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THE SIZE OF THE REPEATING UNIT OF THE REPETITIVE MITOCHONDRIAL DNA FROM A "LOW-DENSITY" PETITE MUTANT OF YEAST

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Summary

1. We have analysed further the properties of mtDNA of RD1A, an ethidium-induced cytoplasmic petite mutant of *Saccharomyces cerevisiae*. RD1A mtDNA contains 3 mole percent (G + C); the H-strand (the strand with the higher buoyant density in alkaline CsCl) contains 3 mole percent G and less than 0.1 mole percent C, the L-strand contains 3 mole percent C and less than 0.3 mole percent G.

2. Analysis of the pyrimidine tracts of RD1A mtDNA on DEAE-cellulose columns shows that the two longest tracts, T_6 and T_5C_2, are present in approximately a 1 : 1 molar ratio, indicating that they occur once only in the proposed repeating unit. Direct sequence analysis of the T_5C_2 tract shows that it is the unique sequence isomer T—T—T—C—C—T—T. Assuming the unique occurrence of T_6 and T_5C_2 in the repeating unit of RD1A mtDNA, we calculate a minimal size of 68 nucleotides.

3. Degradation of the L-strand with the T—C base sequence specific, bacteriophage T_4-induced endonuclease IV yields material which migrates in a single, sharp peak in acrylamide gels; from its mobility in denaturing gels containing 4S and 5S RNA markers, the material is about 70 nucleotides long.

4. We conclude that the minimal repeating unit of RD1A mtDNA is approximately 70 nucleotides long.

Introduction

Growth of yeast in the presence of intercalating dyes such as ethidium, leads to the induction of cytoplasmic petite mutants unable to make functional

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Abbreviations: H-strand, heavy strand; L-strand, light strand.

mitochondria [1]. In such mutants the mtDNA is either altered or completely absent. Detailed analysis [2—4] of one of these mutants (RD1A) has shown that its mtDNA consists of a large number of perfect repeats of a very (A + T)-rich section of wild-type mtDNA, less than 300 nucleotides long.

In this communication we present more detailed information on the composition and size of the repeating unit of this unusual mtDNA. Some of our results have been presented briefly elsewhere [5].

Results

Base composition of RD1A mtDNA

Previous experiments [3,4] had shown a large discrepancy between the mole percent (G + C) determined by direct nucleotide analysis (6%) and by pyrimidine tract analysis (1.2%). Reanalysis of the total RD1A mtDNA and extension of the analysis to the separated complementary strands H and L (Fig. 1 and Table I) showed that the correct mole percent (G + C) is 3% and

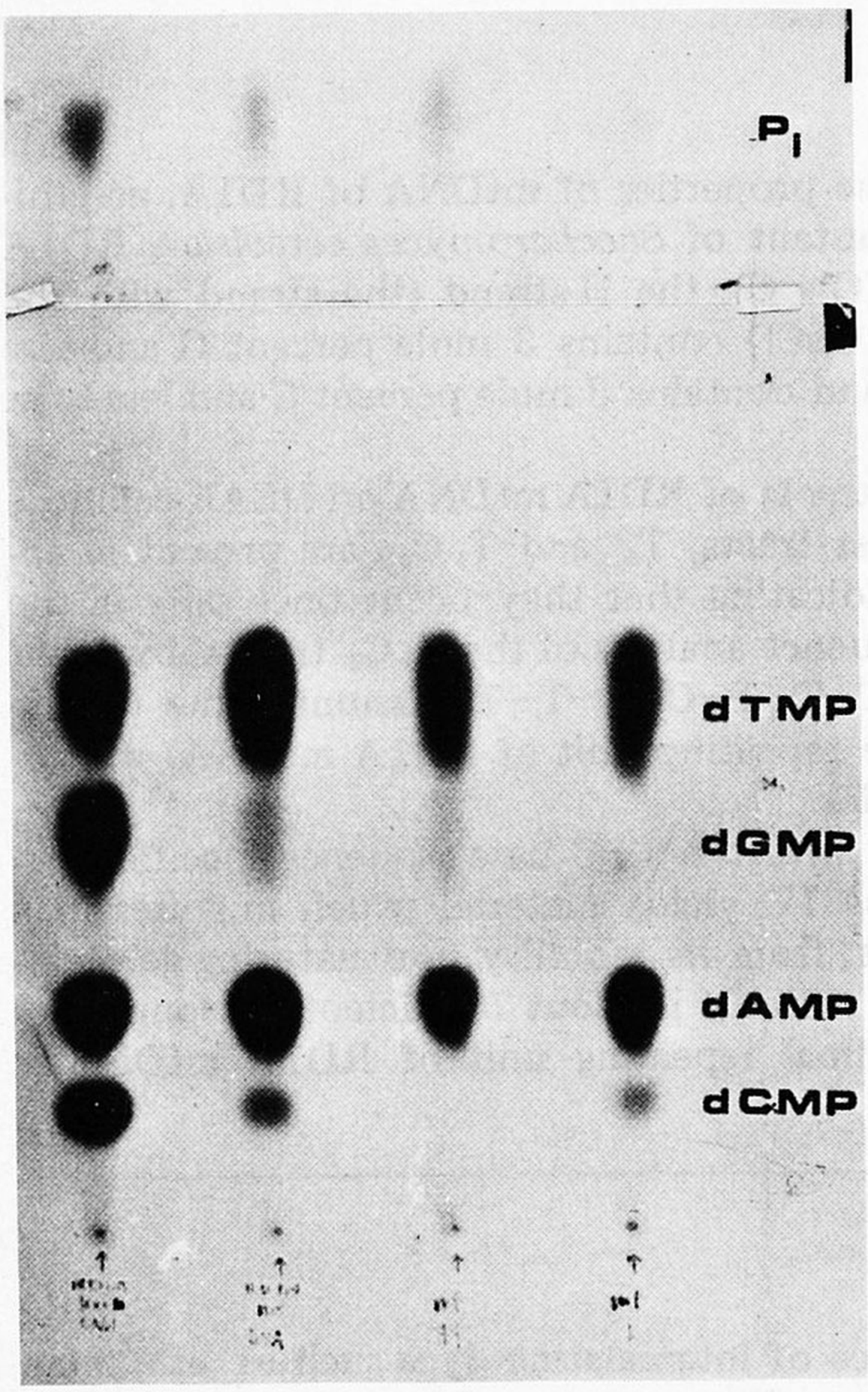


Fig. 1. Autoradiogram of an electropherogram of 5′-deoxyribonucleoside monophosphates released from RD1A mtDNA and nuclear DNA by enzymic digestion. From left to right: RD1A nuclear DNA, duplex RD1A mtDNA, RD1A mtDNA H-strand, RD1A mtDNA L-strand.

TABLE I

BASE COMPOSITION OF RD1A mtDNA AND RD1A NUCLEAR DNA

For details of the enzymic digestion and the separation of 5′-deoxyribonucleoside monophosphates, see Methods. Inputs: duplex mtDNA, 35 000 cpm; H- and L-strand, approx. 8000 cpm; nuclear DNA, 40 000 cpm. Recoveries, > 75%.

DNA	Origin (mole %)	Nucleoside monophosphate (mole %)				P_i (mole %)	(G + C) (mole %)
		dCMP	dAMP	dGMP	dTMP		
Duplex mtDNA	0.1	1.5	50.6	1.5	45.0	1.0	3.0
H-strand	0.5	0.1	43.0	2.8	50.4	3.2	2.9
L-strand	0.2	3.2	52.9	0.3	42.3	1.3	3.5
Nuclear	1.8	20.4	27.8	18.7	30.1	1.2	39.3

essentially all C is in the L-strand and all G in the H-strand. We attribute the radioactivity at the dGMP position in the L-strand electropherogram to streaking of dTMP. The higher T content of the H-strand accounts for its higher buoyant density in alkaline CsCl. RD1A nuclear DNA has 39 mole percent (G + C) in good agreement with the results of Hollenberg et al. [3]. The mole percent (G + C) value of 6% reported previously for RD1A mtDNA [3] was probably due to contamination of the earlier mtDNA preparations with nuclear DNA.

Pyrimidine clusters in RD1A mtDNA

Van Kreijl et al. [4] separated pyrimidine oligonucleotides from RD1A mtDNA on polyethyleneimine cellulose thin layers [6], and found 5 main pyrimidine tracts, T_1, T_2, T_3, T_6, T_5C_2 and 2 minor tracts X_1 and X_2, possibly containing unusual bases. The H-strand DNA had T_1, T_2, T_3 and T_6 as the major tracts and the L-strand DNA T_1, T_2 and T_5C_2 only. These results are qualitatively in agreement with the nucleotide analysis presented in Table I. However, the quantitative evaluation of the chromatograms was not reliable, since only 0.6 mole percent C was recovered [4] from the total of 1.5% present.

For further sequence work a quantitative pyrimidine cluster analysis allows the calculation of the minimal size of a repeating sequence unit. Fig. 2 presents the elution pattern of ^{32}P-labelled pyrimidine oligonucleotides released from RD1A mtDNA by formic acid—diphenylamine hydrolysis [7] and chromatographed according to chain length on DEAE-cellulose [8]. The quantitative analysis of this experiment and comparison with the data of Van Kreijl et al. [4] is presented in Table II. Direct comparison is possible since there are no chain length or sequence isomers in RD1A mtDNA (see below).

The P_i in this analysis (20%) is somewhat higher than the calculated interpyrimidine phosphate content (17.4%) and the uncorrected total moles pyrimidine per 100 moles DNA phosphate (47.9%) show that recovery was better than 96% [9]. The calculated T content from the major component oligonucleotides is 46.6 mole percent, and the C content is 1.25 mole percent; these values compare very favourably with the results from the direct base composition analysis of 45 mole percent T and 1.5 mole percent C. We at-

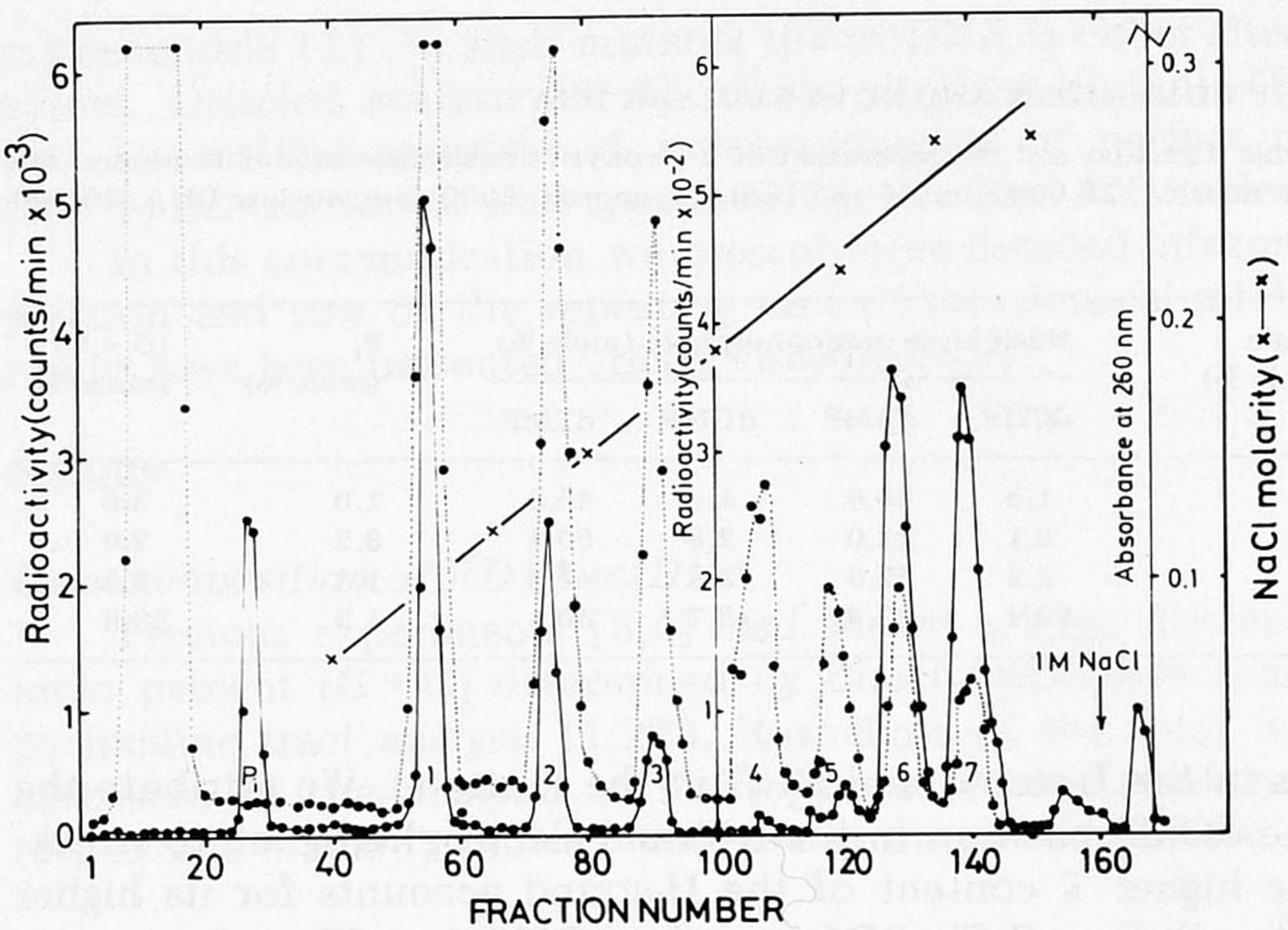


Fig. 2. DEAE-cellulose chromatography of a diphenylamine, formic acid hydrolysate of ^{32}P-labelled RD1A mtDNA and carrier calf-thymus DNA. A mixture of ^{32}P-labelled RD1A mtDNA (85 600 cpm) and calf-thymus DNA (1.5 mg) was degraded as described in Methods. After removal of diphenylamine and formic acid, the pyrimidine oligonucleotides were applied to a column of DEAE-cellulose (20 cm × 1.5 cm) pre-equilibrated with 50 mM sodium acetate in 7 M urea (pH 5.5). The column was washed to remove unabsorbed purines and the oligonucleotides eluted with a linear gradient of NaCl from 0—0.28 M (effluent volume 300 ml; 160 fractions collected). Radioactivity of the fractions was measured by Cerenkov counting; $A_{260\ nm}$ with Zeiss PMQ spectrophotometer and conductivity with a Mullard type conductivity bridge. ●———●, ^{32}P radioactivity; ●- - - - -●, $A_{260\ nm}$; ×———×, NaCl molarity.

TABLE II

PYRIMIDINE TRACTS OF RD1A mtDNA

See Methods for analytical procedure. Input, 85 600 cpm. Recovery, 90%.

Pyrimidine tract	Component	^{32}P (% of total)	Moles pyrimidine/ 100 moles DNA-P	Number of tracts per 100 nucleotides of duplex DNA	
				This paper	Van Kreijl et al. [4]
$n = 0$	P_i	20.0	—	—	—
$n = 1$	T_1	42.0	21.96	21.0	19.5
$n = 2$	T_2	19.1	13.30	6.4	6.8
$n = 3$	T_3	6.7	5.27	1.7	1.9
$n = 4$	prob. X_1*	0.1	0.08		
$n = 5$	prob. X_2*	0.6	0.52		
$n = 6$	T_6	5.1	4.53	0.73	0.51
$n = 7$	T_5C_2	4.8	4.39	0.60	0.30
Extra material ($n > 7$)		0.8	?		
1 M NaCl strip		0.8	?		
		100.0	50.0**		

* Unusual pyrimidine tracts previously reported by Van Kreijl et al. [4].

** Uncorrected total, 47.9.

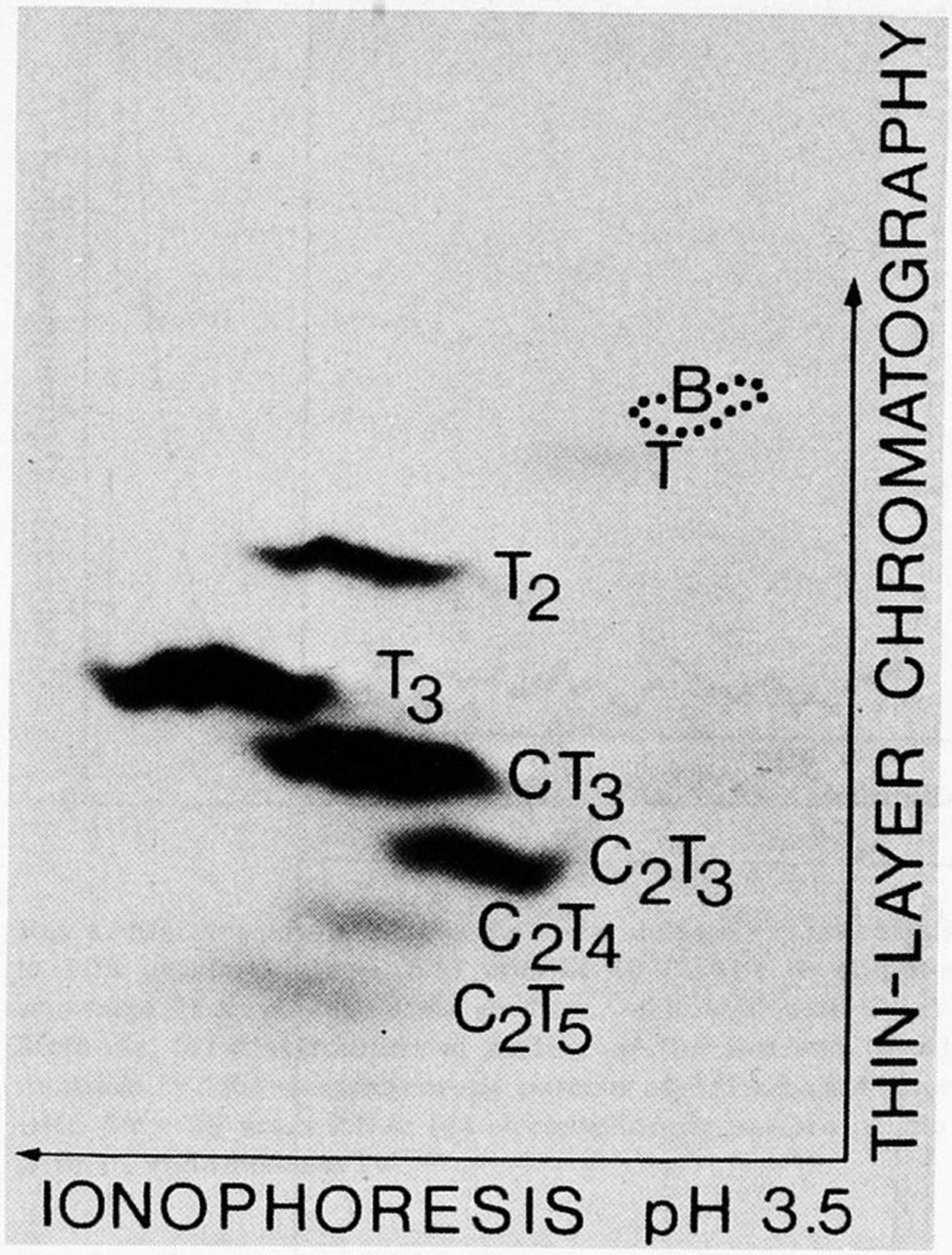


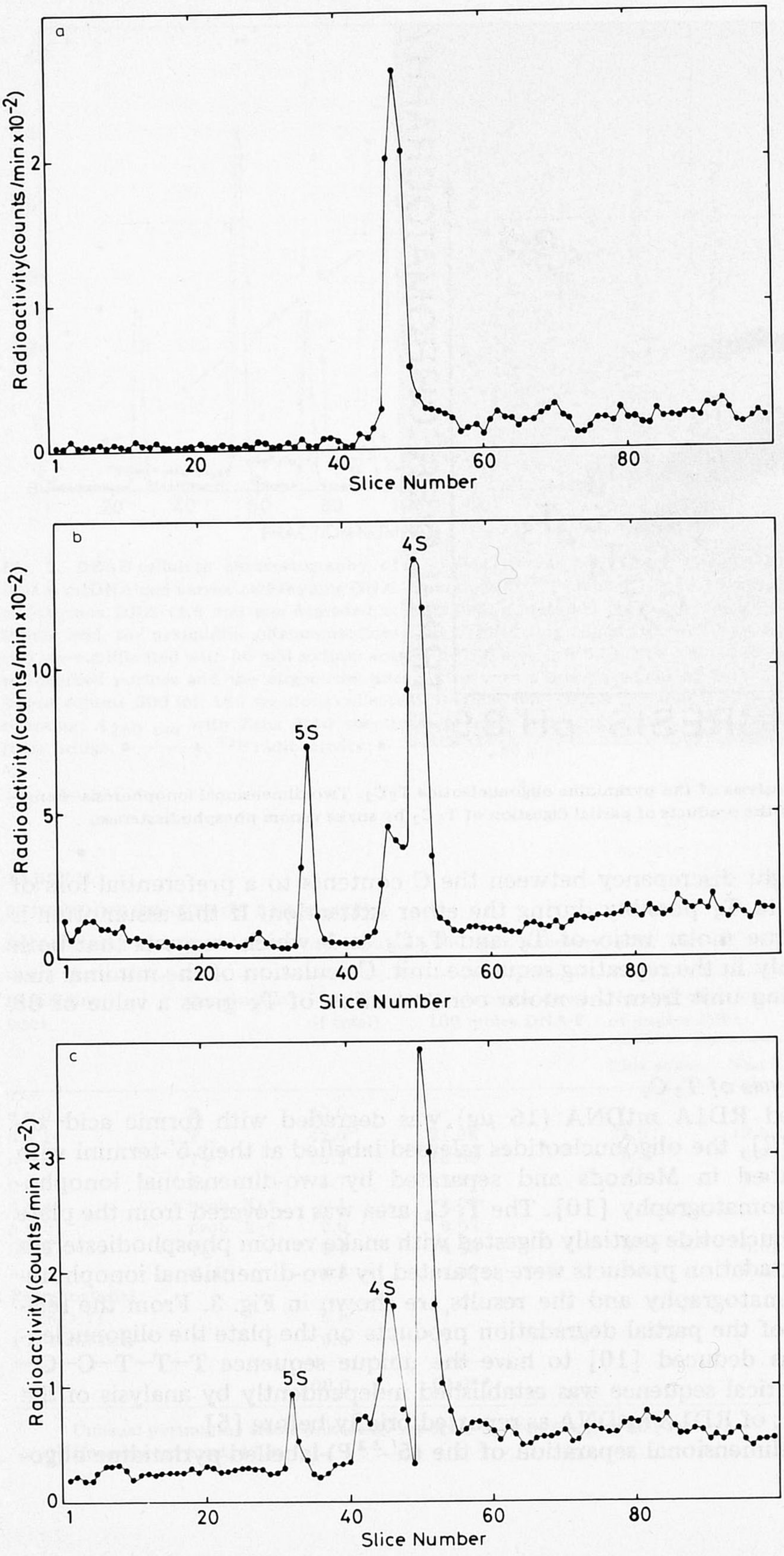
Fig. 3. Sequence analysis of the pyrimidine oligonucleotide T_5C_2. Two-dimensional ionophoresis—homochromatography of the products of partial digestion of T_5C_2 by snake venom phosphodiesterase.

tribute the slight discrepancy between the C contents to a preferential loss of T_5C_2 relative to T_6 possibly during the ether extraction. If this assumption is correct, the true molar ratio of T_6 and T_5C_2 is 1 which suggests that both occur once only in the repeating sequence unit. Calculation of the minimal size of the repeating unit from the molar concentration of T_6 gives a value of 68 nucleotides.

Sequence analysis of T_5C_2

Unlabelled RD1A mtDNA (16 μg) was degraded with formic acid—diphenylamine [7], the oligonucleotides released labelled at their 5′-termini with ^{32}P as described in Methods and separated by two-dimensional ionophoresis—homochromatography [10]. The T_5C_2 area was recovered from the plate and the heptanucleotide partially digested with snake venom phosphodiesterase [11]. The degradation products were separated by two-dimensional ionophoresis—homochromatography and the results are shown in Fig. 3. From the relative positions of the partial degradation products on the plate the oligonucleotide T_5C_2 was deduced [10] to have the unique sequence T—T—T—C—C—T—T. The identical sequence was established independently by analysis of the RNA transcript of RD1A mtDNA as reported briefly before [5].

The two-dimensional separation of the (5′-^{32}P)-labelled pyrimidine oligo-


a
Radioactivity(counts/min x10-2)
0
1
2
1
20
40
60
80
Slice Number
b
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5S
Radioactivity(counts/min x10-2)
0
5
10
1
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60
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Radioactivity(counts/min x10-2)
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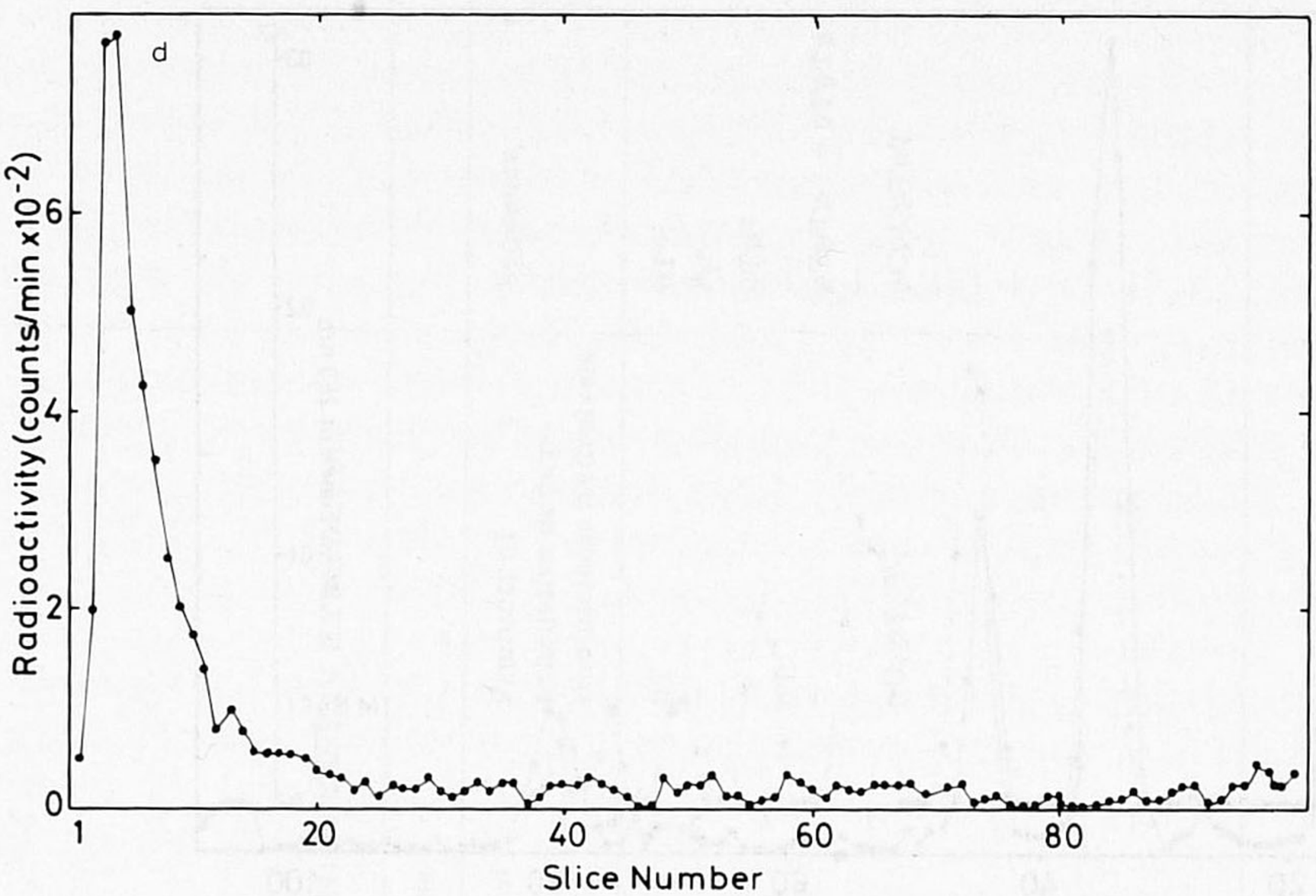

Fig. 4. Electrophoresis of the T_4 endonuclease IV hydrolysis products of single-stranded RD1A mtDNA in 10% acrylamide gels, 8 M urea at 60°C. The H- and L-strands of ^{32}P-labelled RD1A mtDNA were separated in an alkaline CsCl gradient, incubated with T_4 endonuclease IV and processed as described in Methods. (a) Electrophoretic pattern of the L-strand of RD1A mtDNA after treatment with T_4 endonuclease IV; (b) electrophoretic pattern of ^{32}P-labelled 4S + 5S yeast RNA; (c) as (a) but supplemented with 4S + 5S yeast RNA; (d) electrophoretic pattern of the H-strand of RD1A mtDNA after treatment with T_4 endonuclease IV.

nucleotides revealed a number of minor spots in addition to $T_5 C_2$ and T_6. Two of these may correspond to the unusual minor tracts X_1 and X_2 found by Van Kreijl et al. [4] in this DNA. Whether the others are indeed derived from RD1A mtDNA is under investigation.

Fragmentation of RD1A mtDNA by T_4 endonuclease IV

If all the C residues of RD1A mtDNA are present in the sequence T—T—T—C—C—T—T and if the sequence occurs once only in the repeating unit, fragmentation of the L-strand of the mtDNA with the TC-sequence specific T_4 endonuclease IV [12] should yield material of repeating unit length.

This experiment was carried out using separated L or H mtDNA single strands. These were approx. 1000 nucleotides in length based on sedimentation coefficients of approximately 10S in alkaline sucrose gradients. The electrophoretic behaviour of the ^{32}P-labelled T_4 endonuclease IV RD1A H- or L-strand fragments on 10% acrylamide gels under denaturing conditions [13], are shown in Fig. 4: a mixture of ^{32}P-labelled 4S and 5S RNA from yeast was coelectrophoresed as marker. The H-strand material hardly penetrated the gel, showing that it was not extensively degraded, whereas the L-strand DNA migrated as a single, sharp peak, slightly ahead of the 4S RNA marker. From the inverse relationship between the distance moved in the gel and the log of the molecular weight [13], the size of the L-strand fragment is 69 ± 2 bases. This calculation assumes the marker RNAs and L-strand DNA fragment were fully denatured under the conditions of the experiment [13] and behave equivalently in denaturing gels [14].

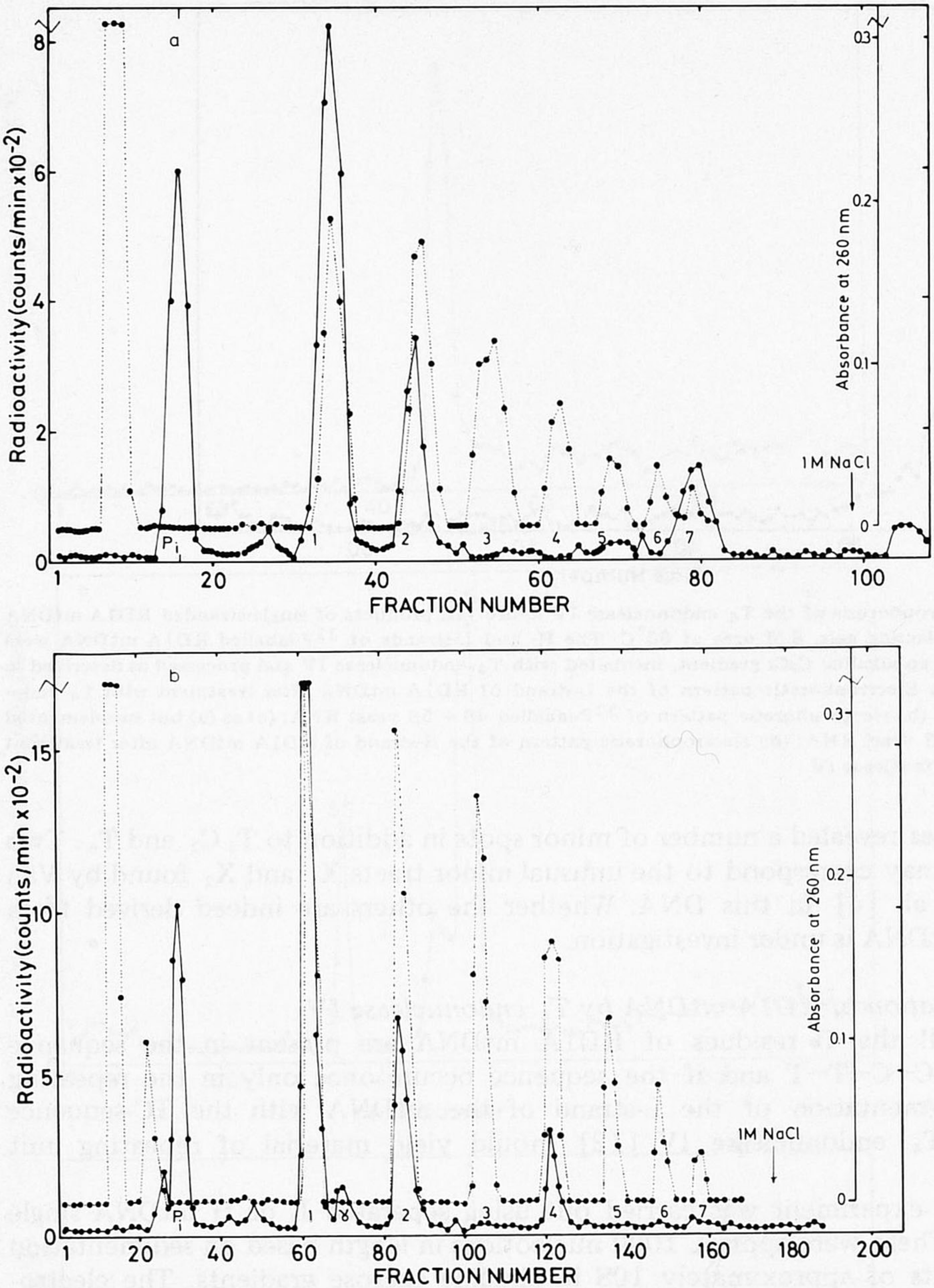

Fig. 5. Chromatography of pyrimidine oligonucleotides from the L-strand of RD1A mtDNA on DEAE cellulose. See Methods for details. (a) Pyrimidine tracts derived from native L-strand; (b) pyrimidine tracts derived from L-strand degraded with T_4 endonuclease IV. ●———●, ^{32}P radioactivity; ●- - - - -●, $A_{260\ nm}$ (from carrier calf-thymus DNA).

The specificity of T_4 endonuclease IV was checked by analysis of the pyrimidine tracts of the L-strand before and after fragmentation with the enzyme. Fragmentation should lead to disappearance of T—T—T—C—C—T—T and appearance of at least two new sequences, T_3p_3 and C—C—T—T [12,15].

Fig. 5 shows a chromatogram of RD1A L-strand pyrimidine tracts before and after T_4 endonuclease IV fragmentation. The quantitative analyses are

TABLE III

ANALYSIS OF RD1A mtDNA L-STRAND PYRIMIDINE TRACTS BEFORE AND AFTER T_4 ENDONUCLEASE IV HYDROLYSIS

Before T_4 endonuclease IV hydrolysis			After T_4 endonuclease IV hydrolysis			
Pyrimidine tract	Component	^{32}P (% of total)	Pyrimidine tract	^{32}P (% of total)	Products of exhaustive deoxyribonuclease hydrolysis	Sequence
			Shoulder preceding P_i	2.6	pT	pT*
$n = 0$	P_i	22.7	$n = 0$	23.9	—	P_i*
$n = 1$	T_1	48.3	$n = 1$	46.6	—	pTp*
			γ	2.9	pT	—
$n = 2$	T_2	14.3	$n = 2$	17.8	—	pTpTp* + pTpTpT
$n = 3$	—	—	$n = 3$	—	—	—
$n = 4$	—	—	$n = 4$	6.1	pC, pT, T_1	pCpCpTpT
$n = 5$	—	—	$n = 5$	—	—	—
$n = 6$	—	—	$n = 6$	—	—	—
$n = 7$	$T_3C_2T_2$	9.7	$n = 7$	—	—	—
Extra material ($0 < n < 1$)		1.6				
1 M NaCl strip		3.4	1 M NaCl strip	0.1	—	—
		100.0		100.0		

* Analysed by direct electrophoresis on Whatman 3 MM in 5% acetic acid.

presented in Table III. The T_4 endonuclease IV fragment lacks $T_5 C_2$ but contains a new $n = 4$ tract, as predicted. In addition, a new small peak and a shoulder preceding the P_i peak were observed. The various ^{32}P-labelled fractions were freed of urea and salt by standard methods and characterized by base composition analysis or by direct electrophoresis on Whatman 3 MM paper in 5% acetic acid [16]. The results are summarized in Table III. The shoulder preceding the P_i peak was identified as pT and pT was released from peak γ by degradation with pancreatic deoxyribonuclease and snake venom phosphodiesterase. Both minor fractions are probably aspecific degradation products generated by T_4 endonuclease IV or by a contaminating enzyme. The $n = 1$ and $n = 2$ material from the chromatogram migrated as a single spot on Whatman 3 MM paper proving the absence of C_1, C_2 and CT. Degradation of the $n = 4$ material with pancreatic deoxyribonuclease and snake venom phosphodiesterase yielded undigested material plus small amounts of pC, pT and T_1 indicating that this is the only C-containing tract after endonuclease IV degradation. The pyrimidine tract at the 3′-end of the T_4 endonuclease IV fragment must, therefore, be C—C—T—T in agreement with the specificity of the T_4 endonuclease IV and the sequence T—T—T—C—C—T—T.

The C content calculated from the $T_2 C_2$ fraction equals 2.45 mole percent in excellent agreement with the value calculated from $T_5 C_2$ (2.42%). The missing oligonucleotide $T_3 p_3$ cochromatographed with T_2; the difference in content of the $n = 2$ fraction before and after endonuclease IV fragmentation equals 3.5% which correlates well with the percentage of $T_3 p_3$ calculated from $T_5 C_2$ (3.64%).

The endonuclease IV experiments provide additional evidence that the sequence T—T—T—C—C—T—T is unique, that the other major pyrimidine tracts in RD1A mtDNA contain no C and that $T_5 C_2$ occurs only once per repeating unit of 69 ± 2 nucleotides.

Discussion

Recent studies on the aberrant mtDNA of cytoplasmic petite mutants in our laboratory [2,5,17] and elsewhere [18—25] have shown that the DNAs arise by a process of deletion, followed by amplification of the remaining wild-type mtDNA segments. Thus the mutation usually does not affect the total amount of mtDNA per cell, but the genetic complexity of the DNA decreases. Previous studies on the RD1A mutant had shown that it is an extreme deletion mutant, the mtDNA representing less than 1% of the wild-type genome in a highly and perfectly repeated form [3—5,17]. In this paper we show by three different methods that the minimal size of the repeating unit of the mtDNA is approximately 70 nucleotides in length.

1. Pyrimidine tract analysis of RD1A mtDNA shows that the molar ratio of the two longest tracts T_6 and $T_5 C_2$ is about 1; based on the moles of T_6 (the most reliable value) calculation of a minimal repeating unit size gives a value of 68 nucleotides.

2. Base composition analysis of the L-strand of RD1A mtDNA gave a value of 3 mole percent C. All the C residues are in $T_5 C_2$ sequences, thus there is one $T_5 C_2$ tract per 67 nucleotides in mtDNA.

3. Cleavage of the L-strand of RD1A mtDNA with the T—C sequence specific T_4 endonuclease IV yields solely fragments with an electrophoretic mobility in acrylamide gels corresponding to a size of 69 ± 2 nucleotides.

The excellent correlation between these three methods indicates strongly that the minimal repeating unit is about 70 nucleotides. We cannot yet exclude the unlikely possiblity that the actual repeating unit is a multiple of 70 nucleotides. This will be determined by the complete sequence analysis that is in progress.

The published pyrimidine tract analysis of RD1A mtDNA [4] determined using polyethyleneimine plates [6] showed a recovery of 0.6 mole percent C and a $T_5 C_2$: T_6 ratio of 1 : 2 indicating loss of $T_5 C_2$. The loss is probably due to poor resolution of longer tracts on PEI plates particularly those containing C.

The way in which the repetitive DNA in petite mitochondria is organized may vary between two extremes. In mutant RD1 described by Hollenberg et al. [26], nearly all the mtDNA was present in the form of small circles; however, in RD1A mtDNA nearly all DNA was linear, heterogeneous in size and much longer than the repeating unit [3] and circles were virtually absent. More recently, a mixture of circles and long linear DNA have been observed in other petite mtDNAs [23]. In these DNAs the repeating units are linked both head to head and head to tail [23]. In RD1A mtDNA the virtual absence of C in the H-strand (Table I) shows the linkage must be mainly, if not exclusively, head to tail. Support for this conclusion is the good recovery of previously separated complementary single strands when chromatographed on hydroxylapatite and eluted by conditions which do not elute duplex RD1A mtDNA [4].

In addition to tandem repeats, RD1A mtDNA should contain a replicator sequence (cf. [17]) and possibly spacer sequences. Candidates for spacer sequences are the minor pyrimidine tracts, observed in the DEAE-cellulose chromatogram which are probably identical with the unusual tracts X_1 and X_2 [4] observed on polyethyleneimine cellulose plates. Their molar frequencies (Table II) are too low to include them in the repeating unit. The sequence of these tracts and analysis of their relative position to the repeating unit and possible role in RD1A mtDNA is under investigation.

Methods

Preparation of DNAs

Mitochondrial and nuclear DNA from RD1A were prepared as described previously [17], except that ribonuclease and pronase treatment were omitted. The complementary strands of RD1A mtDNA were separated by alkaline CsCl gradient centrifugation [3].

Base composition analysis

^{32}P-labelled RD1A DNA plus 10 μg carrier calf-thymus DNA were incubated in 1 mM Tris—HCl (pH 7.0), 8 mM $MgCl_2$ and 1 μg pancreatic deoxyribonuclease I at 37°C (total volume 250 μl) for 60 min. The pH was adjusted to 9 with NaOH, 1 μg snake venom phosphodiesterase added and the mixture incubated for 90 min. The enzymes were removed by filtration

through Sephadex G-50. The included volume was concentrated and analysed by electrophoresis on Whatman 3 MM paper [16] (2 h at 4 kV). Radioactive material was localized by autoradiography, the spots cut out and measured by liquid scintillation counting. Recoveries were 75—90%.

Degradation of DNA to pyrimidine tracts

Pyrimidine tracts were released by hydrolysis with formic acid—diphenylamine according to Burton [7] starting with 1—5 μg ^{32}P-labelled RD1A mtDNA plus 1.5 mg carrier calf-thymus DNA. Formic acid and diphenylamine were removed by ether extraction (10 times an equal volume of ether). Recovery >98%.

Separation of pyrimidine oligonucleotides

Pyrimidine oligonucleotides were separated according to chain length by chromatography on DEAE-cellulose at pH 5.5. The DNA digest was applied to a DEAE-cellulose column (20 cm × 1.5 cm) pre-equilibrated with 50 mM sodium acetate in 7 M urea (pH 5.5) [8]. The column was washed with the same buffer to remove unadsorbed purines and eluted with a linear gradient of 300 ml, 0—0.28 M NaCl in 50 mM sodium acetate, 7 M urea (pH 5.5). Fractions were transferred to scintillation vials and Cerenkov radiation measured. The absorbance at 260 nm was monitored with a Zeiss PMQ spectrophotometer and conductivity with a Mullard type conductivity bridge.

Sequence analysis of $T_5 C_2$

Pyrimidine oligonucleotides were released from 16 μg unlabelled RD1A mtDNA by the method of Burton [7]. Formic acid and diphenylamine were removed by ether extraction and the hydrolysate evaporated to dryness. The oligonucleotides were terminally dephosphorylated using *Escherichia coli* phosphomonoesterase [27] (Worthington) and the 5′-termini labelled with [α-^{32}P]-ATP, using polynucleotide kinase (a gift of A.D. Delaney). ATP was removed by Sephadex G-25 filtration and the oligonucleotides desalted on a small DEAE-cellulose column. The oligonucleotides were then separated by two-dimensional ionophoresis—homochromatography [10]. The $T_5 C_2$ oligonucleotide area was removed from the plate by scraping, the oligonucleotide eluted with triethylammonium carbonate and the solution desalted. Finally, the heptanucleotide was partially digested with snake venom phosphodiesterase [11] and the products separated by two-dimensional ionophoresis—homochromatography [10].

T_4 endonuclease IV fragmentation of single-stranded RD1A mtDNA

^{32}P-labelled RD1A H- or L-strand mtDNA plus 2 μg of the corresponding unlabelled RD1A single-stranded mtDNA were incubated in 10 mM Tris—HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 8.5) with 100 units T_4 endonuclease IV [27] in a total volume of 200 μl, at 45°C for 17 h. The enzyme was removed by phenol extraction. Prior to diphenylamine—formic acid degradation, residual phenol was removed by ether extraction. Prior to electrophoresis 1 μl saturated bromophenol blue solution and 3 μl ^{32}P-labelled 4S + 5S yeast RNA markers were added to 100 μl of the phenol extract.

Gel electrophoresis under denaturing conditions

10% acrylamide gels (10 cm long) were prepared in 20 mM Tris (pH 7.5), 2 mM sodium EDTA, containing 8 M urea. Pre-electrophoresis was for 30 min at 60°C (5 mA/gel). The samples were saturated with solid sucrose and layered on top of the gel. Subsequent electrophoresis was for 3 h at 60°C (5 mA/gel) (the blue dye just ran off the gel). The gels were frozen, cut into 1-mm slices and radioactivity measured by standard liquid scintillation counting procedures. Recoveries were 60—80%.

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