The interactions of the separated strands of satellite DNAs with other DNAs: 1. Conditions for associations of the α-satellite of the guinea pig with heterologous double-stranded DNAs*

Dorothy M. Skinner and Carey A. Chambers

Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37830, USA

Received 25 August 1976

ABSTRACT

The separated H- and L-strands of the α-satellite of the guinea pig, Cavea porcellus, recovered from centrifugation in alkaline CsCl gradients, form complexes with 7 different double-stranded (ds) DNAs including those of 1 bacteriophage, 2 prokaryotes, 2 invertebrates and 2 mammals. The complexes are not artifacts due to in vitro labeling of the satellite, methods of collection, the presence of divalent cations, or the fact that trace amounts of single-stranded (ss) DNAs are used. More complex ssDNAs, such as that recovered from nicked RF M13, do not associate with dsDNAs.

INTRODUCTION

Satellite DNAs are ubiquitous in eukaryotes¹,²; some of those isolated and characterized have been localized by cytological hybridization to specific regions of one or more chromosomes². The primary nucleotide sequences of several have been determined³,⁴,⁵. Physical characterization of satellites is quite advanced; by contrast to such studies, ideas concerning their functions are still largely speculative. These include the provocative but still unproved suggestions as to their roles in "chromosome housekeeping"⁶,⁷ or as "filler" to provide the requisite amount of DNA needed to accompany male-specific DNA and form a discrete Y chromosome⁸ (see also⁹). With the exception of a few redundant DNAs that form distinct bands in CsCl gradients and code for specific proteins¹⁰ there is very little known with certainty concerning the biological functions of satellite DNAs.

We report here data that show the potential for molecular interactions of satellite DNAs. Specifically, we show that the separated strands of the α-satellite of the guinea pig, Cavea porcellus¹¹, form complexes with dsDNAs, some of which are considerably less highly repeated or even unique. We describe the conditions under which such associations occur. In addition, we have characterized the complexes and present evidence that they are not experimental artifacts.

The individual strands of the α-satellite of the guinea pig show a
marked bias in base composition, the L-strand containing 36% C residues and the H-strand 36% G residues\textsuperscript{11,12}. Almost 60% of the sequences in the L-strand are -C-C-C-T-A-A-, the remainder being considered as variations caused by one or two base changes per hexamer; the H-strand is the complementary sequence\textsuperscript{13}.

The separated strands of another satellite, present as a minor component in the DNA of the hermit crab, \textit{Pagurus pollicaris}, form similar complexes\textsuperscript{14}. We have done the more extensive studies described here on the guinea pig satellite because of its greater availability; it comprises 9% of the total DNA\textsuperscript{15} and is isolable in milligram quantities. Several brief summaries of this work have been reported\textsuperscript{14,16}.

\section*{MATERIALS AND METHODS}

\textbf{Purification of DNA.} Satellite DNA of the guinea pig (\textit{Cavia porcellus}) was prepared as described previously\textsuperscript{11,12}. Human thymus DNA, prepared batchwise by adsorption to hydroxyapatite\textsuperscript{17} (HAP; Bio-Gel HTP, DNA grade, Bio-Rad Lab, Richmond, California) was freed of satellites by centrifugation in Cs\textsubscript{2}SO\textsubscript{4} in the presence of Hg\textsuperscript{2+} at a ratio of 0.1/DNA phosphate\textsuperscript{18}. \emph{E. coli} [\textsuperscript{14}C]-DNA was the gift of W. L. Carrier; \emph{M. luteus} DNA was purchased from Miles Laboratories, Inc.; both DNAs were further purified by passage through HAP before use. M13 RF[\textsuperscript{32}P]-DNA, the gift of S. Mitra, was "nicked" by treatment with S\textsubscript{1} nuclease (Sigma; see below) purified by passage through Sephadex G 100 to remove dsDNase activity. T7 DNA was prepared according to Richardson\textsuperscript{19}.

\textbf{In vitro Labeling of DNA.} a. Iodination: Two to ten micrograms of DNA was labeled with \textsuperscript{125}I using the method of Commerford\textsuperscript{20}. Treatment with Na\textsubscript{2}SO\textsubscript{3} was omitted and dialysis was carried out at 4\textdegree{} and 60\textdegree{}. When native \emph{E. coli} DNA was labeled, the specific activity of the product was approximately half that obtained when denatured \emph{E. coli} DNA was labeled under identical conditions. b. DNA polymerase I: DNA was labeled with \emph{E. coli} polymerase I (Boehringer) (3.3 units/\mu g DNA) in a reaction volume of 0.1 ml containing 0.04 m Ci [methyl-\textsuperscript{3}H]dTTP, specific activity 26 Ci/mm mol. Concentrations of unlabelled nucleoside triphosphates were 0.1 mM dGTP and dATP and 0.25 mM dCTP. The reaction mixture was 0.05 M in sodium phosphate (PB), pH 7.55 and 0.01 M in MgCl\textsubscript{2}. After incubation for 3 hr at 15\textdegree{}, the reaction was stopped by adding EDTA to 0.05 M. The volume was brought to 1 ml and protein was extracted by shaking with an equal volume of freshly distilled phenol (equilibrated with Tris buffer, pH 7.8, followed by distilled water) for 15 min at 3\textdegree{}. The aqueous layer was
removed, made 0.3 M in sodium acetate, and precipitated with 2.2 volumes absolute ethanol. The precipitate, collected by centrifugation at 27,000 X g for 30 min, was taken up in 0.5 ml water and the co-precipitated, unreacted NTP’s were removed by passage through a Sephadex G-100 column equilibrated with 2 mM EDTA and 2 mM NaCl. The H-(dG-rich) and L-(dC-rich) strands of the satellite were separated from each other by centrifugation in an alkaline CsCl density gradient as shown in Figure 1b. Pooled fractions of H- and L-strands were neutralized by the addition of 1 M PB, pH 4.4, to 0.1 M and were desalted by dialysis against 0.15 mM NaCl, 0.015 mM NaCitrate.

Density gradient centrifugation. Two step CsCl gradients were centrifuged for 20 hr. Except where noted the amount of ssDNA in the gradients was between 0.04 and 1 µg; siliconized polyallomer tubes were used to prevent its loss. The ssDNAs were heated for 5 min at 1000 before they were added to the gradients. To establish that the companion dsDNAs were not serving as carriers during TCA precipitation of the gradient fractions, parallel gradients were collected either directly on Whatman #17 strips (presumably because of the decreased pore size of the #17 paper) or directly in vials; after dilution, these were counted in Instagel (Packard). In addition, duplicate gradients collected in siliconized tubes containing BSA (100 µg/ml) gave similar or identical recoveries following TCA precipitation, whereas counting in Instagel was approximately 75% as efficient. These experiments led to the routine collection of fractions of gradients in siliconized tubes for absorbancy measurements and their alternates directly on strips for radioactivity assay in a Nuclear-Chicago scintillation counter.

Preparative band sedimentation gradients were in 4.1 M CsCl. Linear 5 to 20 percent sucrose gradients were made 0.1 M in NaCl and 0.05 M in phosphate, pH 7.4. Preparative alkaline sucrose gradients (5 to 20 percent) contained 0.1 M NaOH, 0.9 M NaCl and 0.01 M EDTA. Velocity sedimentation analyses in 0.9 M NaCl, 0.1 M NaOH, were run in a Model E analytical ultracentrifuge.

Treatment with S1 nuclease. Main-component (mc)DNA, either hermit crab or guinea pig, was treated with 5 units S1 nuclease/µg DNA. The reaction mixture, containing 0.03 M sodium acetate, pH 4.5, 0.72 mM ZnSO4, and 0.1 M NaCl, was incubated at 450 for 30 min. The reaction was stopped by the addition of EDTA to 5 mM. The DNA was treated with phenol and
recovered as described above.

**Thermal dissociation determinations.** Selected fractions of neutral CsCl gradients were pooled. Since some fractions had been diluted for observation at 260 nm, all were routinely brought to 2.5 M in CsCl, 0.02 M sodium phosphate buffer, pH 6.8 (PB) and applied at room temperature to HAP columns equilibrated in 0.12 M PB, pH 6.8. The temperature of the column was increased in approximately 5° increments. Six-ml fractions of 0.12 M PB were collected directly in counting vials. After the UV absorbancy was monitored, 10 ml Instagel was added and radioactivity was determined in a scintillation counter.

**RESULTS**

**Preparation and characterization of DNAs.** The dsDNAs used in these experiments were prepared by batchwise adsorption to and elution from hydroxyapatite; their molecular weights determined in alkaline sucrose gradients were on the order of 1-2 x 10^6. When the guinea pig α-satellite was labeled by nick translating with a commercial preparation of DNA polymerase I, it formed one narrow band in neutral CsCl (Fig. 1a); the separated

![Figure 1](image-url)

**Figure 1.** Preparative CsCl gradients of guinea pig α-satellite; (a) 0.05 μg satellite labeled *in vitro* with [3H]dTTP (see MATERIALS and METHODS) was centrifuged in a neutral CsCl gradient (ρ = 1.705 g/cm³). (b) As (a) except 3 μg [3H]dT satellite DNA was centrifuged in an alkaline gradient with 50 μg unlabeled satellite as carrier. The higher specific activity of the H-strand (ρ = 1.778 g/cm³) reflects the twice higher thymine content of that strand as compared to the L-strand (ρ = 1.692 g/cm³).

H- and L-strands formed two sharp bands in alkaline CsCl gradients (Fig. 1b). Band sedimentation in alkaline sucrose gradients showed that the radioactive product contained many molecules considerably smaller than the template. Several electrophoretically pure DNA polymerase I preparations gave similar results with main-component (mc) DNAs of a number of Crustacea. (C. A.
Holland, N. T. Christie, unpublished data). To retard degradation of the separated strands, all glassware to which the purified satellite DNA was exposed was autoclaved, and most was siliconized. Even with these precautions, after some months of storage at 0°C, some of the separated strands of the satellite were as small as 150 nucleotides.

Behavior of iodinated E. coli DNA centrifuged in the presence of unlabeled E. coli DNA. Some of the satellite DNA preparations were labeled with $^{125}$I. Because of the general assumption that iodinated macromolecules tend to "stick" to other macromolecules or to surfaces, we performed a series of control experiments with $^{125}$I-labeled E. coli DNA, selected because the reassociation of in vivo labeled E. coli DNA has been well characterized. In neutral CsCl gradients, such DNA did not associate with any companion DNA, including unlabeled E. coli DNA, as shown by the following experiment (Fig. 2). When 0.05 μg/gradient of extensively iodinated (30% of the dC residues) denatured E. coli DNA with a buoyant density ($\rho = 1.756 \text{ g/cm}^3$) greater than that of native E. coli DNA ($\rho = 1.7035 \text{ g/cm}^3$) was centrifuged in a neutral CsCl gradient in the presence of 50 μg/gradient of native E. coli DNA, there was no evidence for association of the $^{125}$I-labeled DNA with unlabeled DNA. Association of the DNAs should have produced a labeled hybrid DNA having a density less than that of the labeled ssDNA.

As further proof that the associations of the single-stranded satellites and the double-stranded DNAs described below were not due to modifications by the iodination reaction, we have determined the kinetics of reassociation of $^{125}$I-labeled E. coli DNA. Both trace labeled (<0.1% of

---

1. We have designated as "companion DNAs" those double-stranded DNAs with which the separated single strands of the satellite DNAs were centrifuged.
the dC residues labeled) and extensively labeled (30% of the dC residues) DNAs were tested. Their reassociation kinetics were identical to those of control labeled in vivo E. coli [\(^{14}\)C]DNA. The C\(_{50}\)t for half reaction was 5, in agreement with that obtained by others (Fig. 3).

**Figure 3.** The reassociation of fragments of radiolabeled E. coli DNA assayed by binding to hydroxyapatite. DNA sheared in a Virtis 60 blender to 200 nucleotide fragments which were incubated at 60\(^\circ\) C in 0.12 \(M\) PB. The DNA preparations approach 90% reassociation. \(\bullet\) \(\bullet\) \(^{14}\)C-labeled in vivo; \(\bigcirc\) \(\bigcirc\) \(^{125}\)I-labeled in vitro.

**Behavior of H- or L-strands of guinea pig \(\alpha\)-satellite ± double-stranded DNAs in CsCl density gradients.** When a preparation of \([^{3}\text{H}]\)- or \([^{125}\text{I}]\)-H-strand of guinea pig \(\alpha\)-satellite (0.1 \(\mu\)g/gradient) was centrifuged in a neutral CsCl preparative gradient, it formed a broad peak with a density of 1.757 g/cm\(^3\);\(^{11}\) (Fig. 4a,b). The width of the peak at half-height extended over \(\Delta\rho = 50\) mg/cm\(^3\). In the presence of 50 \(\mu\)g/gradient of mcDNA from either guinea pig (Fig. 4c), Pagurus (Fig. 4d,e) or human (Fig. 4g) DNA, or with 50 \(\mu\)g of E. coli DNA (Fig. 4f), the labeled H-strand formed a single sharp peak, which was slightly more dense in each case than the companion dsDNA tested. The peak width at half-height

**Figure 4.** Preparative neutral CsCl density gradients of H-strand DNA alone or in the presence of companion dsDNAs: (a) 0.3 \(\mu\)g \([^{3}\text{H}]\)-dT-H-strand centrifuged in 7.7 M CsCl. (b) As (a) except 0.01 \(\mu\)g \([^{125}\text{I}]\)-H-strand. (c) As (a) except 30 \(\mu\)g guinea pig mcDNA present. (d) As (b), with 50 \(\mu\)g crab mcDNA present. The gradient was collected directly on Whatman \#17 strips without monitoring the \(A\)\(_{260}\)nm of the companion DNA. (e), (f) (g) As (a) except 50 \(\mu\)g crab mcDNA (e) or E. coli DNA (f) or 30 \(\mu\)g human mcDNA (g) present. \(0\) \(\cdots\) \(0\) cpm; \(\bullet\) \(\bullet\) \(A\)\(_{260}\)nm.
extended over $\Delta \rho = 15$ mg/cm$^3$. Partial association of the $H$-strand was also seen with "crab poly(dA-dT)" of the land crab, *Gecarcinus lateralis* 29, which contains 8% G+C residues 30 (Fig. 5a) but not with synthetic, pure poly(dA-dT) (Fig. 5b).

To determine whether the apparent association of iodinated ssDNA with dsDNAs present in O.D. amounts is an artifact due to release of a reactive $^{125}$I species into solution with subsequent binding to the more highly concentrated dsDNA, we made several gradients 2.6 mM in tyrosine as an iodine scavenger before addition of the unlabeled DNA. The presence of tyrosine had no effect on the distribution of the labeled peaks in the density gradients. In the presence of *Pagurus* mcDNA, all the labeled ss-satellite DNA interacted with it. (Data not shown.) In alkaline as opposed to neutral preparative CsCl gradients, no association was observed between labeled satellite ss DNA and companion DNAs present in O.D. amounts. (Data not shown.) Neither did double-stranded satellite labeled by nick translation associate with ds companion DNA.

**Effect of salt concentration on complex formation.** As opposed to synthetic poly(dA-dT), all naturally occurring linear DNAs tested, except *M. luteus* and, to some extent, "crab poly(dA-dT)" satellite DNA, were...
favorable complexing species with \( H \)- or \( L \)-strands of the satellite. When \( M. \) luteus was the companion DNA, the labeled \( H \)-strand formed a relatively broad peak slightly more dense than \( M. \) luteus DNA (Fig. 7a). The \( L \)-strand + \( M. \) luteus complex formed a somewhat narrower band than did the \( H \)-strand \( M. \) luteus complex (data not shown). In the presence of both crab mcDNA and \( M. \) luteus DNA, the \( H \)-strand associated preferentially with the former DNA almost exclusively (Fig. 7b). In similar experiments with \( M. \) luteus DNA + guinea pig or human mcDNA, the \( H \)-strand associated with the eukaryotic DNAs. (Data not shown.) After a four hour incubation of \( H \)-strand with \( M. \) luteus in high salt (7.7 M CsCl), there was slightly more association with \( M. \) luteus (Fig. 7c), while a 20 hr incubation in high salt led to the formation of two complexes with almost equal distribution of the \( H \)-strand between the two companion DNAs (Fig. 7d).

We have taken advantage of the reduced complex formation with \( M. \) luteus DNA to determine the effect of salts on complex formation. \([3H] \)-dT-\( H \)-strand (0.04 \( \mu \)g) was incubated with 50 \( \mu \)g \( M. \) luteus DNA in low salt (0.10 M NaCl, 0.05 M Tris, pH 8) or in high salt (7.7 M CsCl) for one week at different temperatures; then crab mcDNA was added, the CsCl was adjusted to 7.7 M if necessary, and the mixture was centrifuged. After such incubation in high salt, the \( H \)-strand remained complexed with the \( M. \) luteus

---

**Figure 6.** Preparative neutral CsCl gradients of \( L \)-strand alone or in the presence of companion dsDNA. (a) 0.7 \( \mu \)g \([3H] \)-dT-\( L \)-strand. The gradient was collected directly on Whatman \#17 filter paper strips. (b) 1.4 \( \mu \)g \([3H] \)-dT-\( L \)-strand and 50 \( \mu \)g guinea pig mcDNA present. (c) As (b) except 50 \( \mu \)g hermit crab mcDNA present. In (b) and (c) alternate samples were collected in silicon tubes and their absorbancy was measured.

0 - - - 0 cpm; \( \bullet - - - \bullet \) A\text{260nm}
DNA (Fig. 7e). After incubation in low salt with *M. luteus* DNA at 0°, the H-strand associated with crab mcDNA (Fig. 7f). By contrast, in low salt but at room temperature (≈23°), approximately 50% of the H-strand remained associated with the *M. luteus* DNA (Fig. 7g). Clearly, although complex formation is enhanced by high salt, it occurs in physiological salt concentrations as well.

![Figure 7](image.png)

**Figure 7.** Neutral CsCl gradients of H-strand DNA with *M. luteus* and hermit crab mcDNAs: (a) 0.6 μg [³H]-dT-H-strand DNA centrifuged with 50 μg *M. luteus* DNA (ρ = 1.727 g/cm³); (b) 0.04 μg [³H]-dT-H-strand with 50 μg hermit crab mcDNA as well as 50 μg *M. luteus* DNA; (c) 0.6 μg [³H]-dT-H-strand incubated in 7.7 M CsCl for 4 hr at room temperature (≈23°) in the presence of 50 μg of *M. luteus* DNA before the addition of 50 μg hermit crab mcDNA; (d) as (c) except incubation with *M. luteus* DNA was 20 hr; (e) as (c) except 0.04 μg [³H]-dT-H-strand incubated with *M. luteus* DNA for 1 week; (f) as (e) except the incubation solution was 0.1 M NaCl, 0.05 M Tris, pH 8.1 and the temperature was 0°; (g) as (f) except the incubation in low salt was carried out at room temperature. 0 - - - 0 cpm; ○ A260nm.

Other single-stranded DNAs. To determine whether trace amounts of any ssDNA would associate with the 30 to 100-fold excess of companion dsDNA routinely centrifuged in the gradients, experiments were carried out with M13 DNA. Linear pieces of M13 DNA were obtained by treating it with S1 nuclease (see MATERIALS and METHODS) and centrifuging in alkaline sucrose gradients. Selected fractions of different molecular weights were collected and neutralized by the addition of 0.1 M TrisCl to pH 7.8.

Density gradients were run with hermit crab mcDNA as the ds species. The ratio of ss to the ds companion DNA was equal to that used with the ss satellite DNA. Even though degraded to relatively small molecular weight (5 X 10⁵) the phage DNA formed a band at its characteristic density (1.716 g/cm³) in the absence (Fig. 8a) or presence (Fig. 8b) of companion DNA. Because some of the ss satellite preparations were very small (~150 nucleotides) experiments were run with M13 DNA of equivalent size. The bands formed in the absence (Fig. 8c) and presence (Fig. 8d) of companion dsDNA indicate that, again, there was no association. These data show that trace amounts of more complex ssDNAs, such as M13, do not
associate with dsDNAs as do the separated strands of the satellite DNA.

**Complex formation in the presence of chelating agent.** To determine whether trace amounts of divalent cations, such as Mg\(^{2+}\), were causative agents in complex formation\(^{33,34}\), gradients were run in the presence of 0.01 M EDTA; H-strand was the ss species with hermit crab mcDNA as the ds species. The labeled DNA formed a band identical to that formed in parallel gradients without EDTA. (Data not shown.)

Complex formation with ds DNAs treated with \(S_1\) nuclease to remove ss regions. Some of the companion DNAs had been prepared by adsorption to HAP from tissue homogenates that had been blended, a method that shears the DNA\(^{17}\). It seemed possible that the \(L\)- or \(H\)- ss satellites were associating with ss homologous regions in the mc, "crab poly(dA-dT)" satellite, or bacterial, DNAs. To test this possibility, we treated mcDNAs of both hermit crab and guinea pig with \(S_1\) nuclease. Very high concentrations of nuclease were used (5 units/µg DNA), the incubation was carried out at 45°C, the temperature known to be optimal for the enzyme\(^{35}\), even though there is some dsDNase activity at that temperature. Such treatment resulted in an approximate two-fold decrease in the molecular weights of both main component DNAs.

Gradients of typical experiments performed with such \(S_1\) nuclease-treated DNAs (\(S_1\)-mcDNAs) are shown in Fig. 9. \(^3\)H-labeled H-strand of guinea pig satellite was centrifuged with hermit crab (Fig. 9a) and guinea pig (Fig. 9b) mcDNAs. There was a wider than usual distribution of sizes as ss regions (gaps) connecting ds regions were obliterated (compare with Fig. 4c,d,e). In addition, the \(^3\)H-H-strand peak was more than two and one-half times wider than with untreated DNAs. An O.D. shoulder similar to that at \(\rho = 1.711 \text{ g/cm}^3\) (Fig. 9, fraction 25) has been seen in six such gradients, two with guinea pig and four with hermit crab \(S_1\) mcDNAs. In each case, the density at which the complexed H-strand is observed is 8 to
Figure 9. Preparative neutral CsCl gradients of [3H]-dT-H-strand with dsDNA that had been treated with S1 nuclease: Main component DNAs of guinea pig (a) and hermit crab (b) were treated with S1 nuclease. Neutral CsCl gradients were assembled with 0.3 μg [3H]-dT-H-strand and 35 μg guinea pig or 50 μg hermit crab mcDNA. See Figure 4 for gradients with complex formed with untreated companion dsDNAs.

0 - - - 0 cpm; ○ --- ○ A260nm -

10 mg/cm³ greater than the density of the complex with untreated dsDNAs. This shoulder is not apparent in a Model E analytical run of crab mc S1-DNA in the absence of H-strand. All of these data are most easily interpreted by the following model: in large molecular weight DNA, the complexes perturb the density only to a minor extent. With the smaller S1-DNAs, the interspersed non-complexing sequences formerly present in the dsDNAs are no longer attached to the sequences of DNA participating in the complex and the density shift is more pronounced.

Saturation experiments. Because of the limiting quantities of single strands available, saturation experiments could not be performed under ideal conditions. They were also complicated by the fact that high concentrations of single-stranded DNAs aggregate in solutions of high ionic strength such as those used for density gradients or band sedimentation. In one saturation experiment, when 10 μg H-strand was centrifuged with 25

Figure 10. Saturation of binding sites on companion DNA. (a) 10 μg [3H]-dT-H-strand centrifuged with 25 μg hermit crab mcDNA. (b) As (a) except 1 μg H-strand. The arrow indicates the position of H-strand (ρ = 1.757 g/cm³).

0 - - - 0 cpm; ○ --- ○ A260nm.
μg hermit crab mcDNA, 40% of the satellite associated (Fig. 10a). By contrast, in several other experiments, with the same amount of a different preparation of H-strand, aggregation occurred, superseding the association with the double-stranded DNA and the H-strand formed a band at 1.757 g/cm$^3$ similar to that seen in the absence of any ds DNA. In an experiment with 1 μg H-strand, some aggregation was apparent as a slight shoulder on the leading edge of the peak (Fig. 10b).

These data show that the complexes do not involve trace amounts of ss DNAs since the ratio of ss:ds DNA can be as high as 1:6 rather than 1:10,000 as seen with certain other ss:ds nucleic acid interactions$^{37}$.

Characteristics of the complexes formed. We have analyzed the complexes formed between the separated single strands of the satellites and

![Graph](image-url)

Figure 11. Thermal dissociation of ss or ss plus dsDNA complexes from hydroxyapatite. (a) 1 μg [$^3$H]-dT-L-strand brought to 2.5 M CsCl, 0.02 M PB and applied to a 1 cm$^3$ HAP column. Fractions eluted in 0.12 M PB as the temperature was increased were read and counted as described in MATERIALS and METHODS. (c) 1 μg [$^3$H]-dT-H-strand was applied to HAP as in (a); (b) as (a) except fractions of L-strand associated with 50 μg guinea pig mcDNA in a neutral CsCl gradient were pooled, diluted to 2.5 M in CsCl and 0.02 M PB and applied to HAP. (d) As (c) except fractions of H-strand associated with 50 μg guinea pig mcDNA in a neutral CsCl gradient were pooled and adsorbed to HAP as in (c).

0 - - - 0 cpm; $\Delta$ $\Delta$ percent total counts eluted; $\bullet$ $\bullet$ $A_{260nm}$, companion dsDNA.
the double-stranded DNAs by observing their thermal dissociation behavior and their behavior in band sedimentation, which is in solutions of high ionic strength (4.1 M CsCl) and in linear sucrose gradients of lower ionic strength (0.1 M NaCl, 0.05 M Tris, pH 8, 5-20% sucrose).

a. Thermal dissociation. Data from dissociation experiments with L-strand that had not been centrifuged in neutral CsCl gradients are shown in Figure 11. When mixed with CsCl at a final concentration of 2.5 M, equal to that of the diluted ss and ds samples collected from CsCl gradients, and applied directly to HAP equilibrated in 0.12 M PB, more than 80% of L-strand either did not bind or was eluted in 0.12 M PB by 350. Between one and five percent was eluted at each temperature increment thereafter (Fig. 11a). As with L-strand, a fraction of H-strand did not adsorb to HAP (Fig. 11c). By contrast, a large fraction of the G-rich species did adsorb; several different H-strand preparations eluted with a $t_m$ of 460 as shown here or 600 as seen in several other experiments.

The behavior of L-strand dsDNA complexes on HAP differs from that of H-strand dsDNA complexes as shown in Figure 11b and d. Fractions 25 to 34 of a gradient containing 0.3 μg L-strand (similar to that in Fig. 6b) and 50 μg guinea pig mcDNA were pooled, diluted as described in MATERIALS and METHODS and applied to HAP (Fig. 11b). Fifty per cent of the L-strand (radiolabeled) DNA was eluted by 370; another distinct component eluted from 520-750. A pattern similar to that shown in Figure 11b was seen in three thermal dissociation experiments. The main component DNA (monitored at 260 nm) eluted at 900 as it had when applied to HAP by itself.

Data from a typical experiment with H-strand and mcDNA are shown in Figure 11d. Fractions 25 to 39 (see Fig. 4c) and those of a similar gradient, were pooled, diluted, and applied to HAP. No H-strand (labeled) DNA was eluted until 400 and the $t_m$ was approximately 610. A slight shoulder at 700 was seen in each of three experiments. Some ssDNA remained bound until 910, the temperature at which the mcDNA is eluted.

With respect to the tightness of binding, only qualitative statements can be made from the present data. The forces in the centrifugal field are greater than diffusion forces and would tend to separate the DNAs permanently. This does not occur even at low concentrations of ssDNA. Yet the $t_m$ data indicate that the ss + dsDNA complexes are not as stable as the Watson-Crick pairing of the original satellite.

b. Band sedimentation. In band sedimentation gradients, H-strand formed a broad peak with an $S_{20,w}$ of 6 (Fig. 12a); in the presence of crab mcDNA,
(S<sub>20,w</sub> = 10) with which it associated, it moved further into the gradient, assuming an S<sub>20,w</sub> of 12 (Fig. 12b).

![Figure 12. Band sedimentation of H-strand without or with ds companion DNA: (a) 0.3 µg [³H]-dT-labeled H-strand layered on 3 ml of 4.1 M CsCl and centrifuged for 4 hr at 35,000 rpm at 250. (b) As (a) except in the presence of 50 µg crab mcDNA. 0 --- 0 cpm; • • • • A<sub>260nm</sub>.](image1)

![Figure 13. Neutral sucrose gradients of H-strand without or with ds companion DNA: (a) 0.04 µg [³H]-dT-labeled H-strand layered on a 4.8 ml 5 to 20% sucrose gradient containing 0.05 M Tris Cl, pH 8, 0.1 M NaCl and centrifuged for 3 hr at 40,000 rpm at 100. (b) As (a) except incubated 1 week in the presence of 25 µg T7 DNA. 0 --- 0 cpm; • • • • A<sub>260nm</sub>.](image2)

With the exception of the experiments in which complex formation with M. luteus was enhanced by a prior incubation in low salt, the experiments described to this point have all been carried out in high concentrations of CsCl either 4.1 M (velocity sedimentation) or 7.7 M (equilibrium density gradients). To obtain further information on the dependence, or lack of it, of complex formation on high salt, we have examined the sedimentation...
behavior of H-strand in the absence or presence of companion DNA in neutral sucrose gradients. Since there was good complex formation with M. luteus DNA after one week's incubation (Fig. 7g) we incubated T7 DNA (13 nicks/molecule) with H-strand for the same period of time. In the control, H-strand alone formed a diffuse band at the top of the gradient (Fig. 13a) even after preincubation for 2 days (data not shown). Self-association of the ss DNA is thus not detected in these gradients. After incubation with T7 DNA in 0.1 M NaCl, 0.05 M TrisCl, pH 8, approximately 60% of the H-strand associated (Fig. 13b). In four separate experiments, a significant fraction of the complexed DNA pelleted after centrifugation. These data show that the complex can both form and remain stable in low salt.

**DISCUSSION**

The data describe the formation of a complex by the dC-rich or dG-rich separated strands of the α-satellite of the guinea pig with seven different dsDNAs, namely, crab, guinea pig and human main components, T7, E. coli, M. luteus, and, most surprising, "crab poly(dA-dT)." Data on H-strand + dsDNA complexes have been presented in many of the experiments because the density shift is so much more pronounced with it (Δρ = 50 mg/cm³) than that with many L-strand dsDNA complexes (Δρ = 5 mg/cm³). Nevertheless, similar associations are also demonstrable with L-strand, and some have been shown above. A significant fraction of the ssDNA must be involved, since all the radioactive ssDNA forms sharp bands of characteristic densities with each of the companion DNAs. Three different kinds of association can be envisaged. There could be a formation of (partially) ds complexes with ss regions in the companion dsDNAs. A second alternative is that the ssDNAs may displace one strand in dsDNAs as the latter become transiently dissociated or "breathe". Finally, the ss + dsDNAs may form authentic (partially) triple-stranded (ts) structures.

We have presented evidence against the probability that the complexes are artifacts. The participation of the dC-rich strand in complex formation eliminates the possibility that we are seeing nothing more than the aggregation that occurs with dG residues. Complex formation with DNAs labeled in either of two ways, by nick translation or iodination, makes it unlikely that the association is due to artifacts of labeling. Another ssDNA, M13, is considerably more complex than the guinea pig satellite; it has 6000 nucleotides, is unique and codes for eight proteins. The lack of complex formation with M13 DNA indicates that the satellite associations are specific and are not simple physical association of trace amounts of
ssDNA, on the edge of solubility in high salt\textsuperscript{38}, with the dsDNA present in several hundred-fold excess.

While our work was underway\textsuperscript{14,16}, Perlgut et al.\textsuperscript{34} described complexes between native calf thymus DNA isolated as the Mg\textsuperscript{2+} salt and the Na\textsuperscript{+} salt of the homologous denatured DNA; no complex formation occurred with heterologous DNAs such as salmon. The complexes described here differ from those with calf thymus DNA in that Mg\textsuperscript{2+} is not required, either during the preparation of the DNA, during complex formation or during the thermal dissociation studies. Finally, complexes form with heterologous DNAs, even those of T7, two bacteria and "crab poly(dA-dT)."

Various aspects of our experiments resemble earlier work on the association of denatured viral\textsuperscript{39}, bacterial\textsuperscript{40,41}, and vertebrate DNAs\textsuperscript{41} with synthetic ribopolymers or naturally occurring RNAs, specifically rRNA, in CsCl density gradients. In those studies, as in ours, the ratio of dsDNA to ss polymer was very large. In contrast with our studies, in those experiments all the DNAs were heat-denatured before centrifugation. Several types of interaction were noted. In some, namely those with \textit{B. subtilis} and certain phages having marked interstrand bias in base composition, the ribopolymer bound preferentially to one strand and led to strand separation in a neutral CsCl gradient. Both strands of certain other DNAs associated with the ribopolymers to form a single band of increased density. Still other DNAs showed no interaction: the ribopolymer pelleted, leaving the DNA in its usual position in the gradient.

Our data differ markedly from those on complexes formed between mouse satellite cRNA (synthesized \textit{in vitro} with \textit{E. coli} RNA polymerase) and denatured total DNA or DNA isolated from heterochromatin\textsuperscript{37} which in the mouse is enriched in satellite sequences. In those studies, of the 0.01 μg cRNA included in each gradient, more than 90% either pelleted or was considerably closer to the bottom of the gradient than was the complex. The ratio of ss to ds nucleic acid in the complex was 1:10,000, whereas we routinely see ratios of 1:150 and have seen ratios as high as 1:6 (Fig. 10a).

Some of our data argue against the first alternative given above, namely that the ssDNA associates with ss gaps in the companion DNA: (1) treatment of the dsDNAs with S\textsubscript{1} nuclease before centrifugation of the ss + ds DNAs in CsCl gradients should remove some, or, with the high concentrations of S\textsubscript{1} used here, all sites of association. As seen in Figure 9, there was no decrease in the amount of radiolabeled ssDNA bound with S\textsubscript{1}-mc
DNAs. $S_1$ has exonuclease activity only towards ssDNAs. Therefore, even if there were dsDNase activity under our conditions, long ss gaps should not have been created in the companion DNAs. If staggered cuts were made in ds regions of the companion DNAs, in order for the DNA to behave as though a ds cut had been introduced, the cuts must have been 3 or less nucleotides apart in solutions of the ionic strengths used here. "Frayed ends" of only 3 nucleotides seem unlikely as sites for complex formation. (2) treatment of the ss-dsDNA complexes with $S_1$ nuclease before HAP chromatography should not degrade those regions associated in double helical complexes. After $S_1$ treatment of ss + dsDNA complexes, there was no detectable radioactivity adsorbed to HAP hence presumably no double helical complexes. (Data not shown.) It is of course possible that the complexes involve short stretches of satellite sequences, leaving long ss tails susceptible to $S_1$ nuclease and, in that instance, so little of the labeled DNA participated in complex formation as to be undetected after $S_1$ treatment. (3) All seven dsDNAs studied would be required to contain ss regions of homologous sequences. This would require the evolutionary conservation of specific sequences in the DNAs of a bacteriophage, two bacteria, two invertebrates and two mammals, and moreover, that after isolation of the DNA, these sequences are single-stranded and remain so. This seems improbable. Especially puzzling would be the conservation of such sequences in "crab poly(dA-dT)", a DNA comprised of more than 90% alternating deoxyadenylate and thymidylate residues. (4) Both dC-rich and dG-rich strands should be bound in equal amounts to the complementary sequence and the $z_m$ of the complexes should be the same. With both strands, there is a complex dissociable at $\sim 70^\circ$, but there is a quantitative difference in the amount of that complex with each strand.

The second alternative is that the association occurs with transiently dissociated regions in the companion DNAs and is dependent on their "breathing". Since (G+C)-rich DNAs are less easily dissociable than (A+T)-rich DNAs, they may breathe less. At present, we have no other explanation for the lower rate of complex formation with $M. luteus$ DNA, which has the highest (G+C)-content of all the companion DNAs studied. Arguing against this model was again the observation that there was no detectable $H$-strand radioactivity adsorbed to HAP after $S_1$ treatment of the ss + dsDNA complex.

A third alternative is that the ss + dsDNAs form ts complexes. In the past few years, there has been renewed interest in triplex formation be-
cause of the induction of interferon by ribopolymers in the ds form, which, under certain conditions, undergo strand rearrangement to ts complexes and lose activity. Earlier experiments on the formation of nucleic acid triple helices have dealt largely with synthetic ribo- and deoxyribopolymers, especially poly(U) and poly(A). Some have emphasized the reactivity of G-residues in complex formation. Free guanine, guanosine or guanylic acid associates with polyribocytidine. Because the sequence of the H-strand of the α-satellite contains a repeating G-triplet, studies on the interaction of guanine monomers or oligonucleotides with poly(C) seem particularly pertinent here. To avoid the self-bonding that occurs with poly(G) as opposed to that with the trimer, much of the previously published work on such complexes has been done with trinucleotides, such as G-G-Gp, rather than with polymers. The satellite ss + dsDNA complexes resemble ts complexes in certain specific ways. They are favored by long periods of incubation and are rather unstable. Because of the comparatively higher charge density of the three-stranded molecule, they have relatively low \( T_m \) s, e.g. the triplex comprised of G + G + C [the addition of G-G-GP to a double helix comprised of poly (G) + poly (C)] increased the \( T_m \) by only 10° over that of a solution of G-G-GP without poly (C). In the satellite ss + dsDNA complexes, dissociation of the satellite strand occurs at 20 to 25° lower than the dissociation of the helix with which it has complexed (Fig. 11b).

For associations such as those described here to occur in the cell would require that certain regions of a satellite and, depending on the mechanism of complex formation, possibly the companion DNA, become single-stranded. There is recent evidence for ss regions in several eukaryotic DNAs, should such regions in the companion non-satellite DNAs be, in fact, the reacting species.

Finally, although mass action may be secondary in influence to more specific regulatory properties of the intracellular milieu, favoring such interactions, if only by the law of mass action, are the localization of satellite DNAs in specific regions of chromosomes thereby presenting tens of millions of copies of the reactive sequences to (specific?) sequences in the adjacent potential "companion" DNAs. The biological importance of complex formation between the satellite strands with dsDNA may be in the compaction of heterochromatin.

Although specific ts complexes seem to be a reasonable interpretation of our data, it has been claimed that no triple helices have been detected in
any biological system. Experiments in progress should distinguish between the various alternatives.

ACKNOWLEDGMENTS

We thank Ms. M. Schell for isolating the guinea pig α-satellite, Drs. F. J. Bollum, R. J. Britten, J. Cook, D. Crothers, N. Davidson, G. Felsenfeld, S. Mitra and H. Swift for helpful discussions and W. E. Cohn and S. Niyogi for critical reading of the manuscript. This research was sponsored by the Energy Research and Development Agency under contract with the Union Carbide Corporation.

*Research sponsored by the Energy Research and Development Administration under contract with the Union Carbide Corporation.

REFERENCES

1 Arrighi, F. E., Mandel, M., Bergendahl, J. and Hsu, T. C. (1970) Biochem. Genetics 4, 367-376
2 Maio, J. J. (1976) Handbook of Biochemistry, Nucleic Acids, Volume II. The Chemical Rubber Co., Cleveland
3 Gall, J. G. and Atherton, D. D. (1974) J. Mol. Biol. 85, 633-664
4 Skinner, D. M., Beattie, W. G., Blattner, F. R., Stark, B. P. and Dahlberg, J. E. (1974) Biochemistry 13, 3930-3937
5 Biro, P. A., Carr-Brown, A., Southern, E. M., and Walker, P. M. B. (1975) J. Mol. Biol. 94, 71-86
6 Walker, P. M. B., Flamm, W. G. and McLaren, A. (1969) Handbook of Molecular Cytology 15, 52-66
7 Walker, P. M. B. (1971) Progr. Biophys. Mol. Biol. 23, 145-190
8 Cooke, H. (1976) Nature (London) 262, 182-186
9 Kunkel, L. M., Smith, K. D. and Boyer, S. H. (1976) Science 191, 1189-1190
10 Tartof, K. (1975) Ann. Revs. Genetics 9, 355-385
11 Corneo, G., Ginelli, E., Soave, C. and Bernardi, G. (1968) Biochemistry 7, 4373-4379
12 Skinner, D. M. and Beattie, W. G. (1974) Biochemistry 13, 3922-3929
13 Southern, E. (1970) Nature (London) 227, 794-798
14 Chambers, C. A. and Skinner, D. M. (1975) J. Cell Biol. 67, 59a
15 Flamm, W. G., Walker, P. M. B. and McCallum, M. (1969) J. Mol. Biol. 42, 441-455
16 Chambers, C. A. and Skinner, D. M. (1976) J. Cell Biol. 70, 83a
17 Britten, R. J., Pavich, M. and Smith, J. (1970) Carnegie Inst. Washington Yearb. 68, 400-402
18 Corneo, G., Ginelli, E. and Polli, E. (1967) J. Mol. Biol. 23, 619-622
19 Richardson, C. C. (1966) J. Mol. Biol. 15, 49-61
20 Commerford, S. L. (1971) Biochemistry 10, 1993-1999
21 Orosz, J. M. and Wetmur, J. G. (1974) Biochemistry 13, 5467-5473
22 Brunk, C. F. and Leick, V. (1969) Biochim. Biophys. Acta 179, 136-144
23 Vinograd, J., Bruner, R., Kent, R. and Weigle, J. (1963) Proc. Natl. Acad. Sci. USA 49, 902-910
24 Burgi, E. and Hershey, A. D. (1963) Biophys. J. 3, 309-321
25 Markham, A. (1967) in Methods in Virology 2, 1-39, Academic Press, New York
Nucleic Acids Research

26 Ando, T. (1966) Biochim. Biophys. Acta 114, 158-168
27 Miyazawa, Y. and Thomas, C. A. (1965) J. Mol. Biol. 11, 223-237
28 Britten, R. J. and Kohne, D. (1968) Science 161, 529-540
29 Skinner, D. M. (1967) Proc. Natl. Acad. Sci. USA 58, 103-110
30 Gray, D. and Skinner, D. M. (1974) Biopolymers 13, 843-852
31 Szybaliski, W. (1968) Methods Enzymol. 12B, 330-360
32 Marvin, D. A. and Horn, B. (1969) Bact. Revs. 33, 172-209
33 Sander, C. and Ts'o, P. O. P. (1971) J. Mol. Biol. 55, 1-21
34 Perlgut, L. E., Byers, D. L., Jope, R. S. and Khamvinwathna, V. (1975) Nature (London) 254, 86-87
35 Vogt, V. M. (1973) Eur. J. Biochem. 33, 192-200
36 Studier, F. W. (1965) J. Mol. Biol. 11, 373-390
37 Yasmineh, W. G. and Yunis, J. J. (1974) Expt'l Cell Res. 88, 340-344
38 Crothers, D. personal communication
39 Guha, A. and Szybaliski, W. (1968) Virology 34, 608-616
40 Opara-Kubinska, Z., Kubinski, H. and Szybaliski, W. (1964) Proc. Natl. Acad. Sci. USA 52, 923-930
41 Kubinski, H., Opara-Kubinska, Z. and Szybaliski, W. (1966) J. Mol. Biol. 20, 313-329
42 Britten, R. J., Graham, D. E., Eden, F. C., Painchaud, D. M. and Davidson, E. H. In press
43 Freifelder, D. and Trumbo, B. (1969) Biopolymers 7, 681-693
44 DeClercq, E., Torrence, P. F. and Witkop, B. (1974) Proc. Natl. Acad. Sci. USA 71, 182-186
45 Felsenfeld, G. and Rich, A. (1957) Biochim. Biophys. Acta 26, 457-468
46 Riley, M., Maling, B. and Chamberlin, M. J. (1966) J. Mol. Biol. 20, 359-389
47 Lipsett, M. N. (1963) Biochem. Biophys. Res. Commun. 11, 224-228
48 Lipsett, M. N. (1964) J. Biol. Chem. 239, 1256-1260
49 Felsenfeld, G. and Miles, H. M. (1967) Annu. Rev. Biochem. 36, 407-448
50 Hoffman, L. M. and Collins, J. M. (1976) Nature (London) 260, 642-643
51 Swift, H. personal communication
52 Torrence, P. F., DeClercq, E. and Witkop, B. (1976) Biochemistry 15, 724-734

62