybridization was observed to the *Hind*III fragment F in Figs. 13A (slots 7 and 12) and 13B (slots 7 and 12). Hybridization of the same labeled probes to total KDNA digested with *Hpa*II and *Hae*III gave essentially identical results (Fig. 14), but there are some apparent discrepancies, particularly in the case of the 12S RNA. The 9S RNA hybridized mainly to the *Hpa*II A fragment (= RI Maxi A + D fragments) and to the *Hae*III A fragment (= RI Maxi A + D fragments) as expected. The 12S RNA hybridized mainly to the *Hae*III A fragment as expected, but in the case of the *Hpa*II digestion, the 12S RNA apparently hybridized approximately equally to the A and B fragments. One possible explanation for this apparent discrepancy is that it might be an artifact of the Southern transfer method in which large DNA fragments transfer slowly if at all. The KDNA/*Hpa*II A fragment has a molecular weight of $10.6 \cdot 10^6$

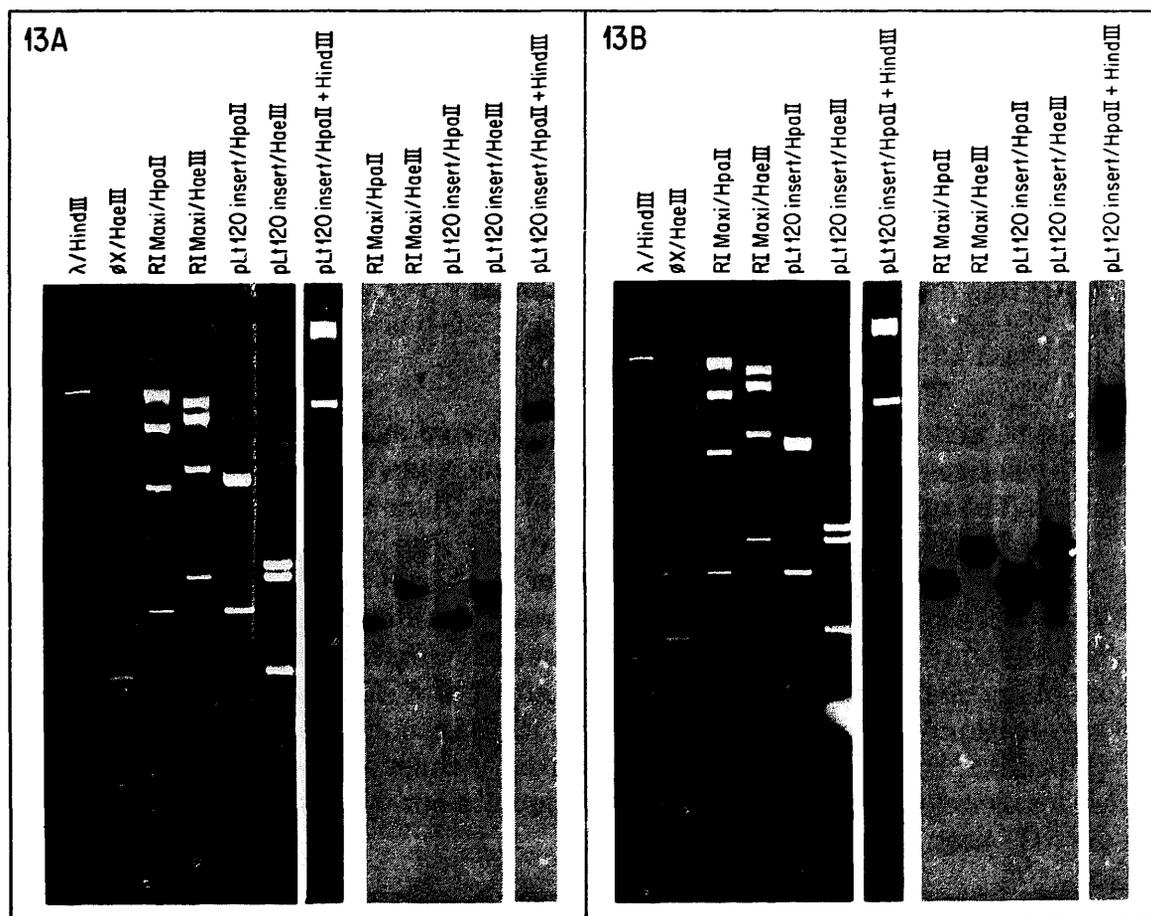


Fig. 13. Southern transfer of agarose gels of RI Maxi DNA and pLt-120 insert DNA digested with several enzymes. (A) Slots 1–6, 1% agarose; slot 7, 2% agarose. The filter was hybridized with ^{32}P -labeled 9S kinetoplast RNA. (B) Slots 1–6, 1% agarose; slot 7, 2% agarose. The filter was hybridized with ^{32}P -labeled 12S kinetoplast RNA.

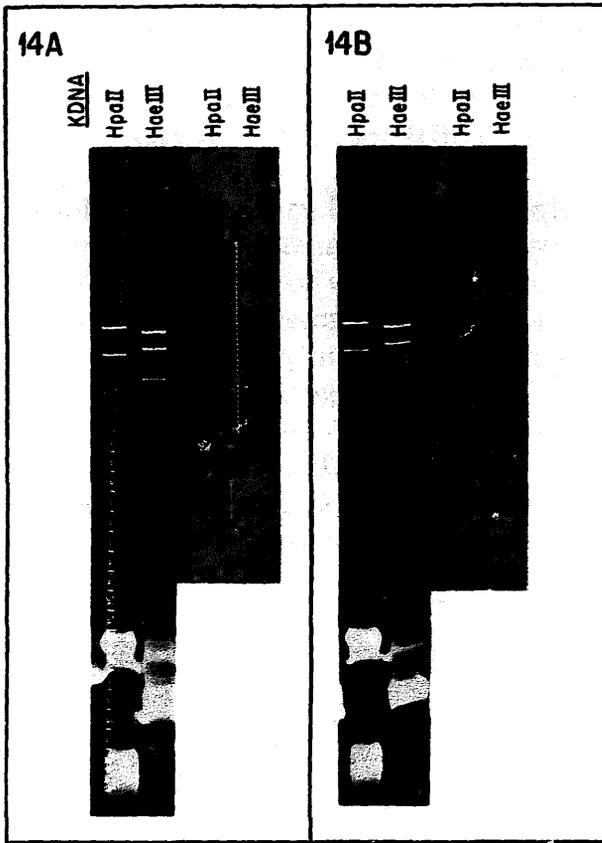


Fig. 14. Southern transfer of 1% agarose gel of total KDNA digested with *HpaII* and *HaeIII*. (A). The filter was hybridized with ^{32}P -labeled 9S kinetoplast RNA. (B). The filter was hybridized with ^{32}P -labeled 12S kinetoplast RNA.

daltons, and therefore may be underrepresented in the transfer and give rise to a relatively smaller autoradiographic signal than the $5 \cdot 10^6$ dalton B fragment. The other possible explanation is that there is a maxicircle heterogeneity. This possibility was tested for the case of the Bam Maxi DNA by hybridizing the labeled 12S RNA probe to a transfer of the gel shown in Fig. 9 of Bam Maxi DNA digested with several enzymes. Hybridization was observed mainly to the following fragments: *BglII* fragment A, *HhaI* fragment A, *HpaII* fragment A, *HaeIII* fragment A, *SalI* fragment A and *HindIII* fragment B. These localizations are in agreement with the RI Maxi and cloned *RI/Bam* fragment B localizations described above. Therefore if there are separate sequence classes of maxicircles, they do not involve molecules containing single *EcoRI* or *BamHI* sites.

DISCUSSION

The availability of a buoyant density method to isolate large quantities of the linearized maxicircle DNA of *L. tarentolae* (Simpson, 1979) has led directly to the development of a restriction enzyme map of the circular molecule and

to the cloning of maxicircle fragments in a bacterial plasmid. An interesting feature of the maxicircle molecule is the intramolecular base composition heterogeneity. More than half of the molecule is extremely high in AT content (85% AT); this portion of the molecule contains all the *SalI* and *HindIII* sites and few if any sites for *HpaII*, *HaeIII*, *HhaI*, and *BglII*.

Transcriptional mapping of the maxicircle has been initiated with the localization of the genes for the kinetoplast 9 and 12S RNAs (Simpson and Simpson, 1978) within a $1.1 \cdot 10^6$ dalton *HindIII/HpaII* fragment located near the single *EcoRI* site.

Cloning of the maxicircle in the bacterial plasmid pBR322 has been initiated with the isolation of two recombinant plasmids containing the *EcoRI/BamHI* B fragment and the *HindIII* D fragment. The 9 and 12S RNA genes are localized within the cloned *EcoRI/BamHI* B fragment. Cloning of the remainder of the maxicircle molecule is in progress. Recombinant plasmids containing unit length minicircle inserts have also been obtained. The availability of cloned maxicircle fragments and cloned minicircles will allow an experimental approach to several important questions such as the complete transcriptional mapping of the maxicircle molecule, subcloning and sequencing of the genes for the 9 and 12S kinetoplast RNAs, determination of the precise extent of minicircle sequence heterogeneity by the cloning and sequencing of the different sequence classes, and determination of the existence or absence of any minicircle transcripts.

The question of maxicircle heterogeneity arises due to the existence of multiple copies (approx. 25 per network in *L. tarentolae*) of the maxicircle molecule within the network. We have shown that maxicircle molecules containing a single *EcoRI* site or a single *BamHI* site give rise to a unique restriction enzyme map with 7 enzymes and are therefore homogeneous at this level. The equivocal results with the transcriptional mapping of the 9 and 12S RNA genes using maxicircle DNA liberated from the networks with *HpaII* may be the result of an artifact of the Southern transfer method in which large fragments transfer poorly and are underrepresented in the blot. Of course, any small scale sequence heterogeneity would not be observed by our methods, and this might in fact be expected assuming independent replication of a population of 25 molecules per KDNA network.

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