Chromatin structure in the macronucleus of the ciliate *Stylonychia mytilus*

H.J.Lipps, A.Nock, M.Riewe and G.Steinbrück

Institut für Biologie III, Abt. Zellbiologie, Universität Tübingen, Auf der Morgenstelle 28,
74 Tübingen, GFR

Received 5 September 1978

ABSTRACT

Evidence is presented that macronuclear chromatin in the hypotrichous ciliate *Stylonychia mytilus* occurs in discrete fragments, each representing at least single genes. The size of these fragments varies between 3 and more than 70 nucleosomes with an average length of about 18 nucleosomes. This observation is discussed with respect to macronuclear structure of hypotrichous ciliates.

INTRODUCTION

The existence of "gene-sized" DNA molecules in the macronuclei of hypotrichous ciliates is now well established /1-4/. The DNA of these nuclei can be separated on agarose gels yielding a typical and reproducible banding pattern /2-4/. In *Stylonychia mytilus* the size of these DNA fragments varies between about 0.3×10^6 and 14×10^6 dalton, and so far, DNA fragments coding for rRNA, 5 S RNA and for the five histone fractions have been identified /3,4/.

While careful analysis of macronuclear DNA has been made in *Oxytricha* /1,2,5,6,/ and *Stylonychia* /3,4,7,8/ little is known about how these DNA fragments occur as nucleoprotein in the macronucleus. All five histone fractions can be demonstrated in the macronucleus of *Stylonychia* /9,10/ and the chromatin is organized in typical eucaryotic nucleosomes with a core particle comprising about 140 base pairs DNA and a nucleosome repeat length of 220 base pairs /11/. However, it has not been possible to demonstrate any kinds of chromosomes in these nuclei; nuclear division occurs not by a typical mitosis but by a process termed amitosis /12/.

In this report we examined the structure and organization of macronuclear chromatin in more detail and present evidence that each "gene-sized" DNA molecule occurs as a discrete chromatin fragment in the macronucleus of Stylonychia mytilus.

MATERIAL AND METHODS

1. Cultures and isolation of nuclei. Stylonychia mytilus (syngen1) /7/ was cultured on Chlorogonium elongatum in neutral Pringsheim solution /7/. One day before isolation of nuclei feeding was stopped. Preparation and separation of macro- and micronuclei was performed as described earlier /7,9/.

2. Preparation of chromatin. For all experiments described here chromatin was prepared by washing the macronuclei several times with 10 mM Tris pH 8.0 and lysing the nuclei in 10 mM Tris, 2.5 mM EDTA pH 8.0. Usually the chromatin solution was adjusted to an OD₂₆₀ of 10. In some preparations 1 mM diisopropylfluorophosphate (Serva) was present during the whole procedure.

3. Electron microscopy of macronuclear chromatin. Electron microscopy of lysed macronuclei was performed essentially according to the method of Miller and Beatty /13/. Between 0.02 μ g and 0.1 μ g chromatin (estimated by absorption at 260 nm) was fixed in 1 % formaldehyde in 0.1 M sucrose pH 8.5 and centrifuged onto a carbon coated grid. After fixation the grid was treated for 30 sec. with 0.4 % Photoflo (Kodak) pH 8.0, stained 2 min. with 0.1 % phosphotungstic acid, dehydrated in ethanol and shadowed with platinum.

4. Nuclease digestion of chromatin. Nuclease digestion with micrococcal nuclease (Worthington) of chromatin and chromatin fractions was performed as described recently /11/ with 180 EU / mg chromatin for 60 sec. at 37 °C. The reaction was stopped by the addition of 1 % SDS and 1 M NaClO₄. The samples were deproteinized with chloroform-isoamylalcohol (24:1) and electrophoresed on a 1.7 % agarose (Miles) gel in 0.04 M Tris, 0.02 sodium acetate, 2 mM EDTA pH 7.8 for 8 hours at constant 40 V. Gels were stained with 0.5 μ g / ml ethidium bromide in electrophoresis buffer and photographed under UV light.

5. Sucrose gradients of macronuclear chromatin. Analysis of macronuclear chromatin on sucrose gradients was done on a 5 - 20 % linear sucrose gradient in 10 mM Tris, 60 mM KCl, 0.5 mM EDTA pH 8.0 with 38 000 rpm for 3 hours in a Beckmann SW 41 rotor. Between 3 and 6 OD₂₆₀ were layered on one gradient. 0.4 ml fractions were collected and the peak fractions were made to 1 % SDS, 0.1 M NaCl and precipitated with 2.5 volumes ethanol. These fractions were then electrophoresed for 16 hours at constant 30 V on a 1.5 % agarose gel in the buffer described above. As a molecular weight marker bacteriophage lambda DNA digested with restriction enzyme EcoRI (Miles) was used. The DNA was transferred from the gels to nitrocellulose filters essentially according to the method of Southern /14/. DNA was denatured for 45 min. in 0.2 M NaOH, 0.6 M NaCl at room temperature. The gel was then neutralized in 1 M Tris, 1.5 M NaCl pH 7.4 for 1 hour at room temperature. The transfer to nitrocellulose filters (Schleicher und Schüll) was done using 20 x SSC in the reservoir.

For identification of chromatin bound proteins peak fractions of the sucrose gradient were precipitated with 2.5 volumes ethanol, dissolved in sample buffer according to Laemmli /15/ and electrophoresed overnight on a 12.5 % Laemmli gel.

6. Preparation of DNA and RNA. DNA from macronuclei was prepared by lysing the nuclei in 0.2 M EDTA, 0.15 M NaCl, 1 % SDS pH 8.0, incubation overnight with 400 μ g / ml Proteinase K (Merck), followed by three extractions with phenol-chloroform (1:1) and three extractions with chloroform-isoamylalcohol (24:1). The DNA was precipitated with 2.5 volumes ethanol /3,4/. Isolation of RNA followed essentially the technique of Kirby /16/. rRNA was further purified on a 5 - 30 % linear sucrose gradient in 0.1 M NaCl, 0.02 M sodium acetate, 1 mM EDTA, 0.1 % SDS. Iodination of rRNA was performed as described by Prenskey /17/.

7. Hybridization. Hybridization of DNA filters with ¹²⁵I rRNA was done in 50 % formamide-5 x SSC at 40 °C overnight with an RNA concentration corresponding to about 10⁶ cpm / ml (about 0.2 μ g RNA). After hybridization filters were washed several times in 5 x SSC, once in 50 % formamide-5 x SSC,

5 x SSC, incubated for 30 min. at 37 °C with 20 µg / ml RNase A and finally washed once more in 5 x SSC. Autoradiography of the filters was done with a Kodak x - omatic film previously sensitized with a short flash and with a Kodak intensifying screen /18/.

RESULTS

Electron microscopic analysis of Stylonychia macronuclei which were lysed under low ionic conditions and under conditions where shearing was largely avoided revealed short chromatin fragments with typical eucaryotic nucleosomes (Figure 1). The length of these fragments varied between about 3 and more than 60 nucleosomes. Since the nucleosome repeat length of Stylonychia macronuclei was found to be 220 base pairs /11/, the size of the fragments corresponds to 660 to more than 13 000 base pairs or to DNAs with molecular weights between 0.44 to more than 8.7×10^6 dalton. We measured 100 chromatin fragments and found the following length distribution (Table):

Figure 1. Electron micrograph of macronuclear chromatin. The nuclei were washed several times in 10 mM Tris pH 8.0, lysed with 10 mM Tris, 2.5 mM EDTA pH 8.0 and the chromatin was prepared for electron microscopy as described in Material and Methods (x 100 000)

Table. A preparation as shown in figure 1 was used for this table. The molecular weight of the DNA was calculated assuming a nucleosome repeat length of 220 base pairs /11/

Number of nucleosomes	Molecular weight of DNA x 10 ⁶		Number of chromatin fragments
0 - 10	0	1.45	13
11 - 20	1.6	2.9	56
21 - 30	3.04	4.35	22
31 - 40	4.5	5.8	5
41 - 50	6.0	7.26	2
51 - 60	7.4	8.7	1
61 - 70	8.86	10.2	1

The average size of the chromatin fragments found in the macronuclei of *Stylonychia* was found to be 18 nucleosomes corresponding to a DNA size of molecular weight 2.61×10^6 dalton. It has been shown in previous reports /3,4/ that the molecular weight of macronuclear DNA isolated under very different conditions ranges between 0.3×10^6 to 14×10^6 dalton with a maximum around 10^6 to 3×10^6 dalton. The length distribution of chromatin fragments fits these data well.

To analyse macronuclear chromatin further we fractionated the chromatin on 5 - 20 % linear sucrose gradients. As shown in figure 2 under the conditions used macronuclear chromatin banded about in the middle of these gradients. More than 60 % of the total OD₂₆₀ were recovered in the peak fractions. As determined by phase contrast microscopy the pelleted material represented macronuclei not or not completely lysed. To make sure that the material detected in the peak fractions is still chromatin we digested total unfractionated chromatin and a fraction from the sucrose gradient with micrococcal nuclease. Figure 3 shows the banding pattern of these digests after separation on 1.7 % agarose gels. Typical nucleosomal bands can be seen although they are not as clear as digests from whole nuclei. As already expected from this result, analysis of chromatin bound proteins in the peak fractions showed all five histone fractions, plus some high molecular weight

Figure 2. Sucrose gradient of macronuclear chromatin. Chromatin was fractionated on a 5 - 20 % linear sucrose gradient as described in Material and Methods.

nonhistone proteins (Figure 4).

When the DNA of the different peak fractions of the gradient was separated by size on agarose gels it became clear that there exists a direct correlation between size of chromatin and size of its DNA (Figure 5). For example, in the high molecular weight fraction of the gradient only high molecular weight DNA was detected. The DNA of the peak fraction (fraction number 13) had an average molecular weight between 10^6 to 3×10^6 dalton which fits again with the data reported above.

To demonstrate that the chromatin fractions represent real genes and not an artefact due to some nonspecific degradation or shearing occurring during its preparation, we hybridized ^{125}I rRNA to the different chromatin fractions of the sucrose gradient. For this purpose the DNA from each fraction was electrophoresed on a 1.5 % agarose gel (Figure 5) and the DNA was transferred to nitrocellulose filters. Hybridization of labeled RNA to these filters was done as described above. Figure 6 shows an autoradiograph of the hybridized filters. There is a strong hybridization band in fraction 15 in which high molecular weight DNA occurs and weaker hybridization bands in fractions 14 and 13. The molecular weight of the DNA frag-

Figure 3. Nuclease digestion of macronuclear chromatin. Fraction 13 from the sucrose gradient (a) and total unfractionated chromatin was digested with micrococcal nuclease under conditions as described in Material and Methods. After deproteinisation the DNA was electrophoresed on a 1.7 % agarose gel and stained with ethidium bromide.

Figure 4. Chromatin bound proteins. Fraction 13 from the sucrose gradient (a) and total unfractionated chromatin (b) was precipitated with 2.5 volumes ethanol. The precipitate was dissolved in sample buffer and electrophoresed on a 12.5 % Laemmli gel. The gel was stained with 0.25 % Coomassie Brilliant Blue.

ment to which the labeled rRNA hybridized was found to be 4.5×10^6 dalton as expected for ribosomal DNA in the macronucleus of *Stylonychia* /3/. This result clearly demonstrates that the observed chromatin fragments are not created by nonspecific degradation and that rDNA occurs at the high molecular weight side of the chromatin gradient.

DISCUSSION

Using several different approaches we have obtained evidence that macronuclear chromatin of the hypotrichous ciliate

Figure 5. DNA from the peak fractions of the sucrose gradient. Peak fractions were made 1 % SDS, 0.1 M NaCl and precipitated with 2.5 volumes ethanol. The precipitates were dissolved in electrophoresis buffer + 0.1 % SDS and electrophoresed on a 1.5 % agarose gel. Up to 20 μ g DNA was loaded on the gel. a) lambda DNA digested with restriction enzyme EcoRI b) - g) DNA from the fractions 10 - 15 of the sucrose gradient h) total macronuclear DNA.

Stylonychia mytilus occurs in discrete fragments. The observation that the material we worked with was still digestible with micrococcal nuclease to discrete nucleosomal bands together with the demonstration of all five histone fractions showed that we are dealing with real chromatin. Since hybridization with 125 I rRNA always revealed hybridization to a defined fragment, it could be demonstrated that the chromatin fragments obtained by sucrose gradients were not produced by nonspecific degradation of the chromatin but that they represent complete genes. To make sure that possible low molecular weight degradation products were not lost on the gel, we used a high percentage agarose gel and loaded up to 20 μ g of DNA on the gel. This may explain the broadness of the hybridization bands observed with whole macronuclear DNA (Fig. 6 g) and fraction 15 of the sucrose gradient (Fig. 6 f). The observation that some

Figure 6. DNA from the gel shown in figure 5 was transferred to nitrocellulose filters and hybridized with ^{125}I rRNA (25 S + 17 S rRNA). a) - f) fractions 10 - 15 of the sucrose gradient g) total macronuclear DNA h) lambda DNA digested with restriction enzyme EcoRI.

hybridization was also obtained in fractions 13 and 14 of the gradient only demonstrates that the separation of chromatin by size on sucrose gradients is not complete. The length of the chromatin fragments varied between about 3 and 70 nucleosomes, with an average around 18 nucleosomes corresponding to a DNA with molecular weight 2.6×10^6 dalton.

Although we only used rRNA as a probe to demonstrate the existence of "gene-sized" chromatin fragments, the observed length distribution of the chromatin fragments is as would be expected if each "gene-sized" DNA piece occurs in the macronucleonucleus as a "gene-sized" chromatin fragment. No stable linkage of several DNA fragments into larger units via theoretical linker molecules has to be postulated.

Since the kinetic complexity of one macronuclear genome unit is about 3×10^{10} dalton and the average molecular weight of macronuclear DNA is roughly 3×10^6 dalton it can be calculated that about 10 000 different DNA fragments corresponding

to 10 000 chromatin fragments should occur in one macronuclear genome unit /7,8,19,20/. The DNA content of the vegetative macronucleus is 4.5×10^{14} dalton /8/; each DNA or chromatin fragment therefore should be present 15 000 times per macronucleus.

This supposed structure of the macronucleus may also lead to some speculations about ageing of these cells, already suggested much earlier by Fauré-Fremiet /12/ and many others. Most ciliates show an ageing process if they are not allowed to mate. During sexual reproduction a new macronucleus is formed from the syncaryon created by the fusion of two haploid gametic micronuclei (for review of macronuclear development in some hypotrichous ciliates see Ammermann /21/). In Stylonychia mytilus this ageing process starts about 800 - 1 000 divisions after conjugation. We have calculated how long it would take until at least one fragment should be lost from the nucleus of a daughter cell. We assumed 10 000 different fragments, each present 15 000 times per macronucleus. All fragments should be duplicated before division and then be distributed during amitosis in a random statistical fashion. Computer analysis of this situation revealed that after about 6 000 divisions there is a high probability that daughter cells have lost one fragment completely. Although this figure is much higher than expected from experimental data, deleterious effects could well occur before total loss of a particular fragment. In our opinion ageing of Stylonychia by gene loss seems to be an attractive hypothesis and should be examined experimentally.

It should be noted that the structure of the macronucleus as discussed above so far seems to be true only for some hypotrichous ciliates. In Paramecium aurelia /22/ or Tetrahymena /23/, where an analysis of macronuclear DNA has been done, there is strong evidence that the DNA occurs as large molecules; therefore the chromatin also has to be of high molecular weight.

The unique structure of the macronucleus of hypotrichous ciliates should not only allow the isolation and analysis of a complete gene, as has been done already in the related ciliate Oxytricha /2/, but also the isolation of complete genes including their associated proteins. It therefore should provide an

ideal system in which to examine structure and regulation of eucaryotic genes.

Acknowledgments. This work was supported by the VW-foundation and the Deutsche Forschungsgemeinschaft.

REFERENCES

- 1 Prescott, D.M., Murti, K.G. and Bostock, C.J. (1973) *Nature* 242, 596-600
- 2 Lawn, R.M., Herrick, G., Heumann, J. and Prescott, D.M. (1978) *Cold Spring Harb. Symp. Quant. Biol.* 42, 483-492
- 3 Lipps, H.J. and Steinbrück, G. (1978) *Chromosoma (Berl.)* in press
- 4 Elsevier, S.M., Lipps, H.J. and Steinbrück, G.: *Chromosoma (Berl.)* in press
- 5 Lawn, R.M. (1977) *Proc. Nat. Acad. Sci. (Wash.)* 74, 4325-4328
- 6 Lauth, M.R., Spear, B.B., Heumann, J. and Prescott, D.M. (1976) *Cell* 7, 67-74
- 7 Ammermann, D., Steinbrück, G., v.Berger, L. and Hennig, W. (1974) *Chromosoma (Berl.)* 45, 401-429
- 8 Ammermann, D., Steinbrück, G., Haas, J. and Hellmer, K.H.: submitted
- 9 Lipps, H.J., Sapra, G.R. and Ammermann, D. (1974) *Chromosoma (Berl.)* 45, 273-280
- 10 Lipps, H.J. and Hantke, K.G. (1975) *Chromosoma (Berl.)* 49, 309-320
- 11 Lipps, H.J. and Morris, N.R. (1977) *Biochem. Biophys. Res. Commun.* 74, 230-234
- 12 Grell, K.G. (1973) *Protozoology*, Springer Verlag, Berlin
- 13 Miller, O.L. and Beatty, B.R. (1969) *Science* 164, 955-957
- 14 Southern, E.M. (1975) *J. Mol. Biol.* 98, 503 - 517
- 15 Laemmli, U.K. (1970) *Nature* 227, 680
- 16 Kirby, K.S. (1968) in: *Meth. Enzymol.* 12 (Grossmann, L. and Moldave, K. eds.) pp. 87-98, Academic Press, New York
- 17 Prenskey, W. (1976) in: *Methods Cell Biol.* 13 (Prescott, D.M. ed.) pp. 121-151, Academic Press, New York
- 18 Laskey, R.A. and Mills, A.D. (1975) *Eur. J. Biochem.* 56, 335-341
- 19 Kloetzel, J.A. (1970) *J. Cell Biol.* 47, 395-407
- 20 Prescott, D.M. and Murti, K.G. (1974) *Cold Spring Harb. Symp. Quant. Biol.* 37, 609-618
- 21 Ammermann, D. (1971) *Chromosoma (Berl.)* 33, 209-238
- 22 Cummings, D.J. (1975) *Chromosoma (Berl.)* 53, 191-208
- 23 Engberg, J., Nilsson, J.R., Pearlman, R.E. and Leick, V. (1974) *Proc. Nat. Acad. Sci. (Wash.)* 71, 895-898