Reduced-stringency DNA reassociation: sequence specific duplex formation

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ABSTRACT

Reduced-stringency DNA reassociation conditions allow low stability duplexes to be detected in prokaryotic, plant, fish, avian, mammalian, and primate genomes. Highly diverged families of sequences can be detected in avian, mouse, and human unique sequence DNAs. Such a family has been described among twelve species of birds; based on species specific melting profiles and fractionation of sequences belonging to this family, it was concluded that permissive reassociation conditions did not artifactually produce low stability structures (1).

We report S_1 nuclease and optical melting experiments, and further fractionation of the diverged family to confirm sequence specific DNA reassociation at 50° in 0.5M phosphate buffer.

INTRODUCTION

In recent years a number of groups have used reduced-stringency DNA reassociation conditions. The most permissive conditions reported were 53° in 1M PB (2); the authors concluded that these conditions were too relaxed. Other laboratories have used conditions ranging from 55° in 0.12M PB to 50° in 0.5M PB (1,3-12). Except for the first and last two reports cited, no effects were noted which were sufficiently unusual as to warrant further characterization of duplexes or study of reassociation criteria.

The criteria-dependent, low-melting components present in the unique sequence mouse, human, and avian genomes are highly diverged families. It was not anticipated from earlier work that such families existed. Ivanov and Markov have characterized the mouse family in terms of S_1 digestion rate and extent, optical melting experiments, and they were able to obtain a partial fractionation of these sequences (9,10). The reassociation criteria were not so permissive, 50° in 0.14M PB, as to raise serious doubts about the results. Somewhat more relaxed conditions were used to demonstrate another family in human unique sequence DNA (50° in 0.24M PB) (11,12). Again, S_1 nuclease experiments were used. Formation of the low-melting

component was sequence specific and a partial fractionation was achieved; formation was also criteria-dependent.

Our conditions, 50° in 0.5M PB are more permissive. In this paper we report additional experiments validating reduced-stringency conditions in DNA reassociation studies. We have used optical melting profiles and S₁ nuclease sensitivity experiments to demonstrate that low-melting DNA duplexes formed in such permissive reactions are sequence specific and are not the result of interactions in which any DNA fragment may participate.

MATERIALS and METHODS

Details regarding DNA sources, purification, shearing, unique sequence chicken DNA isolation, and thermal elution of DNA from HAP columns have been described (1). Some details will be repeated.

DNA Fragment Lengths: DNA fragments eluting from a HAP column were concentrated as follows. Fractions were made 1M NaCl and allowed to flow by gravity through individual Millipore 0.45 μ filters; DNA was quantitatively recovered by briefly agitating (5 min.) the filters in 0.1ml of 30mM NaOH, 2mM EDTA. Electrophoresis was performed in a 1.5% alkaline agarose gel (13) calibrated with an Hae III enzyme digest of pBR322. Markers and DNA were located by scanning a negative photograph of the ethidium bromide stained gel.

Melting DNA Duplexes in Temperature Gradient Block: An aluminum block (14) was used to melt DNA duplexes in solution prior to assaying single stranded DNA on HAP. Thermostated pumps set at 38° and 110° circulated an ethylene glycol-water mixture through the heat exchangers producing a linear temperature gradient (48°-98°) across 25 holes drilled to accept 1.5ml microfuge tubes. Paraffin oil was added to the holes to promote heat conduction to the tubes. Reassociated DNA was diluted to 0.12M PB and 0.5ml aliquots were added to each tube; the tubes were capped and allowed to equilibrate for 20 min. Another set of tubes containing 0.1g HAP in 0.5ml of 0.12M PB was equilibrated at 50°. The samples were transferred to the HAP tubes and incubated at 50° for 15 min., with frequent mixing. The HAP was pelleted and the supernatant optical density was determined at 320nm and 260nm, subtracting the 320nm reading to correct for any HAP fines.

S₁ Nuclease Digestion: The nuclease was purified from Sigma α -amylase (No. 6630) (15). Digestions were performed in 0.36M NaCl, 45mM NaOAc, 0.18mM ZnSO₄, pH 4.4 at room temperature in DNA substrate excess (20ug/ml). Reassociated DNA samples were bound to HAP at 50°, washed with 0.12M PB, and recovered with 0.4M PB to remove single stranded DNA before digestion. In

one series of experiments, digestion rate was followed by observing hyperchromicity at 260nm. A second series measured the rate by increase in TCA solubility of chicken DNA radioactive tracer included in the reassociation; the tracer was labeled with tritium by nick translation (16), and the average single stranded fragment was the same as the driver DNA, 400-600 bases.

Optical Melting Profiles: All optical melting experiments were performed in 0.12M PB. A Gilford 2400-2 spectrophotometer was used and an $0.5^{\circ}/\text{min}$. linear heating rate was controlled by a Gilford 2527 thermal programmer; the data were digitally recorded on magnetic tape. Subtraction of the buffer blank, curve smoothing, computation of the first derivative, and curve plotting control were done in the Carnegie Institution of Washington, Dept. Plant Biology, Hewlett-Packard 1000F computing system. The quantity of DNA melted in each experiment was 4-12 μg in 0.3ml. The curves have been corrected to a constant weight of DNA; thus, the total area under the derivative curves represents the hyperchromicity that would be seen for a fixed amount of DNA reassociated at the various criteria.

Phosphate Buffer Gradient Elution of Duplexes From HAP: Reassociated DNA duplexes were fractionated on the basis of HAP-duplex interaction using a HAP column at 50° and a linear phosphate buffer gradient, 0.12M to 0.33M. DNA was reassociated and loaded onto a HAP column at 50° in 0.12M PB; unbound DNA was washed off with the same buffer. A linear, 50° gradient was pumped over the column and 5ml fractions were collected.

RESULTS

Length of DNA Fragments in Duplexes of Different Thermal Stability: The DNAs used in this and our previous study (1) were randomly sheared using a French pressure cell at 20,000 psi. The fragments had a mass average length of 400-600 base pairs; the distribution was wide and the number average length would be considerably smaller. One explanation for the low-melting component observed in reduced-stringency total and unique sequence chicken DNA duplexes is that rather than reflecting sequence mismatch the decreased thermal stability is due to reassociation of very short but well-paired fragments.

A typical HAP thermal elution profile of chicken unique sequence duplexes formed at 50° in 0.5M PB is shown in Figure 1a. DNA eluting from the column in three temperature ranges, 50° - 65° , 65° - 80° , 80° - 96° , was electrophoresed in an alkaline agarose gel to determine the mass average fragment length and distribution width of fragments in duplexes of different thermal stability. The results are shown in Figure 1b. There were no

Figure 1. Length of fragments in duplexes of various thermal stabilities. (a) Integral HAP thermal elution profile of chicken unique sequence DNA duplexes formed at 50° in 0.5M PB. (b) Single stranded fragment length of fragments eluting from HAP in three temperature ranges; see text and Materials and Methods; A, before reassociation; B, 50° - 65° ; C, 65° - 80° ; D, 80° - 96° .

differences in lengths or distribution widths either among the fractions, or between the fractions and the DNA before reassociation. Thus, the low-melting component is not enriched in shorter fragments and is not an artifact associated with fragment length.

Hydroxylapatite and Thermal Stability Profiles: Studies have shown that DNA duplexes can be washed from HAP without strand separation occurring (17,18). This depends on the phosphate buffer concentration used for washing and the temperature of the column. As duplex sequence mismatch increases, the choice of buffer concentration becomes critical. It is possible that the low stability component seen in Figure 1a eluted as duplexes rather than as single strands. To exclude this possibility, we melted DNA duplexes in solution prior to assaying for single stranded DNA.

Total and unique sequence chicken DNAs were reassociated at standard and reduced-stringency criteria, 60° in 0.12M PB (Cot 3000), and 50° in 0.5M PB (Equiv. Cot 20,000), respectively. A linear temperature gradient was established across a series of tubes into which aliquots of the reassociated DNA were added; see Materials and Methods. The samples were then assayed for single stranded DNA using HAP at 50° in 0.12M PB. This technique does not involve the unknown factors associated with helix unwinding on a HAP crystal surface, and the problems mentioned above. The results are shown in

Figure 2. For duplexes initially formed at standard conditions, no single stranded DNA is produced until 70° . However, considerable amounts of single stranded DNA were produced at lower temperatures when duplexes formed at low criteria were tested. The shape of these curves is very similar to that of the HAP thermal elution profile in Figure 1a. Since the HAP profile can be reproduced by melting duplexes in solution, the lower $T_{e1/2}$ of the less stable component is probably more a reflection of sequence mismatch than of artifacts associated with elution from HAP.

Another aspect of HAP performance is illustrated in Figure 5b. Optical melting profiles of total chicken DNA reassociated at reduced-stringency and single stranded DNA are shown. The significance of these profiles will be discussed later; however it appears that some low stability duplexes are not quantitatively bound to HAP at 50° in 0.5M PB.

S_1 Nuclease Sensitivity of Duplexes Formed At Standard and Reduced-Stringency Criteria: If the $T_{e1/2}$ of the low-melting component in Figure 1a reflects sequence mismatch, one would expect to see different rates of S_1 nuclease digestion when comparing duplexes formed at standard and reduced-stringency conditions, particularly if the rates are estimated in substrate excess. Figure 3 shows the results obtained by following hyperchromicity as base

Figure 2. HAP binding of DNA after heating aliquots of standard and reduced stringency duplexes in 0.12M PB in temperature gradient block. Standard condition, total chicken DNA $\blacktriangle$; same DNA reassociated at 50° in 0.12M PB $\bullet$; unique sequence chicken DNA reassociated at 50° in 0.5M PB $\circ$. The straight line is the temperature gradient.

Figure 3. Hyperchromicity observed during S_1 nuclease digestion of single stranded chicken DNA and duplexes formed at standard and reduced-stringency conditions. See Materials and Methods. Single stranded DNA substrate ▲; HAP bound standard ○, and reduced-stringency duplex ● substrates.

stacking interactions are disrupted, and Figure 4 shows rates and extents of digestion when acid precipitability of reassociated tracer DNA is followed. Completely single stranded DNA was removed from the reassociation reactions before enzyme digestion to insure that the rates would not be artifactually accelerated. Hyperchromicity of duplexes formed at reduced-stringency increased at about twice the rate observed for standard condition duplexes; the rate is well below that of single stranded DNA. Essentially the same results were obtained by measuring acid solubility in the early stages of the digestion (Figure 4a). After two hours of digestion, 20%, 52%, and 80% of standard condition duplexes, reduced-stringency duplexes, and single stranded DNA was acid soluble. During extended digestion at room temperature, the rates were similar although the final extents of digestion were different; 40% digestion was obtained for standard condition duplexes and 68% for reduced-stringency duplexes (Figure 4b).

S_1 nuclease recognizes at least two different substrates among reduced-stringency duplexes. One substrate is digested at a rate similar to that of well-paired duplexes. In early stages of the digestion another substrate structure can be identified as being nuclease sensitive, yet considerably more resistant than single stranded DNA.

Optical Melting Profiles: As another measure of specific base-pairing in the low-melting component of chicken DNA duplexes, we have estimated helical content, i.e. base stacking, by comparing optical melting profiles (Figure 5).

Figure 4. S₁ nuclease sensitivity measured by acid solubility. (a) expanded scale presentation of early time points in panel (b); (b) S₁ digestion for extended time. Double stranded DNA ▲; single stranded DNA △; standard condition duplexes ○; reduced-stringency duplexes ●.

Figure 5a shows the effect of reassociation criteria on total chicken genome DNA duplex formation; single stranded DNA was not removed from the samples before melting in 0.12M PB. Two effects are clear. There is a reduction in the fraction of nucleotides in well-paired duplexes. The second feature is a distinct peak of hyperchromicity between 45° and 55°.

Figure 5b shows the effect of prior HAP binding on optical profiles. The main difference between these two profiles is between 35° and 55°. Prior HAP binding selects against single strands and poorly matched duplexes.

Figure 5c is a comparison of HAP bound, reduced-stringency duplexes formed in total genome and unique sequence chicken DNA. The prominent peak between 45° and 60° in unique sequence DNA suggests an enrichment of such sequences in this fraction of the genome.

Figure 5. Optical melting profiles. (a) Effect of reassociation criteria on duplexes formed with total chicken DNA; the reactions were not bound to HAP before melting; (---), 60°-0.12M PB; (—), 50°-0.5M PB. (b) Effect of HAP binding on melting profile of whole genome DNA reassociated at 50° in 0.5M PB; (---), total reaction mix; (—), HAP bound DNA; (···), single stranded DNA. (c) HAP bound total (---) and unique sequence (—) DNA duplexes reassociated at reduced-stringency. (d) Effect of pre-melting HAP bound unique sequence duplexes formed at 50° in 0.5M PB; duplexes were heated from 25° to 65° at 0.5°/min. and held at 65° for 5 min.; the sample was then cooled to 25° and held for 10 min. before carrying out a normal melt; (---), no pre-melt; (—), pre-melted to 65°.

To confirm that the low-melting component was indeed characteristic of a mismatched set of duplexes, not just a dissociation bubble in well-matched duplexes, a sample of HAP bound, reduced-stringency, unique sequence duplexes was pre-melted to 65°, cooled to 25°, then melted as usual. If the peak were caused by a bubble, the interactions should reform upon cooling since the strands would still be held in register by the more stable interactions. If a duplex subset were dissociating, the peak should not appear in the second melt. The results are shown in Figure 5d. The only profile change was

between 45° and 60° ; the peak was absent and there was a 30% loss of hyperchromicity in this temperature range. Thus, the low-melting hyperchromicity represents irreversible strand separation of a mismatched class of DNA duplexes. The melting temperature range is very similar to that observed for the less stable component in HAP column thermal elution profiles Figure 1a and (1).

Thermal Stability of Duplexes Having Different HAP-Duplex Interactions: To confirm that reduced-stringency criteria produce specific classes of mismatched duplexes, duplexes were fractionated on the basis of strength of adsorption to HAP. Reduced-stringency, unique sequence duplexes were loaded onto a 50° HAP column and unbound DNA was washed off with 0.12M PB. A linear PB gradient was pumped over the column (see Materials and Methods). The elution pattern is shown in Figure 6a. Again, to exclude fragment length

Figure 6. Buffer gradient elution of reduced-stringency, unique sequence duplexes (a), and optical melting profiles of duplexes eluting in fractions 3,4,5,6,8, and 13 (b); curves A,B,C,D,E,F, respectively.

artifacts, we determined the single stranded fragment length of DNA in gradient fractions 2,4,5,6,8, and 13 as described earlier. Average fragment lengths were the same in all fractions.

Optical melting profiles were obtained for several of the gradient fractions and are shown in Figure 6b. Duplexes eluting at low buffer concentration contain very little high-melting helix and are predominantly composed of low stability structures while duplexes eluting at higher concentration are almost exclusively stabilized by well-paired sequences. These results indicate that, in terms of HAP-duplex interaction, the low-melting duplexes are a physically separable class, distinct from stable duplexes.

Fractionation of DNA Sequences Forming Low Stability Duplexes:

Reduced-stringency duplexes eluted from HAP at high and low buffer concentrations (fractions 3 and 10 of Fig. 6a) were the starting material for these experiments. Both fractions were concentrated, adjusted to 0.5M PB denatured, and reassociated to Equiv. Cot 5000. The reactions were diluted to 0.12M PB at 50°, loaded onto a HAP column, and melted as usual; 70% and 90% of fractions 3 and 10, respectively, were HAP-bound after the second reassociation. The results are shown in Figure 7. DNA which originally formed duplexes eluting from HAP at low buffer concentration contained

Figure 7. HAP thermal elution profiles of duplexes formed at reduced stringency using DNA recovered from HAP at low or high PB concentration. Samples were obtained from the gradient shown in Fig. 6a; DNA from fraction 3 ●; from fraction 10 ○.

sequences capable of forming duplexes covering a wide range of stability during a second reassociation. The profile for this fraction is biphasic and reminiscent of the curve shown in Figure 1a. In contrast, only high melting duplexes were formed among sequences in the DNA originally eluted at higher buffer concentration. Thus, it appears that fractionation of reduced stringency duplexes according to HAP-duplex interaction has also achieved a fractionation of DNA sequence content.

DISCUSSION

On the basis of (a) reassociation-dependent melting profiles, (b) criteria-dependent optical and HAP melting profiles, (c) S_1 rate and extent studies, (d) optical melting experiments, (e) fractionation of DNA sequences forming low-melting duplexes, (f) irreversible hyperchromicity in temperature range in which the mismatched family elutes from HAP, we conclude that permissive reassociation conditions (50° in 0.5M PB) are within the limits for sequence specific reassociation suggested by other studies (19,20,21). Briefly, these systematic studies indicate that the minimum information content required to form species specific duplexes is contained in DNA fragments of about 15 bases long, and that interspecies sequence homologies can be detected using fragments about 40 nucleotides long; also reassociation criteria of 40° - 50° in 1xSSC preserve species specific reassociation. A lower limit for reassociation criteria cannot be set which will apply to all situations.

We have excluded certain sources of artifact related to reduced stringency reassociation and the biphasic HAP thermal elution profiles (Fig. 1a). Protein and metal ion contamination of DNA, and random fragment association stabilized by G+C-rich sequences are not involved in formation of the low stability component (1). The effect of base-paired fragment length on thermal stability has been extensively studied (7,22-26); thermal stability declines rapidly for precisely paired sequences of about 50 base pairs and fewer. The results shown in Figure 1b demonstrate that reduced stringency conditions do not favor duplex formation between short single strands; thus, the low-melting duplexes cannot be attributed to fragment length artifacts.

In some instances, well-paired and mismatched duplexes may be washed from HAP during a thermal elution experiment before strand separation occurs (17,18). We have eliminated HAP-duplex interaction as a factor in determining profile shape by heating aliquots of reduced-stringency duplexes at increasing

temperatures in solution before assaying for single stranded DNA, simulating a thermal dissociation experiment in solution. The shape of these profiles is essentially the same as those obtained in HAP column experiments. Binding of mismatched duplexes, however, does not appear to be quantitative. The optical melting profiles in Figure 5b show that low-stability helix containing structures (45° - 55°) are not all bound to HAP at 50° in 0.5M PB. This could lead to an underestimate of the number of DNA fragments involved in low-stability duplexes in a HAP thermal elution experiment.

Structural Organization in Criteria-Dependent, Low-Melting Duplexes: To demonstrate ordered structure in criteria-dependent duplexes, and to bolster the argument for specific, although extensively mismatched, base pairing, two methods were used. S_1 nuclease was used to estimate nuclease sensitive target size, and optical melting profiles were used to demonstrate low stability helix-containing structures.

S_1 nuclease is useful for straightforward estimation of single and double stranded DNAs. Unfortunately, there is a "stringency effect" in the conditions used for digestion (27). To minimize this difficulty, we selected an enzyme:substrate ratio such that the rate of single stranded DNA digestion would be linear for at least four hours, (Fig. 3). Whether TCA solubility or hyperchromicity was measured, the results were similar for the early phase of the digestion. A class of nuclease-sensitive structures is present in reduced-stringency duplexes that is absent among standard condition duplexes, (Figs. 3,4a). When this class has been rendered TCA soluble, the digestion rates for standard and reduced-stringency duplexes are similar, indicating similar substrate structure, (Fig. 4b). Two other laboratories have investigated the S_1 sensitivity of criteria-dependent duplexes (10,12). In both cases more stringent digestion conditions were used, but it was clear from rate and extent of digestion that mouse and human duplexes contain a substrate intermediate in structure between single stranded DNA and well-paired duplexes.

Optical melting experiments are a more powerful means of identifying specific base pairing. Theoretical and experimental studies are in accord and have demonstrated a causal relationship between helical structure and optical activity (28-32). The optical melting curves demonstrate criteria dependent low-melting helical structures (Fig. 5). Similar results have been reported using mouse duplexes (9).

Sequences Forming Low-Stability Duplexes At Reduced-Stringency Are A Specific Subset of the Chicken Unique Sequence Genome: We regard the ability to

fractionate DNA sequences into sets which will and will not form low-melting duplexes as good evidence that reduced-stringency conditions do not stabilize random DNA strand associations, and that the low-melting component of chicken DNA is not a criteria-dependent artifact. Other laboratories have reported that HAP affinity for mismatched duplexes varies as a function of mismatch (18,33,34); the results shown in Figure 6b confirm these reports.

This does not demonstrate different reacting sequence sets, only different HAP-duplex interactions. If the duplexes eluting at higher buffer molarity were a subset of sequences, a melting profile after denaturation and a second reduced-stringency reassociation of the sequences should show no low-melting component. If the conditions artifactually produce low-stability structures, or if these duplexes are not a subset, the low-melting component should be observed. In the case of duplexes eluting at lower buffer concentration, we would expect both the high and low-melting components to form, after denaturation, during a second reassociation. The results shown in Figure 7 are unambiguous; sequences eluting from HAP at high buffer concentration in the first cycle of reassociation are depleted in members of the highly mismatched family. A brief examination of Figure 7 might give the impression that all members of the mismatched family have been removed; this, however, is probably not the case. A sequence member of the mismatched family also has an exact complement in the genome and can form high stability duplexes as well. The rate of mismatched sequence reassociation can be significantly slower than that of exactly complementary sequences (35); the diverged chicken family is about 30% mismatched, and would be expected to reassociate at a rate approximately one-eighth that of precisely paired sequences (1). The fact that the mismatched family can be observed indicates that the size of the family is sufficiently large as to overcome the kinetic disadvantage. Any procedure which changes the balance between family size and reassociation rate will also change the amount of low-melting duplex observed. The most likely explanation for complete absence of low-melting duplexes in the high buffer eluting sample in Figure 7 is that the family size of the mismatched family has been reduced, by the buffer gradient fractionation, to the point where the kinetic factor favors good duplex formation.

Our earlier report demonstrated that fractionation of the chicken genome into repetitive and unique sequence parts achieved fractionation of the low stability component into the unique sequence part (1). It also appears to be a distinct subset among 400-600 base long single copy DNA fragments. Partial

fractionation of highly mismatched mouse and human duplexes has been reported (9-12).

Summary: Standard condition DNA reassociation work has made many contributions to understanding genome organization and evolution. Reduced stringency conditions have not been widely used because of uncertainty about the lower limit for sequence pairing. We feel that reassociation at 50° in 0.5M PB preserves sequence specific reactions and should prove useful in evolutionary studies requiring detection of distantly related DNA sequences.

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Abbreviations: PB, phosphate buffer made of equimolar mono and dibasic sodium phosphate; HAP, hydroxylapatite; $T_{1/2}$, temperature at which half the DNA has eluted from HAP; Equiv. Cot, molar concentration of DNA nucleotides times the incubation time in seconds, corrected for buffer concentration effect on rate; 1xSSC, 0.15M sodium chloride-0.015M sodium citrate.

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