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Hyperpolymer formation during renaturation of DNA from genomes with different sequence organisation

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ABSTRACT

Hyperpolymer formation during the renaturation of DNAs from wheat, calf and E. coli was studied using hydroxyapatite chromatography, electron microscopy and S1 nuclease. Large hyperpolymers could not be eluted from hydroxyapatite with 0.5 M phosphate buffer at 60°C. Large proportions of wheat and E. coli DNAs were incorporated into hyperpolymers when fragments 650 nucleotides long were renatured. A much smaller proportion of calf DNA was incorporated under equivalent conditions. Greater proportions of calf DNA accumulated in hyperpolymers only when longer fragments were incubated. Electron microscopy indicated no obvious differences in the basic structures of hyperpolymers formed by the three DNAs and confirmed the quantitative differences in hyperpolymer formation found by hydroxyapatite chromatography. It is concluded that the proportions and arrangement of the repeated sequences in the chromosomes of higher organisms determine the extent of rapid hyperpolymer formation during DNA renaturation in vitro.

INTRODUCTION

During the last few years we have studied the renaturation characteristics of DNA from many higher plant species, with special emphasis on cereal DNAs (1, 2). Frequently, we noticed that the recovery of renatured plant DNA from hydroxyapatite columns was incomplete when using high molarity phosphate to elute renatured DNA (3). Renatured DNA unable to be eluted from hydroxyapatite with phosphate buffer is in the form of aggregates or hyperpolymers. These hyperpolymers form because the incubated DNA fragments are randomly sheared and the complementary strands that hybridise during incubation do not terminate at the same site. This leaves single stranded ends to the duplexes, which can hybridise with other complementary single stranded fragments or the tails of other duplexes to form multifragment aggregates (4, 5). When incubation is prolonged very large aggregates or hyperpolymers build up.

Hyperpolymer formation during the renaturation of DNA from a number of prokaryotic and eukaryotic species has been reported (4, 5) but in these

studies fragments between 1200 and 30,000 nucleotides long were necessary to detect hyperpolymer formation under the incubation conditions commonly used in DNA renaturation studies. Significant proportions of many plant DNAs formed hyperpolymers in our experiments when much smaller fragments were incubated under similar conditions. Thompson has recently described a similar finding for pea and other plant DNAs (6).

These observations prompted us to examine the appearance of hyperpolymers in the electron microscope and to study the kinetics of their formation during the renaturation of DNAs from wheat, calf and E. coli. These three species were chosen since they were known to have different proportions of repeated and non-repeated sequences in their genomes (7, 8).

The kinetics of hyperpolymer formation are distinctive for each of these representative DNAs. It is concluded that they reflect the organisation of the rapidly renaturing sequences in the chromosomes.

MATERIALS AND METHODS

Isolation and characterisation of DNA

Wheat DNA (Triticum aestivum, var. Chinese Spring) was isolated from green leaves as described previously (9). Calf and E. coli* DNAs were purchased from the Sigma Chemical Company. DNA in ice cold 0.12 M PB* was sheared to the required average fragment sizes by sonication (8). The average single stranded sizes of such fragments were determined by boundary velocity sedimentation in 0.9 M NaCl 0.1 M NaOH as described by Studier (10). For renaturation studies, DNA at various concentrations in 0.12 M PB was denatured by heating to 100°C for five minutes and immediately incubated at 60°C to the required C_0t*. After incubation, the samples were diluted with 0.12 M PB at 60°C and applied to HAP* columns (Bio Rad HTP) previously equilibrated with the same buffer and maintained at 60°C. Unrenatured fragments were eluted from the columns with further washes of 0.12 M PB. Renatured DNA was removed by elution first with 0.5 M PB at 60°C and then with 0.5 M PB at 95°C (see Results) (3). DNA concentrations were estimated by OD_{260} measurements after low speed centrifugation to sediment any HAP. Corrections were made for absorbance found in eluates from columns not loaded with DNA.

S_1 nuclease digestion of DNA

S_1 nuclease was prepared from Takadiastase (Koch-Light Co.) using the method of Sutton (11) and used exactly as described previously (12).

Electron microscopy

The Kleinschmidt spreading technique (13) modified by Davis et al (14) was used to prepare DNA for electron microscopy. Renatured DNA in 0.12 M PB was diluted to 2 µg/ml with 1 M NaCl, 10mM Tris, 5mM EDTA pH 7.8 and a hyperphase solution prepared which contained 50% v/v formamide, 0.2 M Tris, 3mM EDTA, 0.4 M NaCl pH 8.2, 0.5 µg/ml DNA and 50 µg/ml cytochrome C. The hypophase contained 20% v/v formamide in 10mM Tris, 1mM EDTA pH 8.5. Samples were picked up on parlodion coated grids, stained with 50 µM uranyl acetate in 95% ethanol and shadowed with 80% platinum 20% iridium at an angle of 6-10°.

RESULTS

Recovery of renatured wheat DNA from hydroxyapatite

Our initial experiments investigated the effect of fragment size on the recovery of renatured wheat DNA from hydroxyapatite columns. Aliquots of wheat DNA, of different average single strand lengths, were renatured to C_0t 90 in 0.12 M PB. At this C_0t essentially all fragments carrying repeated sequences bind to HAP equilibrated with 0.12 M PB at 60°C (8). The proportions of the bound sequences that could be eluted with 0.5 M PB at 60°C and the proportions that could be eluted only after melting the DNA duplexes at 95°C were estimated. The results, illustrated in figure 1, show that the recovery of renatured wheat DNA from HAP by the commonly used 0.5 M PB (see for example refs. 15-17), is very dependent upon DNA fragment size; with fragments of mean length greater than 1000 nucleotides the recovery was less than half of the DNA applied to the column. However, all the DNA could be recovered after melting the renatured DNA. Subsequent studies revealed that the renatured DNA that could not be recovered from HAP at 60°C was in the form of large aggregates or hyperpolymers which could also be recognised by their rapid sedimentation at relatively low g forces. Two assays which give quantitatively similar results were standardised for these aggregates: (A) the amount of DNA containing renatured regions that cannot be eluted from HAP with 0.5 M PB at 60°C but which is eluted after raising the temperature to 95°C and (B) the amount of DNA that sediments into a pellet in 10 min at 30,000 xg in 1 M NaCl.

Method A was used to obtain the results reported here. Because relatively long double stranded molecules of native DNA can be eluted from HAP with 0.5 M PB, this assay probably measures the formation of branched hyperpolymer networks rather than linear hyperpolymers.

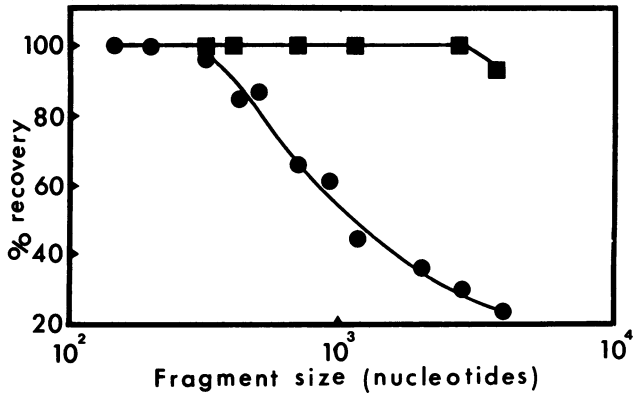

Figure 1. The effect of fragment size upon recovery of renatured wheat DNA from HAP with 0.5 M PB at 60°C and 95°C.

Wheat DNA at 300 µg/ml in 0.12 M PB was sheared to various single stranded fragment sizes. After denaturation samples were incubated to C_0t 90 at 60°C and then diluted to 2 mls with 0.12 M PB at 60°C and applied to HAP columns. After washing off unrenatured DNA with 0.12 M PB at 60°C, duplexes were removed with 0.5 M PB at 60°C or 95°C.

- ● % recovery of DNA when duplex fraction eluted with 0.5 M PB at 60°C
- ■ % recovery of DNA when duplex fraction eluted with 0.5 M PB at 95°C

Hyperpolymer formation by wheat, calf and Escherichia coli DNAs

The kinetics of hyperpolymer formation were studied in wheat, calf and E. coli DNAs to investigate the relationships between hyperpolymer formation and sequence organisation. These three species are known to have differently organised genomes with respect to repeated and non-repeated sequences (7, 1, 18).

Native DNAs at 1.5 mg/ml were sheared to a weight average denatured fragment size of 650 nucleotides. After heat denaturation, the DNAs were separately incubated at 1.5 mg/ml and 100 µg/ml to different C_0t values at 60°C in 0.12 M PB. The proportions of renatured DNA and DNA in hyperpolymers were determined by HAP chromatography and the results are shown in figure 2.

E. coli DNA fragments renatured with second order kinetics with a $C_0t_{\frac{1}{2}}$ = 2.0. Wheat DNA fragments renatured as two major fractions. Those containing repeated sequences (>80% of the genome) renatured by a C_0t of 100, with a $C_0t_{\frac{1}{2}}$ of 0.7 and the overall renaturation curve was not second order. Fragments containing only non-repeated or few copy sequences (<20%

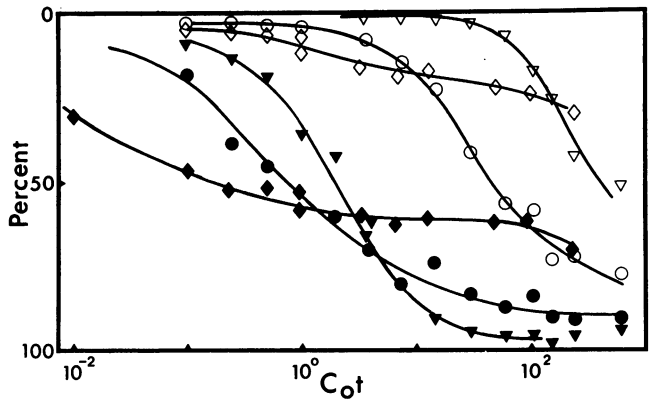

Figure 2. Kinetics of renaturation and hyperpolymer formation of wheat, calf and E. coli DNAs of the same average fragment size.

DNAs at 100 µg/ml and at 1.5 mg/ml in 0.12 M PB were sheared to a weight average single stranded fragment size of 650 nucleotides. Aliquots were melted, incubated at 60°C for different times and fractionated on HAP. Closed symbols are the percentage of total DNA retained on HAP in 0.12 M PB at 60°C. (renatured DNA). Open symbols are the percentage of the DNA in hyperpolymers assayed by their inability to be eluted from HAP at 60°C by 0.5 M PB.

● ○ Wheat ◆ ◇ Calf ▼ ▽ E. coli

of genome) renature much more slowly (8). The repeated sequences of calf DNA renatured on average more rapidly than those of wheat and almost 60% of the genome was in the HAP duplex fraction by C_0t 1, before non-repeated sequences begin to renature. All these results are consistent with other studies (7, 8).

The kinetics of hyperpolymer formation with these DNAs, assayed by hydroxyapatite binding (see figure 2), were slow compared with the kinetics of initial duplex formation. Hyperpolymers of renatured wheat and E. coli DNAs did not appear until most of the wheat fragments containing repeated sequences and most of the E. coli fragments had reassociated. The kinetics of hyperpolymer formation for wheat and E. coli DNAs fit second order curves until around 50% of the renatured DNAs are incorporated into hyperpolymers. Thereafter, the rate of hyperpolymer formation declined due in part, almost certainly, to the high viscosity of hyperpolymers. More than 90% of the wheat genome can become included in hyperpolymers after prolonged incubation (see figure 3).

The kinetics of hyperpolymer formation by renatured calf DNA were

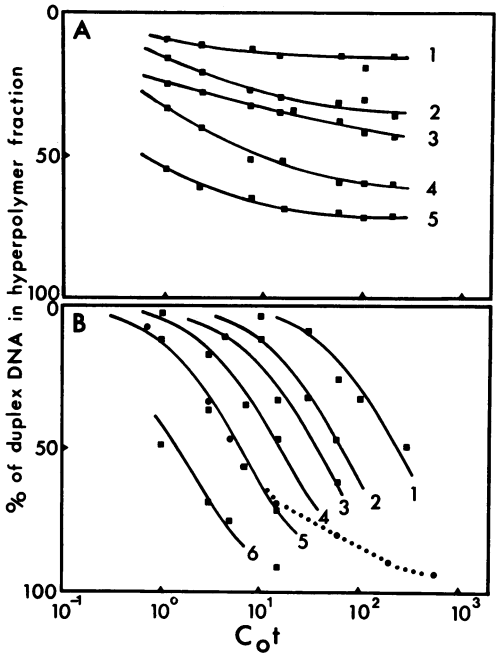

Figure 3. Hyperpolymer formation by different length fragments of calf and wheat DNA.

Aliquots of DNA with various weight average fragment lengths were denatured at 1.5 mg/ml in 0.12 M PB, incubated at 60°C for various times and chromatographed on HAP. Denatured DNA was removed with 0.12 M PB at 60°C. The renatured DNA fraction eluted with 0.5 M PB at 95°C which would not elute at 60°C (hyperpolymer fraction) was estimated in each sample and is expressed as a proportion of the total duplex DNA eluted with 0.5 M PB.

A Calf DNA. The average single strand fragment sizes for curves 1 to 5 were 230, 550, 750, 1250 and 1900 nucleotides respectively.

B Wheat DNA. The average single stranded fragment sizes for curves 1 to 6 were 350, 620, 570, 730, 1100 and 2000 nucleotides respectively. Solid lines are second order curves and the broken line represents hyperpolymer formation in a sample with an average fragment size of 1000 nucleotides after longer incubations.

rather different. About 5% of the DNA appeared in hyperpolymers rapidly (by C_0t 0.1) but the percentage increased to only 24% by C_0t 100.

The effect of fragment size on hyperpolymer formation by wheat and calf DNAs

We have already presented evidence in figure 1 that the amount of renatured wheat DNA that cannot be removed from HAP by 0.5 M PB at 60°C (i.e. hyperpolymer DNA) is very dependent upon the DNA fragment size. The effect of fragment size on the kinetics of hyperpolymer formation was studied for wheat and calf DNAs using various populations of single stranded fragments with mean lengths ranging from 230 to 2000 nucleotides. The results are shown in figure 3.

The incorporation of a large proportion of the renatured, repeated sequences of the wheat genome into hyperpolymers that accumulate with

second order-like kinetics is again evident and contrasts with the kinetics of accumulation of calf DNA hyperpolymers over the equivalent range of C_0t values. With wheat DNA the rate of hyperpolymer formation is very dependent on fragment size while with calf DNA the kinetics of hyperpolymer formation are not greatly dependent upon fragment size over the range of C_0t values studied. What is very evident in the results for calf DNA is that the proportion of the genome incorporated into hyperpolymers increases considerably with fragment size. At a C_0t of 10 with 230 nucleotide long fragments, approximately 12% of the DNA in the HAP duplex fraction was in the hyperpolymer fraction while with 1900 nucleotide long fragments 70% was in hyperpolymers. The DNA in the HAP duplex fraction was 50% of the total DNA with 230 nucleotide long fragments and 78% with 1900 nucleotide fragments, the additional 28% being mostly non-repeated or slow reannealing DNA which is interspersed with the more rapidly renaturing repeated sequences (19). Therefore, with fragments 230 nucleotides long 12 x 0.5 = 6% of the genome was incorporated into hyperpolymers and with fragments 1900 nucleotides long 70 x 0.78 = 55% of the genome was incorporated into hyperpolymers.

Hyperpolymer formation assayed by electron microscopy

DNAs from wheat, calf and E. coli were sheared to weight average single stranded fragment lengths of between 630 and 700 nucleotides. Aliquots at 1.5 mg/ml in 0.12 M PB were denatured, renatured to various C_0t values (see Table 1) and prepared for electron microscopy. These fragment lengths and renaturation conditions were essentially the same as those employed in obtaining the results shown in figure 2. The C_0t values shown in Table 1 were selected relative to the approximate $C_0t_{\frac{1}{2}}$ of the initial duplex formation of all the sequences renatured in figure 2. Several grids of each DNA preparation were prepared and our conclusions are summarised below.

Table 1. Renaturation details of DNAs examined by electron microscopy

Renaturation as a function of $C_0t_{\frac{1}{2}}$	C_0t values and renatured DNA proportions		
	Wheat	E. coli	Calf
$C_0t_{\frac{1}{2}}$ x 1	0.9 (0,53)	3 (0,60)	-
x 10	9 (15,78)	30 (2,94)	0.4 (6,54)
x 100	90 (62,87)	300 (43,96)	4 (14,60)
x 1000	-	-	40 (20,62)
x 2250	-	-	90 (23,64)

Approximate $C_0t_{\frac{1}{2}}$ values are from data in figure 2. The two numbers in parenthesis are (a) the proportion of DNA in hyperpolymers and (b) the proportion of DNA in the duplex fraction, both determined by HAP chromatography (see Materials and Methods).

Some differences were observed between samples of wheat and E. coli DNAs incubated to $C_0t_{\frac{1}{2}}$ (i.e. when approximately 50% of the DNA behaved as renatured DNA on HAP). Higher proportions of renatured fragments with three or four ends and of small multifragment (<10) aggregates with many single stranded ends were present in the wheat DNA. Typical fields are shown in figure 4A (wheat) and 4B (E. coli).

At ten times $C_0t_{\frac{1}{2}}$ small multifragment aggregates were seen in E. coli DNA. In wheat DNA a few large multifragment aggregates were also present. (Fig. 4C). At this C_0t some wheat DNA could not be eluted from HAP with 0.5 M PB at 60°C (see Table 1 and figure 2) so these large aggregates were presumably the hyperpolymers defined by our HAP assays. Most of the wheat DNA fragments, however, were still single stranded, renatured into simple duplexes or in small multifragment aggregates with fewer than ten component fragments. Calf DNA renatured to ten times $C_0t_{\frac{1}{2}}$ showed many small multifragment aggregates and a few larger aggregates but these were smaller than those seen with wheat DNA. Representative fields for E. coli and calf DNAs renatured to ten times $C_0t_{\frac{1}{2}}$ are shown in figures 4D and E respectively.

At one hundred times $C_0t_{\frac{1}{2}}$ some large aggregates were seen in E. coli DNA which were as large or larger than those observed in wheat DNA at ten times $C_0t_{\frac{1}{2}}$. Figure 4F shows four such aggregates in close proximity. It is not difficult to imagine how by hybridisation of the single stranded regions of different aggregates a very large hyperpolymer would be formed. Wheat DNA at one hundred times $C_0t_{\frac{1}{2}}$ is in the form of denatured fragments, small multifragment aggregates or extremely large hyperpolymers. A small part, about 15%, of such a hyperpolymer is shown in figure 4G.

In calf DNA renatured to one hundred times $C_0t_{\frac{1}{2}}$ simple duplexes or denatured fragments were abundant. Many small multifragment aggregates were seen and also a few large hyperpolymers but these were considerably smaller than those seen in wheat DNA at one hundred times $C_0t_{\frac{1}{2}}$. After incubation of calf DNA to one thousand times $C_0t_{\frac{1}{2}}$ and 2250 times $C_0t_{\frac{1}{2}}$, a few very large hyperpolymers were observed of similar size to the largest seen in wheat at one hundred times $C_0t_{\frac{1}{2}}$.

Although such electron microscopy studies cannot be done strictly quantitatively, these observations clearly demonstrated that (1) with increasing incubation time larger and larger aggregates formed with all three DNAs (2) the structures of the aggregates were similar with all three DNAs (3) calf DNA formed some intermediate sized aggregates rapidly but the

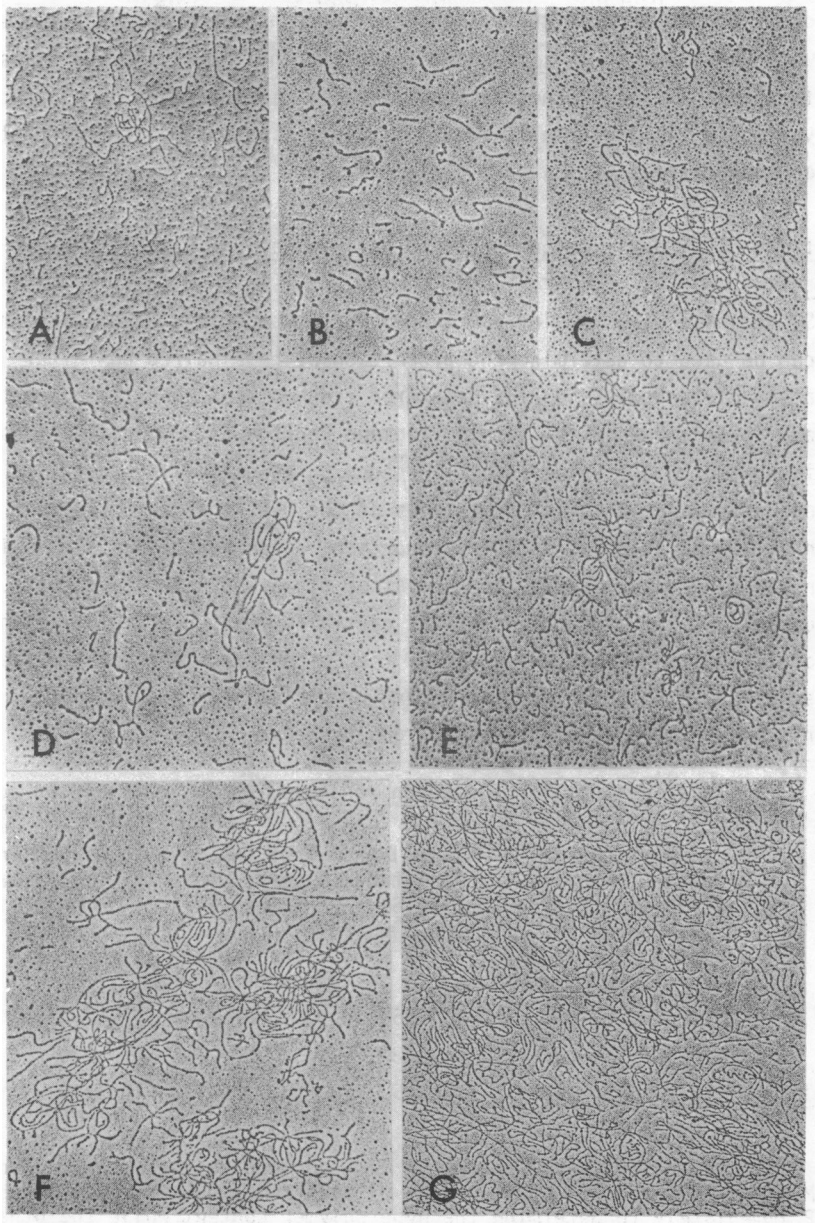

Figure 4. Electron micrographs of wheat, calf and E. coli DNAs after incubation at various C_0t values.

Details of DNA renaturation are given in Table 2. A Wheat, DNA incubated to $C_0t_{\frac{1}{2}}$; B E. coli, to $C_0t_{\frac{1}{2}}$; C Wheat, to $C_0t_{\frac{1}{2}}$ x 10; D E. coli to $C_0t_{\frac{1}{2}}$ x 10; E Calf to $C_0t_{\frac{1}{2}}$ x 10; F E. coli to $C_0t_{\frac{1}{2}}$ x 100; G Wheat, to $C_0t_{\frac{1}{2}}$ x 100, picture shows about 15% of a large hyperpolymer.

subsequent aggregation of these into very large hyperpolymers was slower than with wheat and E. coli DNAs and (4) although at $C_0t_{\frac{1}{2}}$ and ten times $C_0t_{\frac{1}{2}}$ the three DNAs could not be unequivocally distinguished by hyperpolymer observation in the electron microscope, this was possible after incubation to one hundred times $C_0t_{\frac{1}{2}}$.

The effect of S_1 nuclease on renatured calf, wheat and E. coli DNA fragments

S_1 nuclease digests single stranded but not double stranded DNA and thus can be used to determine the proportion of base paired DNA after incubation of randomly sheared, denatured fragments of DNA (11). Wheat, calf and E. coli DNAs with average fragment lengths of 1000 nucleotides were incubated to C_0t values of 75, 70 and 72 respectively as detailed in the legend to Table 2. The proportions of the DNAs that were retained on HAP in 0.12 M PB at 60°C, and the proportions which behaved as hyperpolymer DNA on HAP were determined before and after digestion with S_1 nuclease. Considerably higher proportions of the partially renatured calf fragments were digested with S_1 nuclease than of the E. coli fragments. S_1 nuclease reduced all hyperpolymers to sizes which prevented them from being recognised as hyperpolymers by HAP chromatography.

Table 2. The effect of S_1 nuclease on renatured wheat, calf and E. coli DNAs.

DNA	C_0t of renaturation	% DNA in HAP duplex fraction before S_1 nuclease treatment	% of DNA resistant to S_1 nuclease	% of DNA in hyperpolymers before S_1 nuclease treatment	% of DNA in hyperpolymers after S_1 nuclease treatment
Wheat	75	89	56	60	0
Calf	70	69	38	43	0
E. coli	72	99	85	4	0

DNAs, with average fragment lengths of 1000 nucleotides were reannealed in 0.12 M PB at 60°C and 450 µg/ml. Half of each sample was chromatographed on HAP immediately to determine the hyperpolymer content while the other half was treated with S_1 nuclease (12) before being chromatographed on HAP.

DISCUSSION

The electron micrographs in this paper demonstrate directly the formation of extremely large branched hyperpolymers when DNA fragments from

eukaryotes and prokaryotes renature in vitro. However, hyperpolymers can also be detected by their high molecular weights and their inability to chromatograph through HAP columns once they exceed a certain size. Multi-fragment DNA aggregates are formed by the renaturation of the single stranded ends of fragments already base paired with one or more other fragments (4, 5, 6). Linear multifragment molecules would be expected to form initially from single stranded fragments carrying short identical sequences tandemly arranged or from DNAs such as E. coli which consist entirely of single copy sequences. However the electron microscope pictures and our HAP assay results illustrate that branched networks soon form during renaturation of E. coli DNA fragments. As a consequence of the mode of their formation, hyperpolymers are highly branched networks of double and single stranded DNAs. The presence of single stranded regions can be inferred from the electron microscope pictures as well as the observation that hyperpolymers are degraded to low molecular weight duplexes by S_1 nuclease (Table 2).

The kinetics of the formation of branched hyperpolymers large enough to be retained on HAP at 60°C in 0.5 M PB, appeared approximately second order (figure 2). However, the process is far from being a simple second order reaction; the observed kinetics are probably the results of interactions between many factors which happen to produce approximately second order kinetics (6). Besides being dependent upon the incubation conditions (temperature, salt concentration etc.) and the incubation time, hyperpolymer formation is very dependent upon fragment length (figure 3). Although longer fragments renature more rapidly (20, 21), the major effect is more likely to be due to the effect that fragment length has on the physical dimensions of the resulting hyperpolymers. Hyperpolymers containing the same number of fragments are necessarily larger when the component fragments are longer and thus hyperpolymers with large constituent fragments probably exceed the critical size necessary for retention on HAP with fewer nucleations. Another factor related to fragment length is the length of each single stranded end of a duplex or aggregate which is available for stable nucleation with another fragment. With short fragments, this length is likely to be too small in many cases for a secondary nucleation event thus reducing the rate of accumulation of hyperpolymers. The kinetics of aggregation also depend upon the concentration of homologous, denatured sequences on fragments already partially base-paired. We wish to highlight this factor because it differs during the renaturation of different genomes.

Hyperpolymer formation and the E. coli genome

Randomly sheared fragments of E. coli DNA form duplexes over a single second order C_0t curve (figure 2) because the concentration of essentially all the sequences in the genome is the same (7). The sequences of the single stranded ends to these duplexes must also be in approximately the same concentrations. Thus on average all duplexes would be expected to have a similar probability of hybridising with another single stranded fragment or denatured end of another duplex and eventually being part of a hyperpolymer (21). Consistent with this expectation, at a C_0t of 600 more than 50% of the E. coli DNA had accumulated into hyperpolymers, hyperpolymer formation being approximately 200 times slower on average than initial duplex formation. At higher C_0t values we assume most of the E. coli DNA is in the form of very large hyperpolymers.

Hyperpolymer formation and the calf genome

The calf genome contains approximately 35 to 40% repeated sequence DNA (7 and Table 2). Approximately 10 to 12% consists of highly repeated sequences tandemly arranged in the chromosomes in clusters which can be isolated as "satellites" in Cs_2SO_4/Ag^+ gradients and recognised in restriction enzyme digests (18). The remaining repeated sequence DNA almost certainly consists of short sequences (e.g. less than 450 nucleotide pairs) which are interspersed between non-repeated sequences, a high proportion of which are probably less than 2000 nucleotide pairs long. This interspersion pattern is common to a wide range of animal species (22). A number of observations in this paper support this kind of sequence organisation in the calf genome: (a) the proportion of DNA in the HAP duplex fraction after renaturation of only repeated sequences increases with fragment size (b) after renaturation of repeated sequences on 1000 nucleotide long fragments, large proportions of these fragments can be degraded by S_1 nuclease (Table 2) indicating that many 1000 nucleotide long fragments carry non-repeated as well as repeated sequences.

About 5% of the calf genome was incorporated into hyperpolymers extremely rapidly (by C_0t 0.1) as assayed by hydroxyapatite binding after renaturation of fragments 650 nucleotides long (figure 2). This percentage increased to only 24% by C_0t 100 indicating that only a small proportion (20%) of the renatured repeated sequences can be incorporated into hyperpolymers large enough to be retained on HAP at C_0t values 200 times greater than those required for initial duplex formation. The remainder of the genome which consisted of partially renatured fragments and the fragments

of non-repeated sequence DNA must be incorporated into large hyperpolymers much more slowly.

This behaviour is quite different from that of E. coli DNA where large hyperpolymer accumulation involving most of the genome proceeded with approximately second order kinetics once it had initiated (figure 2). The behaviour of calf DNA is consistent however with the sequence organisation in the genome described above. The rapidly formed hyperpolymers must include predominantly the highly repetitious, tandemly arranged sequences which occupy 10 to 20% of the genome. Randomly sheared fragments from this part of the genome renature very rapidly and the single stranded tails on the duplexes are of similar reiteration frequency so would be expected to continue renaturing with each other to form large aggregates and hyperpolymers.

Renaturation of fragments shorter than 1000 nucleotides from the major part of the genome which consists of short repeats interspersed with non-repeats would leave denatured non-repeated DNA on the ends of many of the duplexes. These non-repeated DNA sequences would be in such low concentration that they would not renature with each other until much higher C_0t values, thus considerably delaying the incorporation into hyperpolymers of the major portion of the genome which consists of interspersed short repeated and non-repeated sequences. However, with increasing fragment length more fragments would contain two regions of repeated sequence DNA separated by an interspersed non-repeated sequence (19). Consequently the tails to duplexes formed from such fragments would frequently include denatured repeated sequence DNA enabling them to form multifragment aggregates and hyperpolymers. The rapid incorporation of more of the calf genome into hyperpolymers when longer fragments are renatured is illustrated in figure 3.

Thus our studies on the calf genome are consistent with the conclusions that with fragments shorter than 600 nucleotides only highly repeated sequence regions of the genome rapidly form hyperpolymers on renaturation but, with longer fragments, the regions of the genome containing short repeated sequences and non-repeated sequences are incorporated into hyperpolymers by repeated sequence DNA renaturation. These results and conclusions are similar to those reported for the behaviour of sea urchin genome repeated sequences (5).

Hyperpolymer formation and the wheat genome

Seventy five percent of the wheat genome consists of repeated sequences renaturing before a C_0t of 100 and a further 20% consists of much more slowly renaturing sequences interspersed with the repeated sequences (1). Some of these interspersed slowly renaturing sequences are responsible for 90% of the wheat genome being retained in the HAP duplex fraction at C_0t 100 (figure 2). With such a high proportion of repeated sequences, at least fifty percent of the genome probably consists of long arrays of repeated sequence DNA (1, 2). Such DNA would be expected to be incorporated into hyperpolymers like the tandemly arranged calf repeated sequences. The finding that some 80% of the wheat genome is incorporated into large hyperpolymers with 650 nucleotide long fragments (figure 2) is consistent with there being long regions of repeated sequences throughout much of the wheat genome. Thompson reached a similar conclusion for the pea genome (6).

Wheat DNA appeared to form hyperpolymers more rapidly than E. coli DNA, as assayed by HAP binding, as a function of the $C_0t_{\frac{1}{2}}$ for initial duplex formation (figure 2). Wheat DNA also appeared to form large aggregates more rapidly than E. coli and calf DNAs as judged by electron microscopy. The reasons for this are probably complex but the differences may indicate that E. coli and calf DNAs initially form more linear multifragment aggregates. Other studies (Rimpau, Smith and Flavell, unpublished) have indicated that short unrelated repeated sequences are interspersed throughout much of the wheat genome. This interspersion pattern would prevent linear aggregates being formed.

With so much repeated sequence DNA in the wheat genome, fragment size would not be expected to influence the proportion of the genome incorporated into hyperpolymers before non-repeated sequence DNA renaturation nearly as much as in the calf genome. This difference was observed (see figure 3). Fragment size however clearly did affect the number of nucleations necessary to attain the size of hyperpolymer required to be retained on HAP in 0.5 M PB at 60°C, since with increasing fragment size, hyperpolymers of the appropriate size are formed more quickly (see above).

Hyperpolymer formation and the use of HAP to study DNA renaturation

The results in this paper have important practical implications for experiments where HAP chromatography is used to study DNA renaturation, especially in genomes which have high proportions of rapidly renaturing, tandemly arranged repeated sequence DNA. In renaturation of the calf and

wheat genomes, hyperpolymers large enough not to be eluted from HAP at 60°C with 0.5 M PB, build up before all the intermediate repeated sequences have reannealed (figures 1, 2 and 3). Therefore, when incubation is continued so that all fragments carrying a repeated sequence form a duplex, significant proportions of the renatured sequences are in very large hyperpolymers. Recoveries decrease very sharply with fragment lengths above 500 nucleotides (see figure 1 and 3) resulting in erroneous estimation of the proportion of DNA in duplex form. Thus when studying the renaturation of plant and animal DNAs, renatured DNA should not be eluted from HAP at 60°C with 0.5 M PB if quantitative estimates of the denatured and non-renatured fractions of the genome are required. As pointed out earlier, this is especially important with DNAs which have more repeated sequence DNA. Other technical implications of hyperpolymer formation for renatured DNA studies have been highlighted by Thompson (6).

Finally, we should like to point out that fractionation of renatured fragments on the basis of their ability to form hyperpolymers contributes information on genome organisation and is a useful way of fractionating repeated sequences arranged differently in the genome. This has recently been illustrated by Marx, Allen and Hearst (23) and Rinehart, Wilson and Schmid (24) in human genome studies.

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* Abbreviations: C_0t - Concentration of DNA (moles of nucleotides per litre) x seconds; HAP - Hydroxyapatite; PB - Equimolar mixture of NaH_2PO_4 and Na_2HPO_4; E. coli - Escherichia coli.