Sequence Organization in Nuclear Deoxyribonucleic Acid from *Physarum polycephalum*

PHYSICAL PROPERTIES OF FOLDBACK SEQUENCES

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(Received 15 June 1979)

An investigation was performed with the use of physical techniques, to determine the nature and organization of inverted repeat sequences in nuclear DNA fragments from *Physarum polycephalum*. From the average size of foldback duplexes (550 nucleotide pairs), and the foldback duplex yield as determined by treatment of DNA with S1 deoxyribonuclease followed by hydroxyapatite chromatography, it is estimated that there are at least 25 000 foldback sequences in the *Physarum* genome. Foldback DNA molecules exhibit properties intermediate between single-stranded DNA and native duplexes on elution from hydroxyapatite with a salt gradient. In addition, thermal-elution chromatography of foldback DNA from hydroxyapatite crystals shows that foldback duplexes are less stable than native DNA. These properties can be explained on the basis that inverted repeat sequences are mismatched when in the foldback configuration. The results of experiments in which the binding of foldback DNA molecules to hydroxyapatite was determined, by using fragments of different single-chain size, agree with previous studies indicating that inverted repeat sequences are located, on average, every 7000 residues throughout the *Physarum* genome. The inverted repeats are derived from both the repetitive and single-copy components in *Physarum* nuclear DNA.

The presence of inverted complementary sequences is a general feature of the organization of nuclear DNA from a wide variety of eukaryotic organisms (Wilson & Thomas, 1974; Cech & Hearst, 1975; Smith & Flavell, 1975; Schmid *et al.*, 1975). Inverted repeats are often referred to as foldback sequences, and sometimes hairpin sequences or palindromes. This is because denatured DNA molecules in which such sequences are present have unusual structural properties, derived from the ability of inverted repeat elements to 'fold back' by intra-chain annealing and form hairpin-like duplexes. Because intra-strand annealing is a fast reaction compared with the rate of formation of intermolecular

duplexes, it is possible to form foldback duplexes selectively by denaturing DNA, and annealing for a brief period under conditions suitable for duplex formation. Thus it is possible to isolate foldback DNA and to study its properties.

In previous work we have characterized foldback molecules in unfractionated nuclear DNA from *Physarum polycephalum* by using the electron microscope (Hardman & Jack, 1977, 1978). In the present paper we extend our preliminary observations (Jack & Hardman, 1977) on the physical properties of foldback sequences in *Physarum* DNA. It is shown that a substantial fraction of the DNA in *Physarum* contains inverted repeat sequences, and that the pattern of distribution of these sequences is in accord with the results obtained in our previous work with the electron microscope. Foldback sequences appear to consist of divergent sets of related inverted repeat elements that form mismatched duplexes in foldback DNA. Reassociation kinetics were used to determine the complexity of the sequences that form foldback duplexes in *Physarum* DNA.

Abbreviations used: C_0t , product of the DNA concentration (mol of nucleotide/l) and time of annealing (s); Pipes, 1,4-piperazinediethanesulphonic acid.

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Materials and Methods

Growth of Physarum cultures and isolation of DNA

For most of this work *Physarum polycephalum* strain LU648 × LU688 (Cooke & Dee, 1975) was used, and was obtained as a gift from Dr. J. Dee, Department of Genetics, University of Leicester, Leicester, U.K. In some experiments strain M₃cVII was used (Daniel & Baldwin, 1964) and was a gift from Dr. T. E. Evans, Case Western Reserve University, Cleveland, OH, U.S.A. This strain gave results similar to those obtained with strain LU648 × LU688. *Physarum* stocks were maintained as submerged shake cultures of microplasmidia. Cultures (30 ml) were grown in 22°C in 500 ml conical flasks in a cooled orbital incubator shaken at 200 rev./min. Isolation of nuclear DNA was performed as previously described (Hardman *et al.*, 1979). Radioactive labelling of DNA was achieved by growing macroplasmidial cultures, as previously described (Hardman & Jack, 1977), or microplasmidial cultures, in the presence of 10 µCi of [*Me*-³H]thymidine/ml (specific radioactivity 20 Ci/mol; The Radiochemical Centre, Amersham, Bucks., U.K.). *Escherichia coli* DNA was labelled with [¹⁴C]-thymidine and extracted as in previous work (Hardman & Jack, 1977).

Controlled shearing of DNA and size fractionation

For use in renaturation experiments, DNA fragments about 300 nucleotide pairs in length were obtained by sonication in an ice-cold bath with an Artek Sonic Dismembrator fitted with a microprobe. A total sonication time of 4 min was used, comprising 15 s bursts of sonication with 30 s cooling intervals. Longer DNA fragments of defined single-chain size were obtained by sonication for brief periods (10–30 s) and subsequent fractionation by centrifuging DNA in 5–10% (w/v) sucrose gradients, containing 0.2 M-NaCl, 0.1 M-NaOH, 1.0 mM-EDTA and 0.1% Sarkosyl NL, in an SW27.1 rotor in the Beckman L2-65B preparative ultracentrifuge. After centrifugation at 27000 rev./min for 24 h, 24 equal fractions were collected from the bottom of the gradients, and the chain size of the DNA in each fraction was determined. For some purposes this was achieved by electrophoresis through alkaline agarose gels. For most experiments, samples were re-sedimented in alkaline sucrose gradients, as described above, with reference to bacteriophage-ϕX174 DNA. Both methods gave similar results, except that average DNA fragment sizes determined by sucrose-gradient analysis were consistently 10% greater than the values obtained by gel electrophoresis (Jack, 1978). The latter values, when obtained, were taken to be more accurate because of the ability to use reliable standards for DNA chain size determination by co-electro-

phoresis of restriction fragments of bacteriophage DNA.

Denaturation of DNA

Unless otherwise stated, DNA was heat-denatured in the presence of 0.12 M-sodium phosphate buffer, pH 6.8, at 100°C for 10 min. In some experiments DNA was denatured with alkali. In these cases 0.1 vol. of 3.0 M-NaOH was added to the DNA solutions, and after standing at room temperature for 10–20 min the solutions were neutralized by the addition of approx. 10 vol. of 0.05 M- or 0.12 M-sodium phosphate buffer, pH 6.8. For DNA samples that were to be treated with S1 deoxyribonuclease (Sigma), heat-denaturation was performed in the presence of 0.18 M-NaCl/0.01 M-Pipes buffer, pH 6.8, instead of phosphate buffer.

Results

Isolation of Physarum foldback DNA with the use of hydroxyapatite

The chromatographic properties of native and denatured *Escherichia coli* DNA on hydroxyapatite are shown in Fig. 1(a). With a gradient of increasing phosphate concentration, denatured DNA chains are eluted over a narrow range of phosphate concentration, i.e. 0.05–0.12 M-sodium phosphate, and most (94%) of the native duplexes are eluted over a similarly narrow range of concentrations, i.e. 0.14–0.25 M-sodium phosphate. A small amount of material (about 6% of the total radioactivity) in the native preparation was shown to be sensitive to the action of single-chain-specific S1 deoxyribonuclease. This is presumably a minor contaminant of single chains in native *E. coli* DNA generated during DNA preparation, corresponding to the peak eluted early in Fig. 1(a).

In similar experiments, native DNA from *Physarum polycephalum* was shown to exhibit properties almost identical with those of native *E. coli* DNA on hydroxyapatite (results not shown). As shown in Fig. 1(b), however, denatured *Physarum* DNA shows properties quite different from those of denatured *E. coli* DNA on hydroxyapatite chromatography, with a significant proportion of material being eluted over a wide range of phosphate concentrations intermediate between those characteristic for native and denatured DNA molecules. In experiments where ¹⁴C-labelled sheared native bacteriophage-λ DNA and ³H-labelled *Physarum* nuclear DNA of average chain length 3000 nucleotide residues were mixed, denatured by heat and then passed through hydroxyapatite crystals at 60°C, less than 2% of the denatured ¹⁴C-labelled bacteriophage-λ DNA was bound to the hydroxyapatite column in the presence of 0.12 M-sodium

Fig. 1. Salt-gradient elution of foldback DNA from hydroxyapatite

In (a) a solution (2 ml) containing $1.5 \mu\text{g}$ of ${}^{14}\text{C}$ -labelled *E. coli* DNA and $40 \mu\text{g}$ of calf thymus DNA in 0.05 M-sodium phosphate buffer, pH 6.8, was heat-denatured. After cooling to 60°C , $1 \mu\text{g}$ of ${}^3\text{H}$ -labelled *E. coli* DNA and $40 \mu\text{g}$ of native calf thymus DNA were added, and the mixture was adsorbed on a 1 ml column of hydroxyapatite, equilibrated previously at 60°C with 0.05 M-sodium phosphate buffer, pH 6.8. DNA was eluted by a 50 ml gradient of 0.05–0.3 M-sodium phosphate buffer, pH 6.8, at a flow rate of 0.5 ml/min. Fractions were collected at 1 min intervals and assayed for radioactivity by liquid-scintillation spectrometry. ●, ${}^3\text{H}$ radioactivity; O, ${}^{14}\text{C}$ radioactivity; ·····, concn. of sodium phosphate. In (b) ${}^3\text{H}$ -labelled *Physarum* microplasmal DNA was sonicated briefly to decrease the average single-chain size of the DNA to 2000 nucleotide residues. Then $2.5 \mu\text{g}$ of this material was added to $40 \mu\text{g}$ of calf thymus DNA. The mixture was denatured in 0.05 M-sodium phosphate buffer, and annealed to an equivalent $C_0t = 1 \times 10^{-4} \text{ M} \cdot \text{s}$. Then $40 \mu\text{g}$ of native calf thymus DNA was added, and the mixture was applied to hydroxyapatite and eluted as described in (a) by a gradient of 0.05–0.4 M-sodium phosphate buffer, pH 6.8. ●, ${}^3\text{H}$ radioactivity.

phosphate, whereas 38% of the ${}^3\text{H}$ -labelled *Physarum* DNA remained bound to the column under these conditions. This bound ${}^{14}\text{C}$ - and ${}^3\text{H}$ -labelled material could be removed completely from the

column by elution with 0.4 M-sodium phosphate buffer, consistent with the results shown in Fig. 1(b). The amount of *Physarum* DNA in this bound fraction is independent of DNA concentration (Jack &

Hardman, 1977; Jack, 1978). In further experiments where shorter *Physarum* DNA chains, 1000 residues long, were used, 17% of the DNA was present in the bound fraction. Of this bound material, 96% remained bound to hydroxyapatite on re-chromatography, and 71% remained bound if re-chromatography was performed after a second round of heat-denaturation. This shows that the hydroxyapatite fractionation step selects a specific fraction of *Physarum* DNA under these conditions. The above control experiments also rule out the possibility that the binding properties of denatured *Physarum* DNA on hydroxyapatite are due to incomplete denaturation or to intermolecular annealing of nucleotide sequences of low complexity in *Physarum* DNA. The observations are complemented by previous work (Hardman & Jack, 1977, 1978), in which it

has been shown that molecules of denatured *Physarum* DNA, after being annealed very briefly to a C_0t value of $1 \times 10^{-4} \text{ M} \cdot \text{s}$, contain intramolecular foldback structures that can form rapidly when conditions favouring duplex formation are restored.

Thermal elution of foldback DNA from hydroxyapatite

The foldback fraction was obtained from moderately long single chains of ^3H -labelled *Physarum* DNA, with a number-average chain length of 1500 residues, by alkali denaturation, neutralization and annealing to a C_0t value not greater than $1 \times 10^{-4} \text{ M} \cdot \text{s}$. ^{14}C -labelled sonicated native *E. coli* DNA was added, and the mixture was applied to hydroxyapatite at 60°C in 0.12 M-sodium phosphate. Extensive washing of the hydroxyapatite

Fig. 2. *Thermal-elution chromatography of foldback DNA*

In (a) 0.12 ml of a solution containing $1 \mu\text{g}$ of ^3H -labelled *Physarum* microplasmodial DNA/ml, of number-average chain length 1500 nucleotide residues, was alkali-denatured by the addition of NaOH to a final concentration of 0.05 M, and neutralized at 60°C by the addition of 1.0 ml of 0.12 M-sodium phosphate buffer, pH 6.8. The solution was annealed to $C_0t = 1 \times 10^{-4} \text{ M} \cdot \text{s}$. Sonicated ^{14}C -labelled *E. coli* DNA (average chain length 300 nucleotide residues) was then added, and the material was absorbed to a 1 ml column of hydroxyapatite at 60°C , previously equilibrated with 0.12 M-sodium phosphate buffer, pH 6.8. Single-stranded DNA was eluted by washing the column with 10 ml of 0.12 M-sodium phosphate buffer, pH 6.8, at 60°C , after which the column was re-equilibrated by washing with 0.1 M-sodium phosphate buffer, pH 6.8, before raising the temperature of the column (Martinson, 1973). The temperature was raised, allowed to equilibrate, and then the column was washed with 4 column volumes of 0.1 M-sodium phosphate buffer, pH 6.8, for each point shown. Samples of the eluted material were assayed for radioactivity by liquid-scintillation spectrometry. ●, ^3H radioactivity; ○, ^{14}C radioactivity. In (b) 0.8 ml of a solution containing ^{14}C -labelled *E. coli* DNA ($2 \mu\text{g}$) and carrier calf thymus DNA ($40 \mu\text{g}$) in 0.18 M-NaCl/0.01 M-Pipes buffer, pH 6.8, was heat-denatured and cooled to 60°C . A 0.25 ml sample containing $1 \mu\text{g}$ of ^3H -labelled *Physarum* DNA/ml, as in (a), in 0.05 M-NaOH to denature the material, was added to the above mixture, followed by $25 \mu\text{l}$ of 0.5 M-HCl to neutralize the alkali. The mixture was annealed at 60°C to a C_0t value of $1 \times 10^{-4} \text{ M} \cdot \text{s}$ with respect to *Physarum* DNA, and then the mixture was added to an equal volume of 0.2 M-NaCl/25 mM- β -mercaptoethanol/1 mM- ZnSO_4 /50 mM-sodium acetate buffer, pH 4.6. S1 deoxyribonuclease (Sigma; 40 units) was added and incubation was performed at 35°C for 40 min. A sample of the mixture was precipitated with 5% (w/v) trichloroacetic acid and assayed for radioactivity. This indicated that less than 1.2% of the ^{14}C -labelled *E. coli* DNA was acid-precipitable, whereas 9.8% of the ^3H -labelled *Physarum* DNA remained acid-precipitable. The remaining portion of the mixture was applied to hydroxyapatite at 60°C , and single-stranded DNA was eluted by 0.12 M-sodium phosphate buffer, pH 6.8, as in (a). Only 5% of the ^3H radioactivity was bound to the column, indicating the presence of single chains of DNA, or that about one-half of the S1-deoxyribonuclease-resistant duplex material was either too short or too unstable at 60°C to bind to hydroxyapatite. Thermal-elution chromatography of the bound material was then performed in the presence of ^{14}C -labelled *E. coli* DNA, as in (a). ●, ^3H radioactivity; ○, ^{14}C radioactivity.

column with 0.12M-sodium phosphate buffer was performed to remove unbound single-stranded DNA.

The phosphate concentration of the eluting buffer was lowered by equilibrating the column with 0.10M-sodium phosphate at 60°C, to prevent premature elution of duplex material at temperatures above 60°C (Martinson, 1973; Bell & Hardman, 1977; Bell, 1978). Thermal-elution chromatography was then carried out to determine the 'melting' temperature of the duplexes in the ³H-labelled foldback fraction compared with the ¹⁴C-labelled *E. coli* DNA duplexes. The results are shown in Fig. 2(a).

The duplexes in the foldback fraction from *Physarum* DNA are known to contain covalently attached single-chain 'tails' (Hardman & Jack, 1977, 1978), with an unknown effect on the 'melting' temperature of foldback DNA. A similar experiment was therefore performed in which foldback DNA was treated with S1 deoxyribonuclease to remove single-stranded 'tails' before thermal-elution chromatography. The result is presented in Fig. 2(b).

Interspersion of foldback sequences in *Physarum* DNA

The amount of *Physarum* DNA that is detected in the foldback fraction shows a strong dependence on the size of DNA chains used, as is clearly indicated by the results shown in Fig. 3. Short fragments, of weight-average length 600 nucleotide residues, yield 14.1% of the DNA in the foldback fraction, whereas fragments 3400 residues in length yield a foldback fraction consisting of 45% of the DNA. This is partly due to the presence of single-chain segments known to be covalently attached to the foldback duplexes, as indicated above (Hardman & Jack, 1978; Hardman *et al.*, 1979), which become longer as the fragment length is increased. However, this is not the complete explanation, since the foldback duplex yield, measured by resistance of foldback DNA to limit digestion with S1 deoxyribonuclease, also increases with chain length (Fig. 3), suggesting that additional segments of foldback duplex are present in longer DNA fragments.

Problems in the recovery of the foldback fraction from hydroxyapatite columns are encountered for DNA fragments longer than 3500 nucleotide residues in length, corresponding to a foldback fraction comprising 45.2% of the total DNA, as indicated in Fig. 3. This can be deduced from the results of the experiments summarized in Table 1. As DNA chain length exceeds 3500 residues, an increase is observed in the proportion of material that binds to hydroxyapatite in 0.12M-sodium phosphate buffer, pH 6.8, but that fails to be eluted with 0.4M-sodium phosphate buffer, pH 6.8. The amount of DNA in this irreversibly bound fraction can be determined by measuring the radioactivity in a sample of the

Fig. 3. Interspersion of foldback sequences in *Physarum* DNA

³H-labelled *Physarum* DNA was sonicated briefly and size-fractionated on alkaline sucrose gradients as described in the Materials and Methods section and Table 1. Samples from appropriate fractions were neutralized by the addition of 0.05M-sodium phosphate buffer, pH 6.8, at 60°C, and the DNA was annealed to $C_0t = 1 \times 10^{-4}$ M·s. The material was then applied, together with 40 µg each of denatured and native calf thymus DNA, to a hydroxyapatite column equilibrated at 60°C with 0.05M-sodium phosphate buffer, pH 6.8. Columns were washed with 10 column volumes of 0.12M-sodium phosphate buffer, pH 6.8, to remove single-stranded DNA, after which the duplex-containing material was eluted by 0.4M-sodium phosphate buffer, pH 6.8, at 60°C. The foldback duplex-containing fraction is defined as the fraction of the total radioactivity bound to the column in 0.12M-sodium phosphate buffer, pH 6.8, and eluted with 0.4M-sodium phosphate buffer, pH 6.8, expressed as a percentage of the total recovered material. Recoveries are discussed further in the text and Table 1. For S1-deoxyribonuclease-resistant material, DNA chains of different average size, as indicated, were digested with the enzyme after annealing to $C_0t = 1 \times 10^{-4}$ M·s, as described in Fig. 2(b). Samples were precipitated with 5% (w/v) trichloroacetic acid, and the S1-deoxyribonuclease-resistant material was taken to be that fraction that was acid-precipitable. ●, % of material in the foldback fraction; ○, % of material resistant to digestion with S1 deoxyribonuclease.

hydroxyapatite-column material after extensive washing with 0.4M-sodium phosphate buffer, pH 6.8. The observation is a special property of denatured *Physarum* DNA, since it is an effect not observed for native *Physarum* DNA of any chain length, or for either native or denatured *E. coli* DNA, or hamster-cell DNA.

Complexity of the nucleotide sequences derived from foldback duplexes

To be capable of forming a foldback duplex it is necessary for a given DNA molecule to contain just

Table 1. *Recovery of Physarum foldback DNA from hydroxyapatite*

^3H -labelled *Physarum* DNA was fragmented, either by sonication or by needle-shearing for fragments of larger average size. In this series of experiments the average DNA fragment size was determined by sedimentation in alkaline sucrose gradients (Studier, 1965) with reference to bacteriophage- ϕX174 single-stranded viral DNA (18.4S). DNA was re-denatured and applied to hydroxyapatite as described in Fig. 3. The duplex-containing DNA fraction is taken to be the fraction of radioactivity bound to hydroxyapatite in 0.12M-sodium phosphate buffer, pH 6.8, and eluted by 0.4M-sodium phosphate buffer, pH 6.8. Irreversibly bound material is that which failed to be eluted by 0.4M-sodium phosphate buffer, pH 6.8. This was determined from the amount of radioactivity associated with a sample of the column material after extensive washing with 0.4M-sodium phosphate buffer, pH 6.8.

Single-chain size of DNA ($10^{-3} \times$ no. of residues)	% of DNA in duplex-containing fraction	% of DNA irreversibly bound to hydroxyapatite	Recovery (% of radioactivity applied)
0.60	14.1	0.8	95
0.85	18.7	0.6	115
1.25	26.3	0.8	90
1.50	28.1	0.6	94
1.70	28.5	1.5	92
2.10	31.6	2.3	100
2.50	37.1	2.4	94
3.00	39.7	1.2	80
3.45	45.2	2.8	98
8.00	56.5	10.1	100
14.00	55.5	16.3	100
20.00	33.9	63.5	85

Fig. 4. *Complexity of foldback sequences in Physarum DNA*

A $0.4\mu\text{g}$ sample of ^3H -labelled S1-deoxyribonuclease-resistant foldback DNA, prepared from *Physarum* macroplasmal DNA as described in Fig. 2(b), was mixed with $2\mu\text{g}$ of ^{14}C -labelled total nuclear DNA from macroplasmidia. The DNA was sonicated in the presence of 0.4M-sodium phosphate buffer, pH 6.8, to decrease the chain size of the DNA to about 300 nucleotide residues. The material was then heat-denatured, as described in the Materials and Methods section, and renaturation was assayed by hydroxyapatite chromatography. With the exception of the points corresponding to a C_0t value of $1 \times 10^{-4}\text{M}\cdot\text{s}$, renaturation was driven by a 80–800-fold excess of unlabelled sonicated total *Physarum* nuclear DNA. ●, ^3H radioactivity; ○, ^{14}C radioactivity.

two inverted complementary sequences. Hence it is conceivable that the inverted repeats could be derived from either the 'repetitive' component or the 'single-copy' component of *Physarum* DNA (Jack, 1978), since in practice it is difficult to distinguish between sequences present with a low copy number from single-copy sequences with any degree of certainty.

To determine whether the sequences present in foldback duplexes in *Physarum* DNA were repetitive or single-copy in character, ^3H -labelled foldback duplexes were isolated after digestion of foldback DNA with S1 deoxyribonuclease, by recovery of the duplexes by using hydroxyapatite chromatography as described in Fig. 2(b). This material was co-sonicated with ^{14}C -labelled total *Physarum* nuclear DNA as a control, then denatured, and renaturation was assayed by hydroxyapatite chromatography. The results are shown in Fig. 4.

General Discussion

In previous studies we have shown that, after denaturation and brief annealing of *Physarum* DNA, the overwhelming majority of duplexes that are formed have a foldback configuration (Hardman & Jack, 1977, 1978; Hardman *et al.*, 1979). DNA molecules containing these foldback duplexes bind selectively to hydroxyapatite in the presence of 0.12M-sodium phosphate buffer, pH 6.8, and are eluted by 0.4M-sodium phosphate buffer, pH 6.8

(Hardman & Jack, 1977). These molecules therefore correspond to the material that is eluted from hydroxyapatite in this range of phosphate concentration in the present work (Fig. 1*b*) with properties intermediate between those of single-stranded and duplex DNA. The duplexes, which bind to hydroxyapatite in 0.12 M-sodium phosphate buffer, pH 6.8, 'melt' over a wide range of temperatures from the annealing temperature of 60°C up to 80°C, the equivalent 'melting' temperature of native *E. coli* DNA under these conditions (Fig. 2*a*). The 'melting' profile is unaffected when the single-stranded 'tails' that are covalently attached to foldback duplexes are removed by the use of S1 deoxyribonuclease (Fig. 2*b*), indicating that the heterogeneity in 'melting' is an intrinsic property of foldback duplexes, and is not an effect related to the presence of the single-stranded segments in foldback DNA molecules. Several factors may contribute to the 'melting' behaviour of the DNA duplexes, namely ionic strength, duplex size, the base composition of the DNA and the degree of sequence mismatching (Schildkraut & Lifson, 1965; Thomas & Dancis, 1973). These parameters are related to the DNA 'melting' temperature, T_m , as follows:

$$T_m = 81.5 + 16.6 \log M + 0.41[\%(G + C)] - 500/b - 315p$$

where T_m is in °C, M is the molar concentration of univalent cation, $G + C$ the average guanine plus cytosine content of the DNA, b its length in nucleotide residues and p the fraction of residues that are mispaired in the duplex. The calculated T_m for 300-residue-long fragments of *E. coli* DNA in solution in 0.1 M-sodium phosphate buffer, pH 6.8 ($M = 0.15$), is 86.2°C, compared with values of 88.0°C and 86.5°C in the two separate experiments shown in Figs. 2*a*) and 2*b*). The thermal-elution behaviour of the *Physarum* foldback DNA duplexes is quite different from the native control DNA, showing a broad 'melting' profile with a T_m value that is 10°C below that of sonicated *E. coli* DNA. Native *Physarum* DNA, with an average $G + C$ content of 41% (Evans & Suskind, 1971), would be expected to dissociate with a T_m value 3.7°C below that of *E. coli* DNA ($G + C = 50\%$). The observed T_m value in separate experiments (results not shown) is 85°C. Only about one-fifth of the foldback duplexes that bind to hydroxyapatite under the conditions described in the present work have a length less than 150 base-pairs, and the average length is about 550 nucleotide residues (Hardman & Jack, 1977; Hardman *et al.*, 1979). By reference to the above equation for T_m it can be deduced that the size of foldback duplexes is unlikely to account for their lack of stability. We are unable to distinguish, however, between the possibility that foldback elements may form unstable duplexes because they are

either comprised of sequences that are especially rich in A and T residues and/or that foldback sequences are mismatched because of sequence divergence. Further experiments are required to investigate these possibilities further, although it would seem likely that different degrees of sequence mismatching in foldback duplexes is the most likely possibility. The above explanation could also account readily for the result shown in Fig. 1*b*), in which foldback duplexes exhibit properties intermediate between those of single-stranded DNA and native duplexes.

Nature and distribution of foldback duplexes

The result shown in Fig. 3 shows that foldback elements are present in at least 45% of DNA fragments 3500 residues long, and that they are interspersed with other sequence components in a substantial portion of the *Physarum* genome. We are unable to determine the distribution of foldback sequences in longer DNA chains by using this method with any degree of certainty because of problems related to the recovery of DNA from hydroxyapatite. The results of previous work with the electron microscope, however (Hardman *et al.*, 1979), show that over 90% of 27000-residue-long DNA chains contain foldback elements, with an average spacing of 7000 nucleotide residues. The results shown in Fig. 3 are entirely consonant with these previous observations. On this basis, it is noteworthy that for longer *Physarum* DNA chains the combined recoveries of DNA in the foldback fraction plus the irreversibly bound fraction are necessary to account for the expected total yield of foldback DNA. This is consistent with the idea that the configuration of foldback-duplex-containing molecules formed from long DNA chains may affect their chromatographic behaviour on hydroxyapatite.

The anomalous binding properties of long foldback DNA may be related to the high density of foldback duplexes in *Physarum* DNA, since *E. coli* DNA, which possesses little or no foldback component (Jack, 1978), and denatured briefly annealed hamster-cell DNA, which contains a low density of foldback sequences (Bell & Hardman, 1977), chromatograph normally. The phenomenon may be related to the formation of tangled foldback structures that are sometimes observed in long denatured chains of *Physarum* DNA (Hardman & Jack, 1978).

Fig. 3 also shows that the yield of S1-deoxyribonuclease-resistant duplex increases in the range 500–3000 residues. This indicates that additional foldback structures are formed from inverted complementary repeated elements that are separated by intervening sequences in this size range. Foldback structures with these properties, termed 'looped hairpins', have been observed in *Physarum* foldback DNA preparations by use of the electron micro-

scope (Hardman & Jack, 1977, 1978; Hardman *et al.*, 1979).

It has been shown previously that repetitive sequences, which comprise 31% of the *Physarum* genome, are closely interspersed with single-copy sequences in a large fraction of the DNA (Jack, 1978). The present results and previous work (Hardman & Jack, 1978) show that foldback sequences consisting of up to 10% of the genome are similarly interspersed with other sequence components. Hence it is reasonable to conclude that foldback sequences are present in segments of DNA occupied by both repetitive and single-copy sequences.

The repetitive component in *Physarum* DNA anneals by a C_0t value of $10\text{M}\cdot\text{s}$ (Jack, 1978), corresponding to about 40% of the ^{14}C -labelled total *Physarum* DNA control shown in Fig. 4. This value is greater than the proportion of repetitive sequences because of the presence of covalently attached single-copy sequence 'tails', resulting from the close interspersed of the two components. Although considerably more of the sequences derived from the ^3H -labelled foldback duplexes anneal by $C_0t = 10\text{M}\cdot\text{s}$ than the control DNA, most of this is due to sequences annealing with a C_0t value of less than $1 \times 10^{-4}\text{M}\cdot\text{s}$, reflecting an enhanced proportion of foldback sequences compared with total DNA. Some of the remaining sequences, detached from foldback duplexes by sonication, anneal with the kinetics characteristic of repetitive sequences. However, single-copy sequences are also present, in similar proportions to total DNA. A noteworthy result is the observation that about one-third of the sequences derived from foldback duplexes, after S1-deoxyribonuclease treatment of foldback DNA followed by sonication, still anneal with the kinetics characteristic of foldback molecules (Fig. 4). Since about one-half of the *Physarum* foldback duplexes are devoid of loops (Hardman *et al.*, 1979) and therefore possess intervening 'turn-around' segments less than 50–100 residues long, a large proportion of these foldback structures would be required to be resistant to the action of S1 deoxyribonuclease to account for the present results. An alternative explanation is that the structure of some foldback elements may be internally palindromic, and consist of sequences of the form ...ss'ss'..., where s and s' are inverted complementary repeated sequences. This type of configuration is known to be a characteristic of the inverted repeated sequences in *Physarum* ribosomal DNA (Vogt & Braun, 1976).

The number of foldback foci in *Physarum* DNA can be estimated from the average stem size of the foldback duplexes, 550 nucleotide residues (Hardman *et al.*, 1979), and the yield of S1-deoxyribonuclease-resistant duplex foldback DNA. This is 9.8% of the total DNA, as determined by precipitation of the material with trichloroacetic acid, or 5%

from the amount of material that binds hydroxyapatite as duplexes (Fig. 2b). The latter value has been used in the following calculations, since it is unaffected by the presence of short partly digested acid-precipitable single chains of DNA that may persist after treatment with S1 deoxyribonuclease. By using a value of 2.7×10^8 nucleotide residues for the size of the haploid *Physarum* genome (Mohberg & Rusch, 1971), it is estimated that a minimum of 1.4×10^7 residues ($2.7 \times 0.05 \times 10^8$) are in the form of foldback duplexes, equivalent to a complement of about 25 000 foldback sequences.

In summary, *Physarum* DNA contains many thousands of potential foldback sequences, which appear to be distributed throughout a large proportion of the genome. As in human DNA, the sequences appear to belong to both the single-copy and repetitive components of the genome, and they exhibit a remarkable degree of organization (Hardman & Jack, 1978; Hardman *et al.*, 1979). If, as seems likely, most of the foldback sequences form mismatched duplexes, then the function of foldback sequences, if indeed such a function exists, is probably not dependent on maintenance of the precise nucleotide sequence of an inverted repeat element at a given foldback locus. More information with regard to the nature and possible function of these sequences will no doubt result from sequence cloning techniques.

P. L. J. is grateful to the Science Research Council for the award of a studentship. N. H. thanks the Medical Research Council, The Scottish Hospitals Endowments Research Trust and the Cancer Research Campaign for supporting various aspects of this work, Professor H. M. Keir for providing facilities in the Department of Biochemistry, and Ms. Julie Spear for skilful typing of the manuscript.

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