

Fractionation of the gene-size macronuclear chromatin fragments of the binucleated eukaryote *Oxytricha*

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Summary

The macronuclear chromatin of *Oxytricha nova* consists of chromatin fragments which are fully soluble in 0.2 mM EDTA and whose DNA length varies from 500–25 000 bp. The DNA migrates electrophoretically as a series of discrete bands, with specific genes present in only one or a few bands. The chromatin fragments are composed of nucleosomes and migrate electrophoretically in proportion to their DNA length. These results suggest schemes for the fractionation of undigested chromatin in order to enrich for specific genes, facilitating analysis of changes in chromatin structure associated with changes in gene expression.

Introduction

The chromatin of most eukaryotic nuclei, including the micronuclei of the hypotrichous ciliates, consist of chromosome-size DNA molecules organized into long nucleosomal chains (1). Unless digested with nucleases or mechanically sheared, such chromatin is insoluble in most buffers. In contrast, the macronuclear chromatin of the hypotrichous ciliates, such as *Oxytricha*, exhibits a nucleosomal substructure but consists of DNA fragments 500–25 000 bp in length and is highly soluble in low ionic strength buffers (2, 3). In this report we describe our utilization of these unique features to develop procedures for the fractionation of soluble macronuclear chromatin without the use of nucleases or mechanical shearing.

Experimental procedures

Cell culture

The *Oxytricha nova* cell line used here was a gift from David Prescott (University of Colorado, Boulder, Colorado). The methods used to maintain the cell stocks and to grow large-scale cultures in 50-liter fermentation vats have been recently described (4).

Chromatin isolation

The chromatin isolation procedures used here have been described in detail elsewhere (5). The basic procedure consists of the following steps: 1) filter cultures through cheesecloth to remove aggregated algae; 2) utilize a continuous flow centrifuge to sediment cells onto a cushion of 10% sucrose, 10 mM Tris (pH 7.0), 50 mM bisulfate, 0.12% spermidine, 0.5% Triton X-100, and 1 mM PMSF

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(buffer A); 3) resuspend nuclei in buffer A without the sucrose (buffer B); 4) separate macronuclei and micronuclei by differential centrifugation in 10% sucrose gradients in buffer B; 5) band macronuclei according to buoyant density in a 40–50% metrizamide gradient in buffer B; 6) dilute samples seven-fold with buffer B and pellet the nuclei; 7) resuspend the pellet in buffer B; 8) dialyze overnight against 10 mM Tris-HCl (pH 7.0), 60 mM NaCl, 0.2 mM EDTA, 0.1 mM PMSF (buffer C) to remove the spermidine; 9) dialyze against 0.2 mM EDTA (pH 7.0) to lyse nuclei; 10) complete lysis by vigorous pipetting; 11) remove insoluble material by centrifugation. The soluble chromatin (supernatant) in 0.2 mM EDTA (pH 7.0) was stored at 4 °C.

Solubility studies

The solubility of the macronuclear chromatin was determined by adjusting the buffer conditions to the described composition and measuring the A_{260} of the supernatant following brief centrifugation. The 0.2 mM EDTA–chromatin solution was adjusted by adding $MgCl_2$, spermidine, KCl, or NaCl from a concentrated stock solution. The mixture was vortexed vigorously and left at room temperature for 20 min. The insoluble material was pelleted by centrifugation in an Eppendorf microfuge for 15 min. The absorbance of the supernatant at 260 nm was measured with a Zeiss UV spectrophotometer. All samples were run in triplicate.

Samples were prepared for DNA gel electrophoresis by precipitating the soluble chromatin with 3 volumes of absolute ethanol, centrifugation for 15 min in an Eppendorf microfuge, lyophilizing the pellet, and resuspending the pellet in 20% Ficoll 400, 0.5% sarkosyl, and 0.02% bromophenol blue (DNA sample buffer). The proteins were digested overnight at 37 °C with 200 μ g/ml proteinase K. Electrophoresis of the DNA was performed in agarose gels prepared as described by Levinger et al. (6), using buffer E (40 mM Tris-HCl, 5 mM Na acetate, and 1 mM EDTA, pH 7.9).

Sucrose gradients

Macronuclear chromatin in 0.2 mM EDTA was dialyzed overnight against 60 mM NaCl, 2 mM EDTA, 5 mM Tris-HCl (pH 7.0) (buffer D). One ml of a 700 μ g/ml chromatin suspension was layered

over a linear 5–20% sucrose gradient in buffer D. Centrifugation was in a SW41 rotor at 25 000 rpm for 3 hr at 4 °C. The gradient was fractionated using an Isco gradient fractionator and the absorbance at 254 nm was monitored. For DNA gels, aliquots were incubated at 37 °C for 1 hr with 200 μ g/ml proteinase K in DNA gel sample buffer. The DNA electrophoresis in agarose gels was performed as described by Levinger et al. (6).

Two-dimensional gel electrophoresis

The two-dimensional chromatin/DNA gels were adapted from those described by Levinger et al. (6). In the chromatin first dimension, samples were prepared by the addition of one tenth volume of 90 mM Tris, 2.5 mM EDTA, 90 mM boric acid (pH 8.3) and electrophoresed in 1.0% agarose containing 3.0 mM EDTA and 2 mM Na acetate (pH 5.5). A 3.0 mm wide strip was excised from the central region of the chromatin containing lane and soaked two hours in 0.5% sarkosyl and 0.025% bromophenol blue. The second dimension gel consisted of 1% agarose containing 40 mM Tris-HCl (pH 7.9), 5 mM Na acetate, 1 mM EDTA and 0.5% sarkosyl. Both dimensions were run 16 hr at 70 volts using water cooling (r.t.) and buffer exchange.

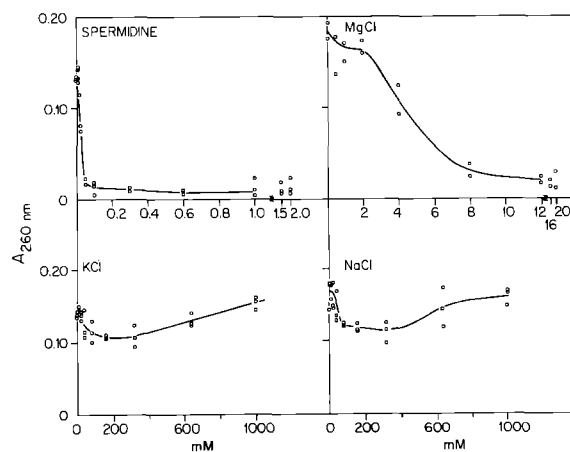


Fig. 1. Solubility of macronuclear chromatin. The indicated levels of spermidine, Mg, K, or Na ions were added to macronuclear chromatin in 0.2 mM EDTA. The samples were incubated at room temperature for 20 min and the insoluble material removed by centrifugation. The absorbance at 260 nm was determined for the soluble fraction. A similar study was performed using $CaCl_2$; the solubility curve resembled that of $MgCl_2$, but was almost twice as effective a precipitating agent on a molar basis.

Results

The overall chromatin recovery rate of our isolation procedure is typically 60–80%. The major loss of material occurs in the macronuclear purification by buoyant density sedimentation in metrizamide gradients. The recovery from the gradients is about 70–85%. The second major loss occurs during the differential sedimentation steps which segregate the macronuclei and micronuclei. The segregation is very effective, leaving less than one micronuclei per

100 macronuclei (0.025% micronuclear chromatin contamination), and yields an 80–95% recovery. The efficiency of recovery of soluble macronuclear chromatin following nuclear lysis in 0.2 mM EDTA and sedimentation of the insoluble material is 90–100%.

Our earlier work and that of others demonstrate that the macronuclear chromatin has normal nucleosomal substructure, with a nucleosome repeat length of 220 bp (3, 5, 7). We observe four inner histones analogous to histones H2A, H2B, H3 and

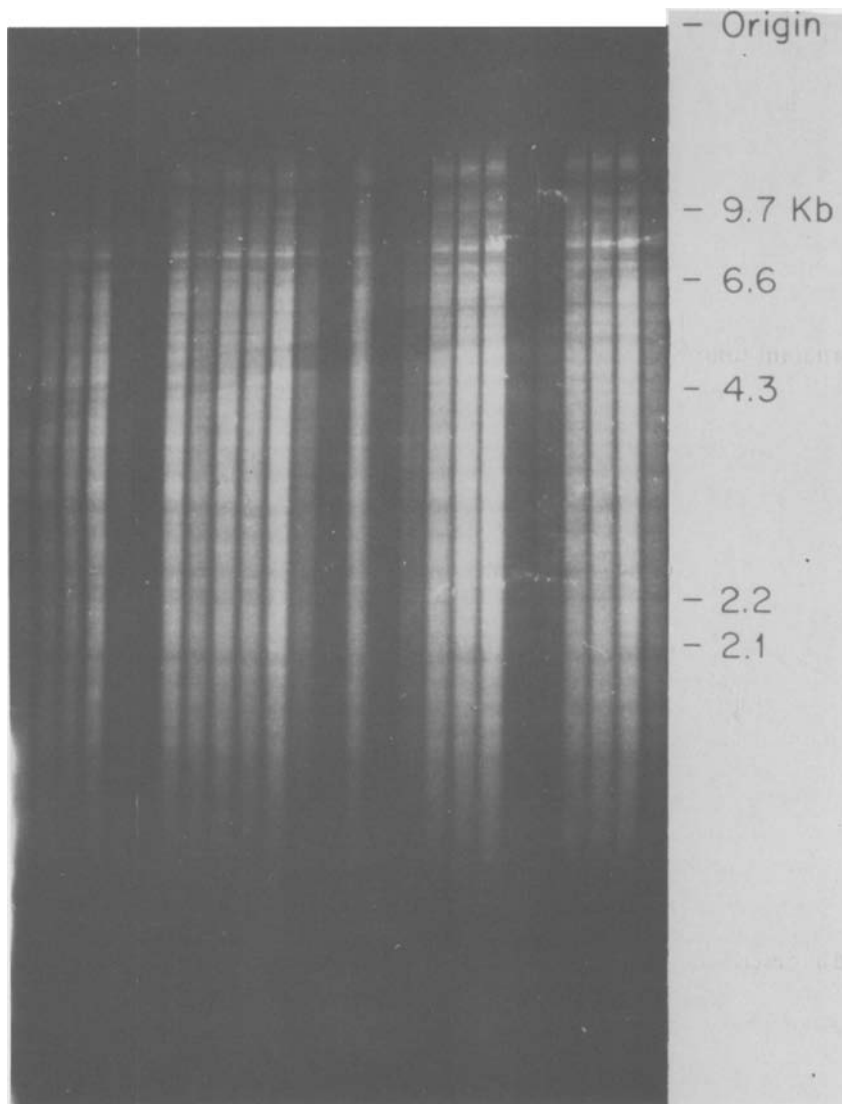


Fig. 2. DNA gel (1.5% agarose) of macronuclear DNA precipitated from the soluble fractions in solubility studies described earlier. From right to left: lanes 1–5: 0, 0.5, 1.0, 2.0, 4.0 mM CaCl_2 ; lanes 6–11: 0, 0.5, 1.0, 2.0, 4.0 and 8.0 mM MgCl_2 ; lanes 13–19: 0, 10, 20, 40, 80, 160 and 320 mM NaCl ; and lanes 20–25: 0, 10, 20, 40, 80, and 160 mM KCl .

H4 of other eukaryotic cells, but no classical H1.

The solubility of the macronuclear chromatin with varying spermidine, $MgCl_2$, KCl and NaCl concentrations is shown in Fig. 1. One hundred percent solubility corresponds to an A_{260} in the range of 0.14–0.18 O.D. units. This chromatin precipitates almost completely at low spermidine concentrations (0.05 mM or 0.003%). In the $MgCl_2$ solubility decreases much more gradually, with 50% solubility at about 5 mM. In the presence of

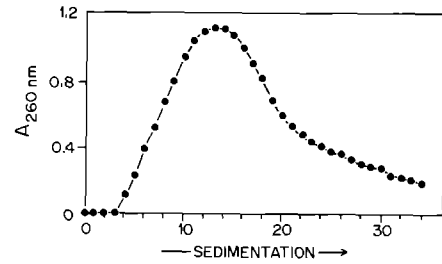


Fig. 3. Profile of the macronuclear chromatin distribution as measured by its DNA absorption at 260 nm from a 5–20% sucrose gradient centrifuged at 38 000 rpm for 3 hr in an SW41 rotor.

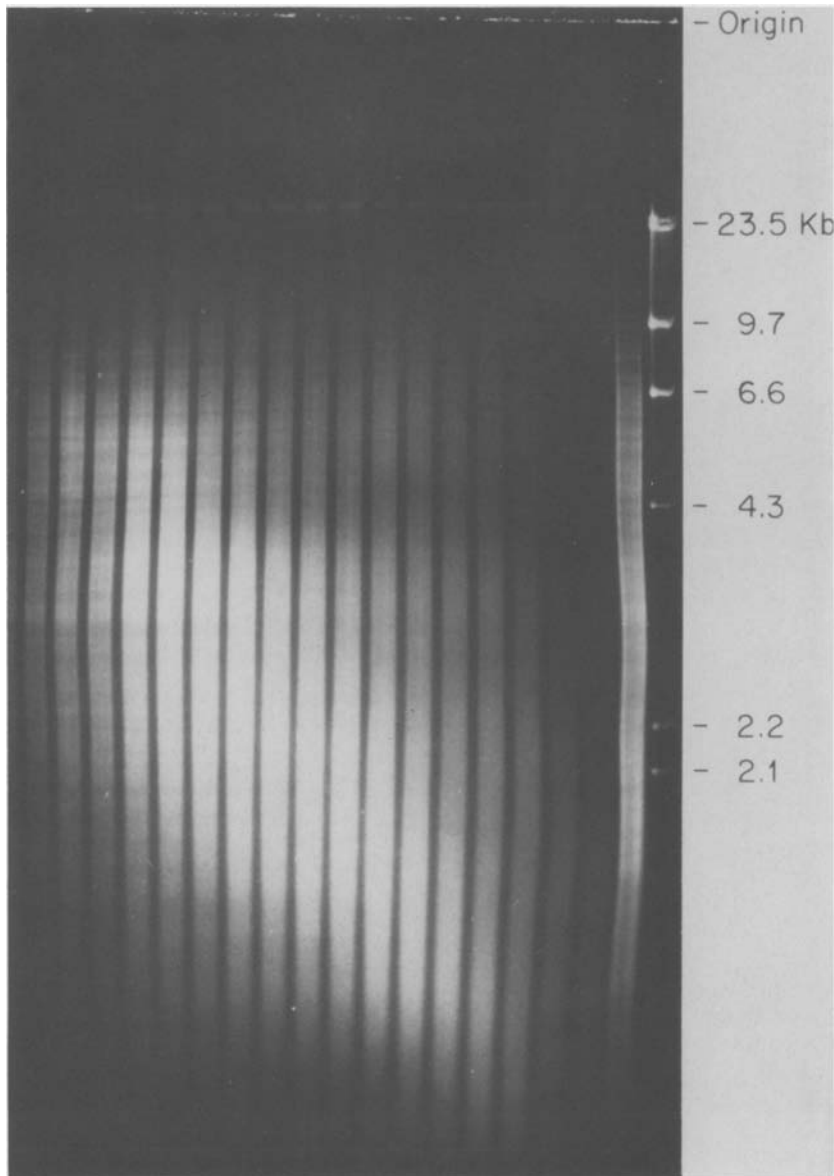


Fig. 4. DNA gel (1.5% agarose) demonstrating the DNA distribution of the fractions 5–21 of the sedimentation shown earlier. From right to left, Hind III digested DNA marker, total macronuclear DNA, followed by fractions 5–22.

KCl or NaCl, the solubility decreases by about 30% at 200–300 mM, then increases to near 100% solubility at 1.0 M.

DNA gel electrophoresis was performed on the DNA of the soluble chromatin in the MgCl₂, KCl and NaCl solubility experiments to test for the preferential precipitation of specific genes. As shown in Fig. 2, there was no apparent difference in the DNA length distribution at various concentrations of any of the ions.

The results of the fractionation of macronuclear chromatin based on sedimentation in sucrose velocity gradients are shown in Figs. 3 and 4. It is clear from the DNA gels of the central 18 fractions (Nos. 5–22) of the gradient that there is a fractionation of chromatin fragments by size, but that specific DNA bands are present across several sucrose fractions. In Fig. 5, we plot the relative distribution of a segment of the profile in the regions of 7 500 bp (Fig. 5a), 2 200 bp (Fig. 5c) and 1 000 bp (Fig. 5e) as a function of gradient fraction. The width of the segment analyzed corresponds to about 1 mm on the 200 mm gel. The enrichment for these bands are shown in the subsequent plots (Figs. 5b, 5d, 5f). These data indicate that this sedimentation procedure does provide a crude fractionation of chromatin based on size, but the enrichment for genes in specific regions is less than 3.5-fold. We have also sedimented chromatin into sucrose with varying concentrations of EDTA (2–12 mM) and NaCl (0–60 mM) and observe changes in the gradient profile, but little or no decrease in the spread of specific genes across the gradient.

The electrophoretic mobility of unfractionated macronuclear chromatin is shown in Fig. 6a. The smallest fragment electrophoreses as expected for dimer nucleosomes. Based on the 500–25 000 bp size distribution on the DNA gels (Figs. 2 and 4) and the 220 bp nucleosome repeat length, we estimate a size range from dimer to 114-mer. Using similar chromatin isolation procedures, undigested micronuclear chromatin and avian erythrocyte chromatin does not even enter the gel. We excised a narrow strip from the central region of the chromatin lane, deproteinized the chromatin, and ran the material in a second DNA dimension. The results, shown in Fig. 6b, were quite dramatic. The diagonal line indicates a good correlation between electrophoretic mobility of the chromatin and its corresponding DNA length. In cases in which

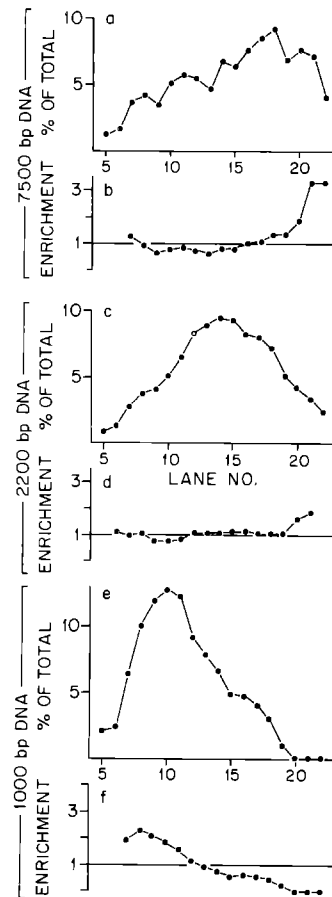


Fig. 5. Analysis of the DNA distribution in fractions 5–22 of the sucrose gradient described in the previous figure. Densitometer tracings were made from a negative of the profiles of each fraction. The percent represented by a 1 mm wide band corresponding to 7 500, 2 200, and 1 000 bp fragment are plotted in Figs. 5a, c, and e, respectively. The enrichment ratios for each size fragment are shown in Figs. 5b, d, and f. Ratios were calculated as the relative increased content of a particular size fragment, compared to its content in unfractionated chromatin; a ratio of 1.0 represents no enrichment.

chromatin aggregation was observed, the diagonal line was replaced by a smear. A spot was sometimes noticed along the diagonal line for some of the more prevalent bands, such as the 7 500 bp rRNA gene.

Discussion

Our data clearly indicate the utility of macronuclear chromatin of the hypotrichs for the fractionation of specific genes in their soluble chromatin

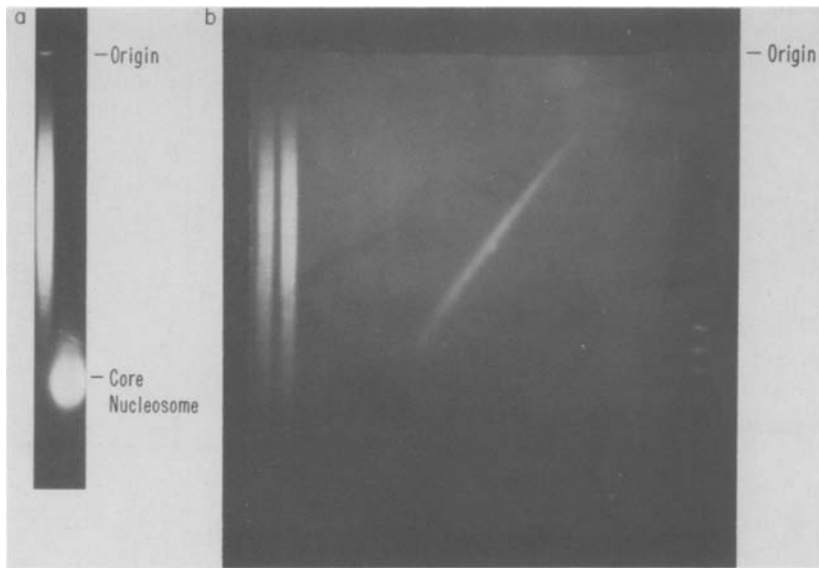


Fig. 6. a. Chromatin gel of macronuclear (left) and chicken 146 bp core nucleosome particles (right); b. Two-dimensional chromatin/DNA gel as described by Levinger et al. (6). From left to right, lane 1 – macronuclear DNA; lane 2 – macronuclear chromatin; central region – DNA from a first dimension macronuclear chromatin gel; lane 3 – Hae III digested ϕ X molecular weight marker.

form. To obtain gene-size fragments from most other eukaryotic nuclei, the chromatin must be digested with nucleases. This digestion may perturb the chromatin organization (Cartwright et al., 1983). The presence of macronuclear genes as relative short DNA fragments *in vivo* circumvents the need for nuclease digestion. Also, the nuclease cleaves each gene into a wide range of sizes and with little register, whereas the fragments containing each type of macronuclear gene are homogenous in length and sequence (3, 8, 9, 10, 11, 12, 13).

The solubility properties of the macronuclear chromatin (Fig. 1) indicate that under certain ionic conditions we can precipitate a fraction of the chromatin, for example: 30% at 3 mM MgCl₂, 0.2 mM EDTA (pH 7.0); 200 mM KCl, 0.2 mM EDTA (pH 7.0); or 200–300 mM NaCl, 0.2 mM EDTA (pH 7.0). This precipitation proved not to be the result of specific genes being preferentially precipitated (Fig. 2). Evidently, salt-dependent solubility is not a function of DNA (and chromatin) length. Thus, this scheme does not appear applicable for the fractionation of specific genes, but may yet prove useful for enrichment of transcriptionally active chromatin fragments, or chromatin species with varying protein content.

The analysis of macronuclear chromatin fractionated by size using sucrose velocity gradients (Figs. 3–5) indicates a very limited enrichment for the relatively large or small genes (a 2.5-fold enrichment for 3 fractions containing 19% of the total 7 500 bp material and a 2-fold enrichment for 5 fractions containing 54% of the total 1 000 bp material) and no enrichment for medium size (2 200 bp) genes. Thus, this method offers only limited potential as a fractionation scheme.

The narrow diagonal line seen in the 2D chromatin/DNA gel (Fig. 5) indicates a very good correlation between the electrophoretic mobility of the chromatin fragments and their respective DNA length. These results suggest that preparative electrophoretic methods could be employed to enrich for specific populations of genes in their soluble chromatin form. Of particular interest would be the 7 500 bp band, since its primary constituent is the rDNA gene (11). This band represents about 0.2% of the genome. Since the region within 1 mm on each side of the fraction represents about 0.4% of the total macronuclear DNA, this population would contain about 50% DNA from the 7 500 bp band. Average sized gene, such as actin (2 200 bp), represent one of 17 000 different gene types (or 0.006% of

the total). By fractionation of 1 mm segments containing about 0.24% of the total macronuclear DNA, we could get a (400)fold enrichment for this gene. Thus, we could isolate a fraction containing 2.4% actin genes. Repeated cycles of preparative gel electrophoresis should further improve the enrichment.

The tight correlation between the electrophoretic mobility of the chromatin fragments and their respective DNA also has potential as an assay for changes in chromatin structure. Possible applications include assaying for structural changes in chromatin associated with changes in gene expression and monitoring the addition or removal of nucleoproteins. In these cases, Southern blots (14) of the two-dimensional chromatin/DNA gels will be hybridized to genes of interest to detect deviations from the diagonal line. The only biochemical assays currently capable of detecting changes in chromatin organization with changes in transcriptional activity are the DNase I assays. Weintraub and Groundine (15) have demonstrated that active chromatin is hypersensitive to DNase I. Wu et al. (16) have shown a DNase I hypersensitive site on the 5' end of transcribing genes. Since it has been suggested (1) that DNase I may alter the chromatin conformation it is assaying, an independent and non-destructive assay could be of great value. We are currently seeking inducible genes in *Oxytricha* to test the ability of these two-dimensional gels to detect changes in transcriptional activity.

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