The structure of nucleolar chromatin in Physarum polycephalum

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Received 26 January 1978

ABSTRACT

The nucleolar DNA of Physarum polycephalum has been differentially labelled with \(^{3}H\)-thymidine and the structure of the nucleolar chromatin investigated by digestion with micrococcal nuclease. Nucleolar chromatin which had been labelled in G2 phase of the cell cycle and then digested before mitosis had an identical DNA repeat length to main band DNA (165 ± 5 base pairs) but there was a definite indication that the rate of digestion was faster for nucleolar DNA than for main band DNA. Nucleolar chromatin which had been labelled in G2 and the label chased through mitosis into G2 phase of the next cycle showed an identical DNA repeat length to main band DNA and was also digested at the same rate. We conclude that nucleolar chromatin, at least 25 per cent of which is maximally transcriptionally active in G2, has a nucleosome-like structure.

INTRODUCTION

The major fraction of eukaryotic chromatin is thought to be composed from a repeating substructure made up of about 200 base pairs of DNA and eight core histones (the nucleosome) to which one or two molecules of H1 are bound [1]. The most common way of revealing the substructure has been partially to digest the chromatin in nuclei with exogenous micrococcal nuclease [2] which gives rise to a characteristic repeat pattern for the DNA. Although it is not clear how much of the genome is contained within such a repeating substructure it seems probable that this structure is characteristic of inactive, non-transcribing chromatin. Transcriptionally active chromatin also shows a basic repeat when the chromatin is digested with micrococcal nuclease [3,4].

In the following we report some preliminary observations on the structure of nucleolar chromatin in the slime mould Physarum polycephalum. The nucleolar DNA contains the ribosomal RNA (r-RNA) genes and probably no others since the only transcription products obtained from in vitro transcription in isolated nucleoli are r-RNA precursors [5]. Physarum
has several advantages over other systems for investigating the nucleolar chromatin, among which are the natural synchrony of its nuclear divisions [6] and the differential synthesis of nucleoplasmic and nucleolar DNA which enables the latter to be differentially labelled [7]. There are several hundred copies of each r-RNA gene per nucleolus [7,8,9]. Two copies each of the 18S and 26S r-RNA genes are arranged symmetrically along a linear DNA molecule of molecular weight $39 \times 10^6$ [10,11]. At least 25% of the total genome is used to synthesise the primary gene product, a precursor r-RNA, molecular weight $4-5 \times 10^6$, the rest presumably being non-transcribed spacer.

A complication arises from the possibility that the newly synthesised and the newly labelled r-DNA may not be transcriptionally active [12]. This problem is overcome in the present study by chasing the newly synthesised, newly labelled r-DNA into the next cell cycle when it must be transformed into a transcriptionally active state.

With these points in mind we have followed the digestion of nucleolar chromatin with micrococcal nuclease under conditions where the nucleolar DNA is newly synthesised, and possibly transcriptionally inactive, and where the DNA is certainly active in the next cell cycle. The results suggest that in the former case there may be a distinct difference in the rate of digestion of r-DNA and bulk chromatin although identical nucleosome-like lengths of DNA are protected in each case, whereas in the latter case the rates of digestion are identical as well as the nucleosome repeat lengths.

**EXPERIMENTAL METHODS**

**MATERIALS**

$6-^3H$ Thymidine (specific activity $26.4$ mmol$^{-1}$) was obtained from the Radiochemical Centre Amersham. Micrococcal nuclease was purchased from Worthington Biochemicals and Proteinase K from Boehringer. Hind III restriction endonuclease was prepared essentially by the method of Smith and Wilcox [13] from a cell paste of H. influenzae obtained from M.R.E. Porton.

**Culture Conditions**

Liquid cultures of Physarum (strain M CVIII) were grown at 26°C as described by Daniel and Baldwin [14]. Mitotically synchronous plasmodial cultures were grown on filter paper in petri dishes [15]. Observations on mitosis were made according to Guttes et al. [16].
Preparation of Nuclei

Nuclei were prepared as described previously [5].

Micrococcal Nuclease Digestion

Nuclei were washed in digestion buffer (0.25M sucrose, 10 mm Tris, pH 8.0, 1 mM CaCl₂, 1 mM Phenyl methyl sulphonyl fluoride) and resuspended in buffer at a concentration of 5 x 10⁷ nuclei ml⁻¹. The nuclei were preincubated at 37°C for 5 min. before the addition of micrococcal nuclease to a final concentration of 25 units ml⁻¹. Aliquots (1 ml) were removed after 1, 2 and 5 min, cooled on ice and made 10 mM in EDTA to terminate the enzymic activity. Pancreatic RNAse A was added to a concentration of 50 µg ml⁻¹ and the samples reincubated at 37°C for a further 30 min. Proteinase K was then added (200 µg ml⁻¹) and the samples were deproteinised for 2-3 h at 37°C.

DNA was purified for electrophoresis by extraction with chloroform: isoamylalcohol (24:1) and dialysed against electrophoresis running buffer.

Hind III Digestions

10 µg of S or G₂ phase labelled deproteinised DNA was incubated in 200 µl of Hind III restriction endonuclease for 30 mins. The reaction was terminated by the addition of 20 µl 1% SDS. Samples were applied directly to a 0.6% agarose gel and electrophoresed at 1 volt cm⁻¹ overnight. Molecular weights of DNA fragments were determined by comparison with undigested λ DNA and Hind III/ λ DNA fragments as described by Murray and Murray [17].

Gel Electrophoresis

The (Tris/EDTA/acetate) buffer system of Loening [18] was used with a resolving system of 2% acrylamide:0.5% agarose in a 30 cm x 29 cm horizontal gel. Gels were run at 4 volts cm⁻¹ for 3-6 h and the DNA visualised using 10 µg ml⁻¹ ethidium bromide.

After the gel had been photographed it was sliced and the gel slices counted for radioactivity as described previously [5]. The nucleosome repeat sizes of Physarum main band and ribosomal DNA were determined by comparison with a limit digest of PM2 DNA digested with restriction endonuclease Hae III and sized according to Noll [19].

RESULTS

The synthesis of main band DNA in Physarum is confined to the first three hours of the mitotic cycle whereas nucleolar DNA is synthesised from 1 h after mitosis throughout interphase [7,20]. The behaviour of
Physarum main band DNA and nucleolar DNA towards micrococcal nuclease can therefore be analysed separately by differential labelling in S and G2 phase of this organism.

It is important to establish that the level of radioactivity incorporated in G2 phase is indeed present only in nucleolar DNA and does not represent label incorporated into main band DNA as a result of asynchrony in the surface cultures. The S phase and G2 phase-labelled DNA were therefore digested with the restriction endonuclease Hind III and the products examined on gels (Figure 1). The G2 phase-labelled DNA was not digested to products corresponding to the bulk of main band DNA (Figure 1a). More than 85% of the G2-labelled DNA is present in fragments of $20 \times 10^6$ and $5-6 \times 10^6$ molecular weight, which correspond to the products characterised previously by Molgaard et al. [10] for nucleolar DNA of Physarum. Under identical conditions main band DNA is digested to products of higher molecular weight than the G2-phase labelled DNA, only 10% of the count appearing in the expected r-RNA size bands of molecular weight $20 \times 10^6$ or less. At least some of this must arise from label incorporated into nucleolar DNA during S phase since synthesis of nucleolar DNA takes place throughout interphase. This demonstrates, therefore, that greater than 90% of nucleolar DNA has been labelled in the G2 pulse.

A similar conclusion may be drawn from the results of an experiment where label incorporated in G2 is chased through one cell cycle to the following G2. Isolation of the DNA followed by Hind III restriction nuclease digestion yields the fragments shown in figure 1b which again correspond to partial or complete products observed by Molgaard et al. [10].

Synchronous cultures of Physarum were labelled for 3 h immediately after mitosis III (that is, in early S) at the end of which time nuclei were isolated and digested with micrococcal nuclease. The pattern of DNA digestion products is shown in figure 2. The typical pattern of nucleosomal chromatin is observed. The radioactivity profile and fluorescently stained bands correspond exactly. The size of the monomer repeat was 165 b.p. in agreement with previous estimates [21-23].

Synchronous cultures from Physarum were labelled between 2 h and 1 h before mitosis III (that is in mid to late G2) to label differentially the nucleolar DNA. The nuclei were isolated after this time, treated with micrococcal nuclease and the DNA products analysed as before. The
ethidium bromide staining pattern in this case corresponds to the nucleo-
plasmic (main band) DNA, whereas the labelled DNA corresponds only to the
digestion products of nucleolar DNA. Figure 3 shows the profile obtained
in such an experiment. The ethidium bromide pattern is similar to the
one shown in figure 1 for S phase-labelled DNA. The third digestion time
point demonstrates that the bulk of the DNA has been digested to monomer
and dimer nucleosome products but trimer and tetramer are also visible.
The presence of significant amounts of dimer, trimer and tetramer was
confirmed by scanning negatives of the gel photographs with a Vitatron
Flying Spot Densitometer, TLD 100. In contrast, the G2 phase-labelled
DNA shows a similar nucleosome pattern but the rate of digestion appears
to be faster. The third digestion time point contains only mononucleosome
fragments, label corresponding to dimer or higher multimers being entirely
absent. This result was obtained in three separate experiments.

In order to investigate whether this was a characteristic of newly
replicated nucleolar DNA or whether it reflected the structure of total
Figure 2. Analysis of DNA after digestion of S phase-labelled nuclei with micrococcal nuclease. Radioactivity profiles: (a) — control; — — after 1 min digestion. (b) — after 2.5 min digestion; — — after 5 min digestion. (c) — after 10 min digestion. Ethidium bromide stained profile: I control; II after 1 min digestion; III after 2.5 min digestion; IV after 5 min digestion; V after 10 min. Arrows indicate positions of mono-, di-, tri- and tetra-nucleosomes from right to left.
nucleolar DNA, a chase experiment was performed. Synchronous cultures of Physarum were labelled between 2 h and 1 h before mitosis, washed and transferred to cold medium. They were then allowed to grow for a further 7 h (6 h after the next mitosis) before the nuclei were isolated and digested. The DNA products of micrococcal nuclease digestion are shown in figure 4. The ethidium bromide and radioactive-label patterns are similar. In this experiment the rate of digestion of the nucleolar DNA appears to
be the same as main band DNA. The third digestion point shows the presence of label in monomer, dimer and higher multimer nucleosome products although the bulk of material has been digested to the mononucleosome limit. This is consistent with the ethidium bromide-stained bands corresponding to the bulk DNA digestion pattern.

Figure 4. Analysis of DNA after digestion of G2 pulse-chased labelled nuclei with micrococcal nuclease. Radioactivity profiles: (a) control; (b) after 1 min digestion; (c) after 2 min digestion; (d) after 5 min digestion. Ethidium bromide stained profile: I control; II after 1 min digestion; III after 2½ min digestion; IV after 5 min digestion. Arrows indicate positions of mono-, di-, tri- and tetra-nucleosomes from right to left respectively.
DISCUSSION

The nucleolar DNA in Physarum is made up of several hundred identical copies of a linear DNA molecule of molecular weight $39 \times 10^6$. Each of these molecules contains a centre of symmetry with two copies each of the 19S and 26S r-RNA genes arranged in a palindrome [10]. The primary transcript has a molecular weight of $4-5 \times 10^6$ and contains both the 19S and 26S RNA species. Each gene (19S + 26S) is apparently separated by a non-transcribed spacer of molecular weight about $9-10 \times 10^6$. Thus when all the genes are being transcribed, about 25-30% of the nucleolar DNA is transcriptionally active.

In synchronised cultures of Physarum the replication of main-band DNA takes place exclusively in S-phase whereas the replication of nucleolar DNA takes place throughout interphase [20]. It is therefore possible differentially to label nucleolar DNA by adding label in mid G2. That this is the case is confirmed by comparing the sizes of the radioactively labelled DNA fragments produced by a Hind III digest of total DNA and DNA labelled in G2. The molecular weights of the fragments observed agreed well with those predicted from the results of Molgaard et al. [10] and confirm that the nucleolar DNA has been specifically labelled.

The DNA digestion patterns of cultures labelled in G2 phase enable the following conclusions to be drawn regarding the structure of newly-synthesised nucleolar DNA. Firstly, a nucleosomal pattern of fragments is produced which are similar, if not identical, in size to those produced by main band DNA. The nucleolar DNA thus appears to be folded into a nucleosome-like structure with a repeat length of 165 b.p. Secondly, there is a definite indication that the digestion of newly synthesised nucleolar-DNA is greater than bulk chromatin DNA which implies that either this chromatin is in a different conformation from bulk chromatin or possibly that the enzyme has a greater accessibility to the newly synthesised chromatin.

These observations and the conclusions we have drawn from them are similar to those of Seale [26] who also found an increased rate of digestion of newly replicated chromatin in Hela cells. Seale concluded from a detailed kinetic analysis of oligosome formation that this increase in rate was not due to an increase in spacer length between nucleosomes in newly replicated DNA but rather due to an increase in accessibility to the nuclease. A detailed kinetic analysis of the data presented here was not
attempted: the quantitative assessment of the relative amounts of oligosomes was subject to large error, due to the high background fluorescence (not evident on the photographs) between nucleosomes on scans of the gel photographs.

On the other hand, after the labelled nucleolar DNA has gone through mitosis it appears to have a structure identical to bulk chromatin both with respect to the size of the nucleosomal fragments and the rate of digestion. Since this DNA must be in a transcriptionally active state, we may conclude that certainly the spacer regions in an active r-DNA gene contain nucleosome-like lengths of DNA. It is less certain whether the actively transcribing r-DNA genes themselves contain the characteristic nucleosome-like lengths of DNA because transcription only occurs from 25-30% of the total nucleolar DNA and it is possible that the methods used here are not sufficiently sensitive to detect an altered pattern of DNA lengths in a small proportion of the total population. Having said that, it is apparent that the radioactivity profiles show in most cases a fairly discrete set of DNA fragments superimposed on a relatively low background with no obvious evidence of heterogeneity. Further studies are necessary to clarify the situation and we are at present attempting to purify the active fraction from the bulk nucleolar DNA. This result is consistent with similar studies on the nucleolar genes of T. pyriformis [3]. It implies that in Physarum histones are present along the total length of the genome and protect the DNA from nuclease digestion to give a nucleosome-like ladder pattern. However, because mononucleosome lengths of DNA can also be obtained from chromatin in which the intact nucleosome structure is at least partially unfolded by urea or low ionic strength [24], it is not possible to say whether or not the individual nucleosomes are unfolded in this case. Electron microscope pictures of active genes of r-DNA from Oncopeltus [25] show a smooth fibre rather than the typical beaded appearance characteristic of folded nucleosomes. The ladders generated by micrococcal nuclease digestion are characteristic of native chromatin and are thought to be dependent upon the proper maintenance of HI-histone interactions involved in higher order folding. Since the ladders are observed in active chromatin, we may conclude either that such chromatin is superfolded, or, if unfolded, is unfolded in such a way as to preserve the HI interactions responsible for the generation of the ladders. In this respect unfolded active chromatin is unlike sheared chromatin.
In summary, therefore, our experiments indicate that there is a difference in the rate of nuclease digestion of bulk chromatin and newly replicated nucleolar chromatin which may be attributed entirely to conformational differences associated with replication. This being so, the results obtained with pulse-chased nucleolar chromatin, which is not newly replicated, are consistent with other studies which show that transcriptionally active regions of the genome are not differentially degraded, by micrococcal nuclease [27, 28]. Such regions of the genome are, however, more susceptible to digestion with DNase I [27, 28]; it remains to be seen whether the same is true in the nucleolar chromatin in Physarum.

ACKNOWLEDGEMENTS

M.J.B. acknowledges support from the Medical Research Council.
K.E.D. acknowledges Wolfson College for a Guy Newton Research Fellowship.

REFERENCES