S_1 nuclease definition of highly repeated DNA sequences in the Guinea pig, Cavia porcellus

Howard R.Hubbell*, Donald L.Robberson and T.C.Hsu

Department of Biology, University of Texas System Cancer Center M.D.Anderson Hospital and Tumor Institute, and University of Texas Health Science Center Graduate School of Biomedical Sciences, Houston, TX 77030, USA

Received 22 August 1979

ABSTRACT

Native DNA of the Guinea pig, Cavia porcellus, purified from liver or tissue culture cells, was heat denatured and reassociated to a C_0t value of 0.01 (equivalent C_0t value of 7.2×10^{-2}). The reassociated DNA was isolated by digestion with the single-strand DNA specific enzyme S_1 nuclease. Spectrophotometric and radioactivity assays demonstrated that 24% of the total DNA was resistant to S_1 nuclease treatment. Zero-time reassociation indicated that approximately 3% of the DNA was inverted repeat sequences. Thus, highly repeated sequences comprised 21% of the total genome. CsCl buoyant density ultracentrifugation indicated that this fraction was composed of both main band and satellite sequences. Although actinomycin D - CsCl density gradients failed to give significant separation of the repetitive sequences, distamycin A - CsCl gradients were able to fractionate the DNA into several overlapping bands. The heterogeneity of the repetitive DNA was further demonstrated by the first derivative plots calculated from their thermal denaturation profiles. This analysis revealed six major thermalytes which indicate that there may be at least six discrete components in the repetitive DNA.

INTRODUCTION

Investigations of Guinea pig DNA have demonstrated that three highly repeated satellite sequences exist which, in CsCl density gradients, are slightly denser than the main band DNA (1). Satellite I has been shown to consist of the hexanucleotide sequence 5'-CCCTAA-3' (2). Although satellites II and III may have been derived from the same ancestral sequence as satellite I, they appear to be more complex (3). No molecular studies, however, have been carried out to determine whether highly repeated sequences are present in the main band region of CsCl or $Ag^+Cs_2SO_4$ density gradients, but are obscured by the bulk of the non-repeated DNA.

Unfortunately, in only two (4,5) of the many molecular studies conducted on Guinea pig DNA was the taxonomy of the species identified. As illustrated by studies of the genera Mus (6), Drosophila (7) and Dipodomys (8), closely related species may have different amounts of

the same highly repeated satellite or completely different satellites. In the Guinea pigs, genus Cavia, nearly 20 species have been on record, and two of them, Cavia porcellus and C. cobaya, are used in clinical and biological laboratories. Most investigators working with Guinea pigs simply refer to the common name or assign a species name without taxonomic verification. Thus reported data may represent the mixture of at least two different species. We have examined the DNA of one particular species of Guinea pig, C. porcellus, defined by its karyology (9,10), and report some of the physical properties of the total complement of the repeated DNA sequences. The chromosomal locations of these same sequences, as demonstrated by in situ hybridization have previously been reported (10).

MATERIALS AND METHODS

DNA preparation

Guinea pig livers were removed and quickly frozen on dry ice. The DNA was isolated by the Marmur method (11), with several modifications. The frozen tissue was sliced and homogenized in saline-EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0). The nuclei were lysed by the addition of pronase (200 µg/ml; CalBiochem) and SDS (final concentration 0.13%; Matheson Coleman and Bell). After overnight incubation at 37°C, the solution was deproteinized by shaking with redistilled phenol saturated with saline-EDTA. After centrifugation, the DNA was precipitated from the aqueous layer with 95% ethanol and redissolved in 0.1 X SSC. The salt concentration was then raised to 1 X SSC. Pancreatic RNase (50 µg/ml; type I-A, Sigma), T_1 RNase (300 U/ml, Sigma) and α-amylase (100 µg/ml; Bacillus subtilis type II-a, Sigma) were added and the mixture was incubated at 37°C for 90 min. Pronase (100 µg/ml) was added and the incubation was continued for 1 hr. The mixture was deproteinized with a solution of equal parts SEVAGS (chloroform-isoamyl alcohol, 24:1) and saturated phenol. After centrifugation, the DNA was precipitated and redissolved, as above. Sodium acetate-EDTA (2.0 M sodium acetate, 1 mM EDTA; 0.1 volume) was added to the DNA and the DNA was precipitated with 0.54 volume of 2-propanol. The DNA was redissolved in 0.1 X SSC. Each liver yielded about 20 mg of DNA.

An aliquot of DNA was dialyzed against 1.0 M NaCl, 10 mM Tris, pH 8.0 buffer and 0.1 volume of 1.0 N NaOH (12) was then added. The molecular weight of the denatured DNA was determined by velocity sedimentation in a Beckman Model E analytical ultracentrifuge equipped with an automatic ultraviolet scanner.

Tissue culture

Fibroblast cell lines were derived from ear biopsies of the same animals from which the liver tissue was obtained. The cultures were maintained in a modified McCoy's 5a medium supplemented with 20% fetal calf serum (GIBCO). Confluent cultures were subcultured by detachment of the cells from the culture flask with a 0.01% trypsin (3X purified; Worthington) solution and replating the cells into two or more flasks.

Preparation of tritium labeled DNA

Radioactive Guinea pig DNA was isolated from tissue culture cells. Tritiated thymidine (0.5 µCi/ml, specific activity 6.7 Ci/mM; New England Nuclear) and unlabeled thymidine (10.0 µg/ml; CalBiochem) were added to tissue cultured cells in exponential growth. After 54 hr, the cells were washed with saline-EDTA, detached from the flasks with 0.1% pronase and centrifuged. The cell pellet was resuspended in saline-EDTA, and pronase (50 µg/ml) and SDS (final concentration 0.25%) were added. The mixture was incubated and the phenol extraction was performed as described above. The specific activity of the [^{3}H]DNA was 1250 cpm/µg.

Assay of S_1 nuclease activities

The *Aspergillus oryzae* S_1 nuclease purified from takadiastase powder (Sankyo) by DE-52 and G-100 column chromatography (13) was assayed for single-strand activity by using 15 µg of a heat denatured mixture of [^{3}H]*B. subtilis* DNA (5000 cpm/µg) and non-radioactive human placental DNA (1:14) as substrate. The reaction was carried out in 10^{-2} M NaCl, 3 x 10^{-2} M sodium acetate and 3 x 10^{-5} M $ZnCl_2$, pH 4.5, in a total volume of 0.5 ml. After incubation of 50°C for 10 min, the reaction was stopped by the addition of 25 - 50 µg of nonradioactive carrier DNA and an equal volume of 10% trichloroacetic acid (TCA; Fisher). The TCA precipitable material was loaded onto glass fiber filters (Reeve Angel) with gentle suction and washed with 5% TCA and then 95% ethanol. The filters were counted in a Beckman LS-100C scintillation counter. One unit of activity was defined as the amount of enzyme solubilizing 1% of the denatured [^{3}H]DNA in 10 min (13). The specific activity of the enzyme preparation was 9.9U/µg. The protein concentration was measured by the Folin phenol reagent method (14).

The enzyme was assayed for double-strand activity using unlabeled bacteriophage PM2 DNA or tritium labeled polyoma DNA (specific activity 24,000 cpm/µg). The polyoma DNA was prepared by the Hirt procedure, as described in Fried (15), using a low multiplicity infection of secondary

mouse embryo cells in the presence of tritiated thymidine. The PM2 DNA was incubated in the S_1 buffer with 104 U S_1 at 50°C for 10 min. The reaction was stopped by chilling in an ice water bath and adding EDTA to a final concentration of 20 mM. The PM2 digestion mixture was layered on 1.4% agarose (Bio-Rad) gels and electrophoresed in E buffer (40 mM Tris-HCl, pH 7.9, 5 mM sodium acetate, 1 mM EDTA) with 0.5 µg/ml ethidium bromide (16). Molecular weight markers for the 1.4% agarose gels were generated by digestion of the PM2 DNA with either Hpa II (16) at 37°C for 2 hr or Hind III (17) restriction enzymes. The Hind III digestion was carried out in 6 mM Tris, pH 7.5, 6 mM $MgCl_2$ and 40 mM NaCl at 37°C for 24 hr. The [^{3}H]polyoma DNA was incubated at similar enzyme to DNA ratios and the TCA precipitations were washed and counted as previously described.

Isolation of repeated DNA sequences

Native Guinea pig DNA, with an average length of 15,500 $\pm$ 600 base pairs, was heat denatured and immediately added to buffer kept at 60°C (final salt concentration 1 X SCC). The DNA was reassociated to a $C_o t$ value of 0.01, which corresponds to an equivalent $C_o t$ value of 7.2×10^{-2} (18). The reassociation was stopped by chilling to 4°C and 15 - 20 µg aliquots of DNA were added to S_1 buffer with 104 U S_1 (final volume 0.5 ml). The digestion was carried out for 10 min at 50°C and then stopped by placing the mixture into an ice water bath. The unlabeled DNA was dialyzed extensively into 1 X SSC.

The amount of renatured DNA remaining after digestion was determined by either TCA precipitation and scintillation counting of the [^{3}H]DNA or by spectrophotometry of the unlabeled DNA. Because of the significant ultraviolet absorbance of the S_1 at 260 nm, a set of simultaneous equations were derived whose solution allows the determination of the DNA absorbance (A) at 260 nm in the presence of the protein.

If we assume that the absorbance of nucleic acids (DNA) and proteins (P) are additive at both 260 and 280 nm, then

$$A_{260} = A_{260}^{DNA} + A_{260}^{P} \text{ and} \quad [1]$$

$$A^{280} = A_{280}^{DNA} + A_{280}^{P} \quad [2]$$

where A_{260} and A_{280} are the final observed absorbance at 260 and 280 nm, respectively. If we also define the ratios

$$\alpha = A_{260}^{DNA} / A_{280}^{DNA} \text{ and} \quad [3]$$

$$\beta = A_{260}^{P} / A_{280}^{P} \quad [4]$$

then $A_{260}^{DNA} = A_{260} - A_{260}^{P} = A_{260} - \beta A_{280}^{P} = A_{260} - \beta(A_{280} - A_{280}^{DNA})$. [5]

Substituting α into the equation gives

$A_{260}^{DNA} = A_{260} - \beta A_{280} + \beta A_{260}^{DNA}/\alpha$. [6]

Solving for A_{260}^{DNA} vives

$A_{260}^{DNA} = [A_{260} - \beta A_{280}]/(1 - \beta/\alpha)$. [7]

By independent determination of the ratios α and β we were able to determine the absorbance of the DNA at 260 nm in the presence of the enzyme. From the absorbance we were able to calculate the concentration of the double-stranded DNA in the unlabeled sample.

Analytical ultracentrifugation

DNA samples were examined by analytical ultracentrifugation in CsCl density gradients (42,040 rpm, 25°C, 20 - 24 hr). Bacteriophage ϕ2C DNA, $\rho = 1.742$ g/cm^3 (19), was used as a reference density marker. In gradients without a marker, the density of the DNA was calculated by the root-mean-square method (20), using β values interpolated from the data of Ifft et al. (21). The ultraviolet absorbance at 265 nm was recorded on Kodak electron microscope film and the negatives were traced on a Joyce Loebl double beam recording microdensitometer, Model E12 MKIIIB.

Antibiotic - DNA density gradients

Actinomycin D (Cosmegen®, MW 1254.74; Merck, Sharp and Dohme) and distamycin A HCl (MW 517.96; Farmitalia) were suspended in buffer (1 X SSC or 0.025 M Tris HCl, pH 7.95), at final concentrations of 500 μg/ml and 1000 μg/ml, respectively. These antibiotics were added to both native and repetitive DNA samples at different r_f values (range 0.33 - 2.0, w/w). The samples were then examined by analytical ultracentrifugation.

DNA denaturation analysis

Thermal denaturation was carried out in a Zeiss PM QII spectrophotometer equipped with an automatic recorder. Sample temperature was increased 0.5°C per min by a circulating water bath controlled by a Lauda linear temperature programmer. The melting temperature (T_m) was determined in either 0.1 X SSC or 1 X SSC.

The thermal denaturation curves were smoothed by connecting the midpoints between two consecutive data points. At each temperature point, the hyperchromicity was obtained from the constructed midpoint lines. After this smoothing, the change in hyperchromicity vs. change in temperature ($\Delta H/\Delta T$) was calculated and plotted at the temperature of the

corresponding midpoint.

RESULTS

Buoyant density analysis of Guinea pig DNA

In neutral CsCl buoyant density gradients, native C. porcellus DNA, isolated either from the liver (Fig. 1a) or cultured fibroblasts cells (Fig. 1b), displayed a density distribution similar to those previously reported (27-29). The density of the main band was 1.700 g/cm^3 and that of the satellite, which appeared as a shoulder on the dense side of the main band, was 1.703 g/cm^3. Although there might have been differences in the relative amounts of particular sequences, no new density classes were apparent. The profiles of the liver and fibroblast DNA were very similar.

After thermal denaturation at 100°C for 15 min, the liver DNA profile showed a characteristic density shift to 1.713 g/cm^3 (Fig. 1c). Upon

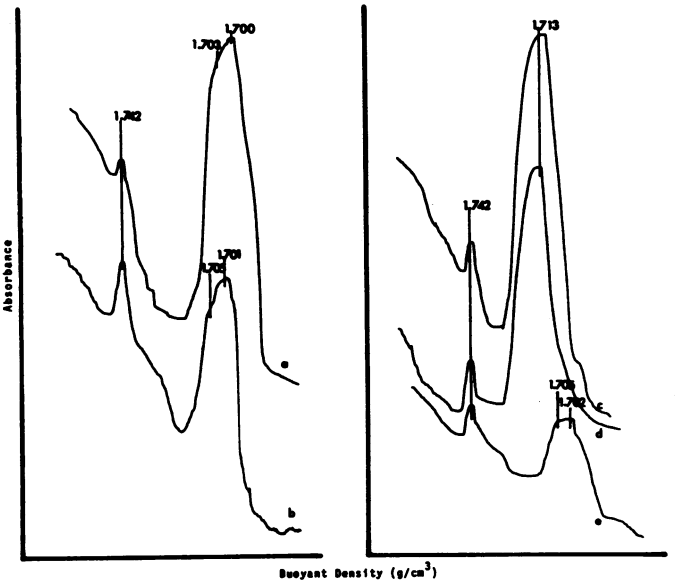

Figure 1. Analytical ultracentrifugation of Guinea pig DNA. DNA samples were centrifuged to equilibrium in CsCl at 42,040 rpm and 25°C for 20–24 hr. Bacteriophage φ2C (ρ = 1.742) was used as a density marker. a) native liver DNA, b) native fibroblast culture DNA, c) denatured liver DNA, d) denatured liver DNA reassociated to an equivalent C_0t value of 7.2 x 10^{-2} and e) denatured liver DNA reassociated to an equivalent C_0t value of 7.2 x 10^{-2} and then digested with 104 U S_1 nuclease.

reassociation to an equivalent $C_o t$ value of 7.2×10^{-2}, a small amount of the DNA was observed at the density of native DNA (Fig. 1d). When the reassociated sample was digested with S_1 nuclease, the remaining DNA had densities of 1.705, 1.702 and 1.701 g/cm^3 (Fig. 1e). No DNA was found in the region previously occupied by denatured sequences, indicating that the digestion went to completion. Tissue culture DNA, treated in the same manner as the liver DNA, behaved similarly in CsCl density gradients.

Quantitation of highly repeated sequences

Earlier studies have indicated that approximately 10% of the Guinea pig genome was contained in the three highly repeated satellites which were expected to be present in our reassociated fraction (1). This figure did not account for any highly repeated sequences present in the main band DNA. We have determined the total amount of our reassociated DNA by two different methods. The [^{3}H]DNA isolated from fibroblast culture cells was reassociated to an equivalent $C_o t$ value of 7.2×10^{-2}. After complete digestion of 17.06 µg of DNA with S_1 nuclease, 24.3% of the total DNA remained as TCA precipitable material (Table I).

Since the liver DNA could not be uniformly labeled for these experi-

TABLE I

RADIOACTIVITY ASSAY OF REASSOCIATED GUINEA PIG DNA

REMAINING AFTER S_1 NUCLEASE TREATMENT

Post-Reassociation Treatment*	TCA Precipitable cpm	% TCA Precipitable cpm
A. Untreated	21,100	100.0
B. S_1 Nuclease	5,300	25.1
C. S_1 Nuclease	5,110	24.2
D. S_1 Nuclease	5,000	23.7
E. Average of values in B, C and D	5,140 (S.D. $\pm$ 150)	24.3 (S.D. $\pm$ 0.7)

*All samples were denatured and reannealed to an equivalent $C_o t$ value of 0.072.

Results of experiments in B, C and D are independent S_1 nuclease digestions of different reassociated samples from the same DNA preparation.

ments, we developed a spectrophotometric method to measure the fraction of highly repeated sequences in this sample (see MATERIALS AND METHODS). In order to avoid DNA loss during the extraction of S_1 from the sample, the digestion mixture was extensively dialyzed against 1 X SSC and the optical density and volume of the dialysate was measured. Using the extinction coefficient of double-stranded DNA at 260 nm we found that, in three separate experiments, 24.1% of the DNA was present in the reassociated, highly repeated fraction (Table II).

The reassociated DNA fraction contained inverted repeat sequences. The quantity of these sequences was analyzed by assaying the amount of double-strand DNA present after denaturation and zero-time reassociation. The snap-back DNA constituted 2.7% of the total genome. Highly repeated sequences, therefore, comprised 21% of the total DNA.

Fractionation of the reassociated sequences by antibiotic-DNA density gradients

Our reassociated sample apparently was made up of several families of DNA sequences, as seen by its CsCl pattern (Fig. 1e) and large percentage

TABLE II

SPECTROPHOTOMETRIC ASSAY OF REASSOCIATED DNA REMAINING AFTER S_1 NUCLEASE TREATMENT

Post-Reassociation Treatment*	Micrograms of Reassociated DNA+		% of DNA Resistant to S_1 Nuclease
	Before Digestion	After Digestion	
A. S_1 Nuclease	1,220	274	22.5
B. S_1 Nuclease	419	110	26.3
C. S_1 Nuclease	193	45	23.6
D. Average of experiments in A, B and C	-	-	24.1 (S.D. $\pm$ 2.0)

*All samples were denatured and reannealed to an equivalent C_0t value of 0.072.

+Quantities of DNA were determined using equation [7], as described in MATERIALS AND METHODS, and a specific extinction coefficient of 50 µg per one absorbance unit at 260 nm.

Results of experiments in A, B and C are independently determined S_1 nuclease digestions of different reassociated samples from the same DNA preparation.

of the genome (Tables I and II). In order to examine the degree of heterogeneity, the DNA was complexed with antibiotics and analyzed in CsCl gradients. Actinomycin D, which intercalates preferentially into the GpC dinucleotides of double-stranded DNA (30), was complexed with native DNA in various antibiotic/DNA ratios (r_f, w/w). At ratios of 0.33, 0.50 or 1.00, the DNA was fractionated into a minor and a major component with buoyant densities of 1.689 and 1.671 g/cm^3, respectively (Fig. 2a). The reassociated sample did not display any fractionation at the same antibiotic/DNA ratios (Fig. 2b).

Distamycin A HCl, which preferentially binds to AT rich sequences (31), was used with greater success to separate different DNA classes in both the native and repetitive samples. At r_f = 0.50, native Guinea pig DNA was fractionated into at least four components with buoyant densities of 1.622, 1.626, 1.637 and 1.648 g/cm^3 (Fig. 3a). The reassociated highly repeated sequences were also separated into several components with den-

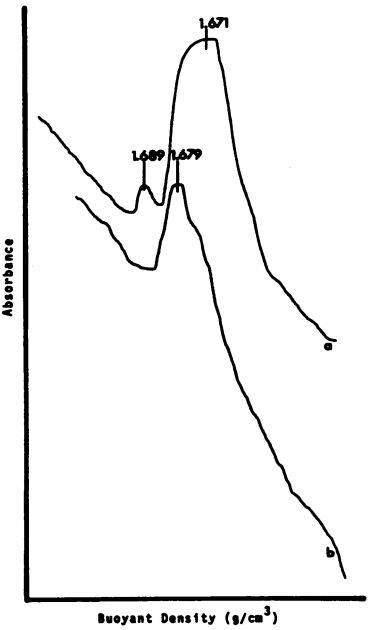

Figure 2. Actinomycin D–CsCl density gradients. Actinomycin D in either 1 X SSC or 0.025 M Tris HCl, pH 7.95, was added to DNA samples and the mixture was analyzed by CsCl buoyant density ultracentrifugation. a) native DNA, r_f = 0.50, b) highly repeated sequences, r_f = 0.50.

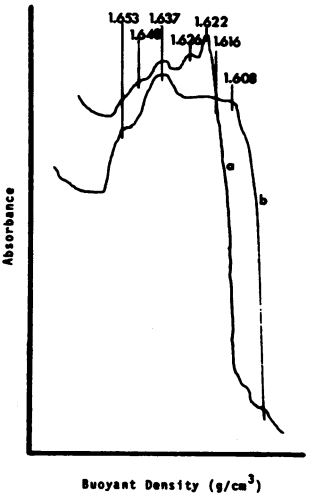

Figure 3. Distamycin A-CsCl density gradients. Distamycin A in either 1 X SSC or 0.025 M Tris HCl, pH 7.95, was added to DNA samples and the mixture was analyzed by CsCl buoyant density ultracentrifugation. a) native DNA, r_f = 0.50, b) highly repeated sequences, r_f = 0.50.

sities of 1.608, 1.616, 1.637 and 1.653 g/cm^3, at r_f = 0.50 (Fig. 3b).

Thermal denaturation analysis

Heterogeneity can also be demonstrated by thermal denaturation profiles if the base composition of the various DNA families are sufficiently different. The denaturation profile of Guinea pig liver DNA possessed a single sharp transition when the denaturation was performed in either 0.1 X SSC (T_m = 67.0°C; Fig. 4a) or 1 X SSC (T_m = 82.6°C; Fig. 4b). In contrast, the denaturation profile of the reassociated highly repeated DNA displayed a broad transition in both 0.1 X SSC (T_m = 65.3°C, Fig. 4a) and 1 X SSC (T_m = 78.3°C; Fig. 4b). Both the native and repetitive DNA denaturation profiles showed a characteristic increase in their respective melting temperature and decrease in their transition breadths when the denaturations were performed in 1 X SSC as compared to 0.1 X SSC.

In both 0.1 X SSC and 1 X SSC, the reassociated repeated DNA displayed some stepwise melting, indicative of the presence of several different sequence or base composition classes of the DNA. Calculation of the first derivative of the denaturation hyperchromicity as a function of temperature in both 0.1 X SSC and 1 X SSC clearly revealed the heterogeneity of DNA

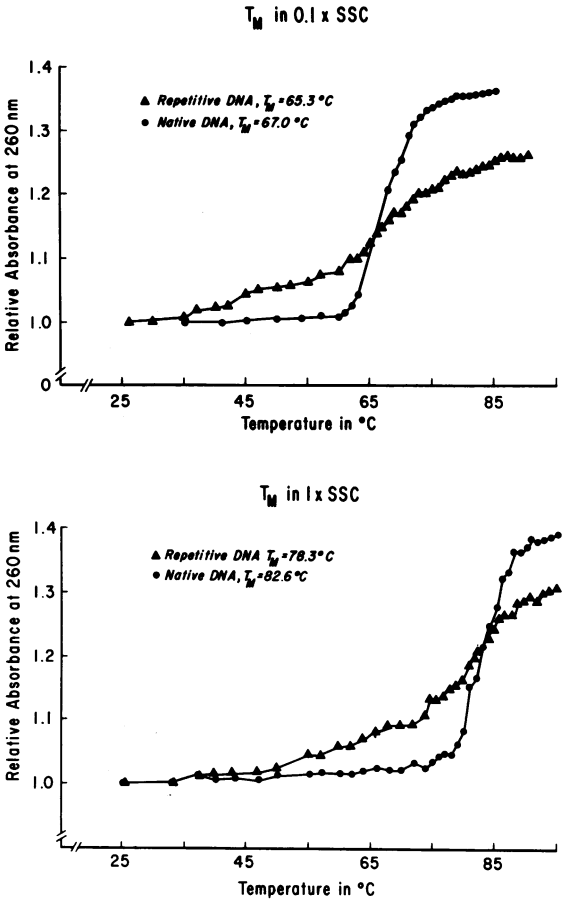

Figure 4. Thermal denaturation of Guinea pig DNA. The melting temperatures of native (●) and highly repeated (▲) DNA sequences were determined in a) 0.1 X SSC and b) 1 X SSC.

sequences in the reassociated repetitive fraction (Fig. 5a and b). In both 0.1 X SSC and 1 X SSC, we detected a minimum of six thermal transitions, reflecting the presence of at least six classes of repeated DNA sequences. It should be noted that the relative positions of these thermalytes may vary with ionic strength (32).

Characterization of S_1 nuclease activities

Before using the S_1 nuclease preparation to define the highly repeated sequences in the Guinea pig DNA, we examined two aspects of the enzyme digestion procedure which could significantly effect the interpretations

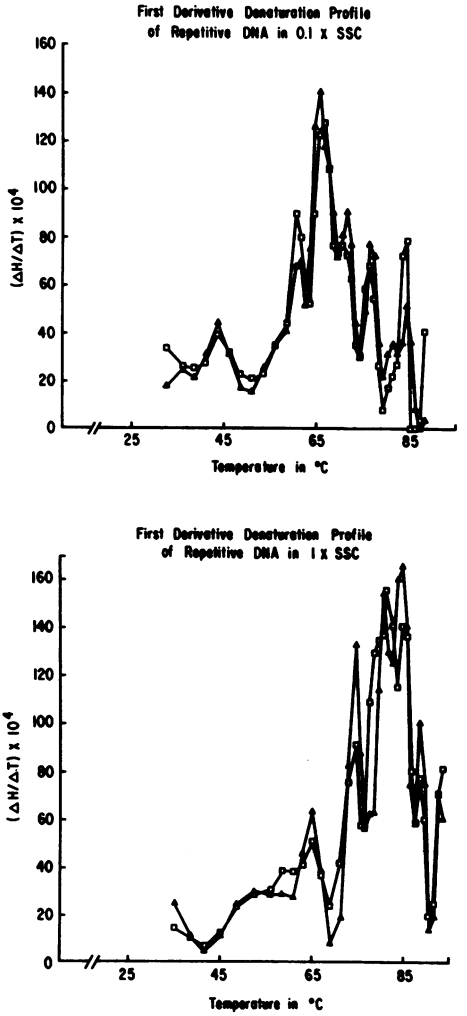

Figure 5. First derivative profiles of the thermal denaturation of Guinea pig highly repeated DNA. Thermal denaturation of the samples shown in Fig. 4 (▲) and of duplicate repetitive samples (□) were analyzed by the change in hyperchromicity as a function of the change in temperature in a) 0.1 X SSC or b) 1 X SSC.

of our results. Since crude preparations of S_1 nuclease possess double-strand deoxyribonuclease activity (22), we considered it necessary to test for such activity in our purified preparation. DNase activity was assayed using bacteriophage PM2 DNA. Covalently closed ciruclar (form

I) or nicked circular (form II) PM2 DNA should be converted to linear unit length duplex DNA (form III) by the single-strand specific nuclease activity of the enzyme, as seen in experiments done on SV40 (23) and polyoma (24) DNAs. Degradation of the linear duplexes to shorter lengths, however, would result from the double-strand DNase activity. Samples of PM2 DNA, which contained a mixture of form I and II DNAs, were incubated under the conditions used for the isolation of the reassociated repetitive Guinea pig DNA, and then electrophoresed in 1.4% agarose gels. In Fig. 6, gels a and b demonstrate, respectively, the relative positions of form III DNA, generated by Hpa II digestion (25), and of forms I and II, from the untreated sample. The form II band, found above the form I band, is very faint. After incubation of the untreated PM2 DNA in the S_1 buffer alone, a significant amount of the form I molecules were converted to form II (Fig. 6c). Incubation of 14.7 µg of PM2 DNA in the presence of 104 units of S_1 resulted in a reduction of the molecular weight of the DNA (Fig. 6d). Molecular weight markers, generated by Hind III digestion of PM2 DNA (17), gave several bands, the top 5 of which correspond to 5348, 4200, 2115, 1750 and 924 base pairs, respectively (Fig. 6e; M. T. Kuo, personal communication). The bands of 4200 and 1750 base pairs were due to mutant PM2 molecules in our sample which were not found in similar Hind III digestion of wild type PM2 DNA (25). Comparison of gels d and e demonstrated an approximate two- to five-fold reduction in molecular weight of the PM2.

Radiolabel analysis of the S_1 double-strand deoxyribonuclease activity was carried out using covalently closed circular polyoma DNA. Incubations performed under the same DNA/enzyme ratio as previously described demonstrated that only 2.9% of the native DNA was rendered TCA soluble.

Taken together, these results indicated that the molecular weight reduction of the PM2 DNA was not due to large amounts of double-strand exonuclease activity in the S_1 sample. Rather, it appears that a low level of endonuclease activity was present. Alternatively, it is possible that the nicking of one strand, introduced by the S_1 buffer alone, provided single-stranded regions which may be attacked by the S_1 and fragment the DNA molecule. Also, weakly hydrogen-bonded or strained regions in the closed circular DNAs examined, opened by the relatively high incubation temperature, may have been subject to S_1 digestion. In either case, it is apparent that these activities did not lead to a selective loss of sequences during the enzyme digestions.

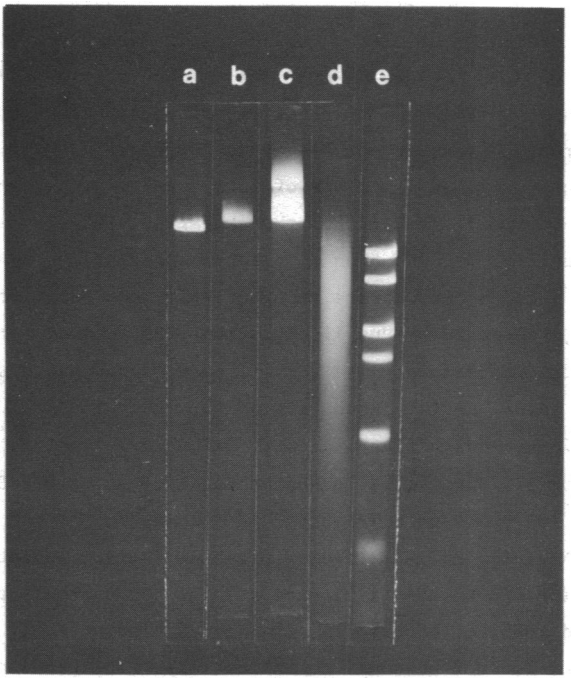

Figure 6. Double-strand deoxyribonuclease activity of S_1 nuclease. Native bacteriophage PM2 DNA was digested with either Hpa II (16) at 37°C for 2 hr, Hind III at 37°C for 24 hr in 6 mM Tris, pH 7.5, 6 mM $MgCl_2$, 40 mM NaCl or S_1 nuclease at 50°C for 10 min in 10^{-2} M NaCl, 3 x 10^{-2} M Na acetate, 3 x 10^{-5} M $ZnCl_2$, pH 4.5. The samples were electrophoresed through 1.4% agarose gels and the DNA was visualized by staining with ethidium bromide (16). a) linear, unit length form III PM2 DNA (0.5 µg) generated by Hpa II digestion, b) untreated PM2 DNA (1.0 µg) comprised mainly of form I with a small amount of form II, c) PM2 DNA (14.7 µg) incubated in S_1 buffer without enzyme, d) PM2 DNA (14.7 µg) incubated with 104 U S_1 nuclease, e) molecular weight markers of 5348, 4200, 2115, 1750 and 924 base pairs, (top 5 bands, top to bottom) generated by digestion of PM2 DNA (0.5 µg) with Hind III.

We also considered it necessary to assess the effect of SSC on the S_1 single-strand activity. The S_1 activity is increased by Zn cations (26), however, the citrate anion in the SSC was expected to chelate the Zn^{++}. As seen in Fig. 7, low concentrations of SSC apparently activated the enzyme and no inhibition occurred at concentrations below 0.5 X SSC (7.5 mM citrate). At 0.25 X SSC, the final concentration in the S_1 digestions after DNA reassociation, the S_1 activity was greater than 95% and the reaction essentially went to completion.

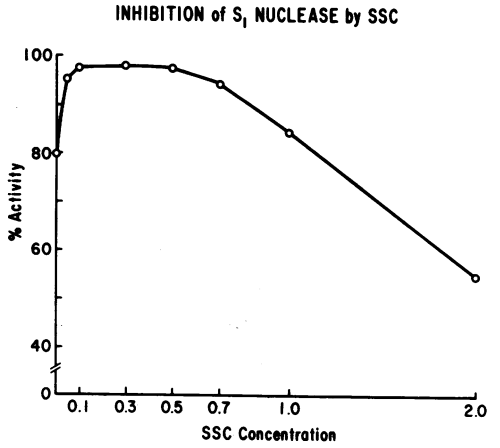

Figure 7. Inhibition of S_1 nuclease by SSC. A heat denatured mixture of [^{3}H]B. subtilis and unlabeled human placental DNAs (1:14) was incubated with 104 U S_1 nuclease at 50°C for 10 min in the presence of different concentrations of SSC. At 0.25 X SSC, the concentration used for digestion of Guinea pig DNA, the reaction went to completion.

DISCUSSION

Three classes of highly repeated DNA sequences have previously been isolated from the Guinea pig genome. These sequences appear as satellites of the main band in $Ag^+Cs_2SO_4$ buoyant density gradients and together comprise 10.5% of the total DNA (1). By utilizing a procedure which is non-selective in the isolation of highly repeated sequences, we have obtained a fraction which constitutes 24% of the genome. The amount of snap-back DNA found in our sample, 2.7%, is in line with similar assays of three widely divergent DNAs. Analysis of Drosophila melanogaster (33), rat (34) and human (35) indicates that inverted repeats make up 3, 4 and 6% of these respective genomes. Allowing for these inverted repeat sequences, we find that the highly repeated sequences of the Guinea pig comprise 21% of the total DNA. Thus, about half of the highly repeated DNA has previously been masked by the main band of the buoyant density gradients. The high frequency of these sequences is compatible with the distribution of these same sequences in the C-band positive centromeric and short arm regions of Guinea pig chromosomes (10). The main band position, thermalyte distribution and fractionation in distamycin A-CsCl gradients possibly reflect the interspersion of these repeated DNAs, fairly high in AT content, with

non-repeated sequences. These adjacent unique DNAs would be removed during the S_1 nuclease digestion of the renatured duplexes. The occurrence of at least six distinct thermalytes indicates that there may be six or more different classes of highly repeated sequences in the Guinea pig genome. It may be possible to utilize thermal elution chromatography to obtain purified fractions of these reassociated DNAs for further studies.

The small amount of contaminating double-strand deoxyribonuclease activity present in the S_1 nuclease preparation may have an effect on the number of different density classes seen in the repetitive sample. A reduction in molecular weight of a molecule of DNA containing two adjacent classes of highly repeated sequences may yield two peaks of differing buoyant densities rather than one peak. Similarly, such cutting within one class of repetitive DNA may yield two DNAs of different density if the average AT content of the two halves of the sequence differ significantly. In order for this to occur, the action of the DNase would have to be non-random. Digestion of PM2 DNA in a non-random manner should yield at least one discrete band somewhere within the PM2 DNA digestion seen in Fig. 6d. Such a band is not seen in this digest. The site specific DNase activity cannot be ruled out, however, because the proper sites for the DNase attack may not be present within the PM2 DNA.

The double-strand DNase activity also does not appear to cause significant losses in the DNA. The average 2- to 5-fold reduction of molecular weight seen in the digestion of PM2 DNA (Fig. 6d) can most likely be attributed to nicking by the S_1 buffer alone (Fig. 6c) and the subsequent digestion of the single-strand region opposite the nick, rather than the double-strand activity. If the double-strand activity was responsible, the extent of the degradation of the PM2 DNA would not be expected to be so extensive. Although the nick and digest mechanism could also occur in the reassociated samples, it would not lead to a loss of DNA classes unless the nicking appeared only in particular sequences. Sequences which are weakly bonded or have a melting temperature below the 50°C used in the S_1 nuclease digestions may be expected to be particularly susceptible to such attack. Since the melting temperatures of the repeated sequences show a significant amount of DNA which denatures below 50°C we do not feel that the nicking is limited to the weakly bonded, early melting sequences.

It is noteworthy that our estimate of the amount of reassociated DNA sequences determined by spectrophotometry (Table II) is in excellent agreement with that derived by conventional radiochemical techniques (Table

I). The procedure of spectrophotometrically determining DNA concentrations in the presence of the enzyme may have general utility since the significant losses of DNA, commonly observed during deproteinizing procedures, can now be avoided.

ACKNOWLEDGEMENTS

We wish to thank Drs. Cynthia Chuang, Allen Ansevin, Frances Arrighi and Grady Saunders for their comments and suggestions during the course of this work. We also thank Dr. Chuang for the S_1 nuclease preparation, Drs. Horace Gray and Roger Hewitt for the purified PM2 DNA, and Dr. Saunders for the use of the Zeiss PM QII spectrophotometer. We also express our gratitude to Misses Mary Bea Cline and Dot Holitzke for their help in the preparation of this manuscript.

This work supported in part by Public Health Service Training Grant (CA-05047) and Research Grants (CA-16527) from the National Cancer Institute and VC-21 from the American Cancer Society.

*Present address: The Wistar Institute of Anatomy and Biology, 36th Street at Spruce, Philadelphia, PA 19104, USA

REFERENCES

1. Corneo,G., Ginelli,E. and Polli,E. (1970) Biochemistry 9, 1565-1571.
2. Southern,E.M. (1970) Nature 227, 794-798.
3. Harbers,K., Harbers,B. and Spencer,J.H. (1976) Fed. Proc. 35, 1677, Abs.
4. Guttann,T., Votavova,H. and Pivec,L. (1976) Nucleic Acids Res. 3, 835-845.
5. Mazrimas,J.A. and Hatch,F.T. (1977) Nucleic Acids Res. 4, 3215-3227.
6. Sutton,W.D. and McCallum,M. (1972) J. Mol. Biol. 71, 633-656.
7. Hennig,W. and Walker,P.M.B. (1970) Nature 225, 915-919.
8. Mazrimas,J.A. and Hatch,F.T. (1972) Nature New Biol. 240, 102-105.
9. Cohen,M.M. and Pinsky,L. (1966) Cytogenet. 5, 120-132.
10. Hubbell,H.R., Sahasrabuddhe,C.G. and Hsu,T.C. (1976) Exptl. Cell Res. 102, 385-393.
11. Marmur,J. (1961) J. Mol. Biol. 3, 208-218.
12. Studier,F.W. (1965) J. Mol. Biol. 11, 373-390.
13. Sutton,W.D. (1971) Biochim. Biophys. Acta 240, 522-531.
14. Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) J. Biol. Chem. 193, 265-275.
15. Fried,M. (1974) J. Virol. 13, 939-946.
16. Sharp,P.A., Sugden,B. and Sambrook,J. (1973) Biochemistry 12, 3055-3063.

17. Danna,K. and Nathans,D. (1971) Proc. Nat. Acad. Sci. U.S.A. 68, 2913-2917.
18. Britten,R.J., Graham,D.E. and Neufeld,B.R. (1974) Methods Enzymol. 29, 363-418.
19. May,P., May,E., Granboulan,P., Granboulan,N. and Marmur,J. (1968) Ann. Inst. Pasteur (Paris) 115, 1029-1046.
20. Flamm,W.G., Birnstiel,M.L. and Walker,P.M.B. (1969) in Subcellular Components: Preparation and Fractionation (Birnie, G.D. and Fox,S.M. Eds.), pp. 125-155, Plenum Press, New York.
21. Ifft,J.B., Voet,D.H. and Vinograd,J. (1961) J. Phys. Chem. 65, 1138-1145.
22. St. John,T., Johnson,J.D. and Bonner,J. (1974) Biochem. Biophys. Res. Commun. 57, 240-247.
23. Mechali,M., de Recondo,A.-M. and Girard,M. (1973) Biochem. Biophys. Res. Commun. 54, 1306-1320.
24. Germond,J.-E., Vogt,V.M. and Hirt,B. (1974) Eur. J. Biochem. 43, 591-600.
25. Brack,C., Eberle,H., Bickle,T.A. and Yuan,R. (1976) J. Mol. Biol. 104, 305-309.
26. Ando,T. (1966) Biochim. Biophys. Acta 114, 158-168.
27. Kit,S. (1961) J. Mol. Biol. 3, 711-716.
28. Corneo,G., Ginelli,E., Soave,C. and Bernardi,G. (1968) Biochemistry 7, 4373-4379.
29. Flamm,W.G., Walker,P.M.B. and McCallum,M. (1969) J. Mol. Biol. 42, 441-455.
30. Sobell,H.M. and Jain,S.C. (1972) J. Mol. Biol. 68, 21-34.
31. Zimmer,Ch., Reinert,K.E., Luck,G., Wahnert,U., Löber,G. and Thrum,H. (1971) J. Mol. Biol. 58, 329-348.
32. Vizard,D.L. and Ansevin,A.T. (1976) Biochemistry 15, 741-750.
33. Schmid,C.W., Manning,J.E. and Davidson,N. (1975) Cell 5, 159-172.
34. Szala,S., Michalska,J., Paterak,H., Bieniek,B. and Chorazy,M. (1977) FEBS Letters 77, 94-98.
35. Dott,P.J., Chuang,C.R. and Saunders,G.F. (1976) Biochemistry 15, 4120-4125.