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Concanavalin A-reactive nuclear matrix glycoprotein

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ABSTRACT

The binding capacity of concanavalin A (Con A) to condensed euchromatin and heterochromatin was investigated in chicken erythrocyte nuclei (CEN), mouse liver cells, *Zea mays mays* meristematic cells and *Drosophila melanogaster* polytene chromosomes after 4 N HCl hydrolysis to determine whether binding was preferentially occurring in bands and heterochromatin. Dry mass (DM) variation was investigated in CEN by interference microscopy. Feulgen and Con A reactions were employed for all materials to correlate the loci of the two reactions. Quantifications and topological verifications were carried out by video image analysis (high performance cytometry). It was observed that 4 N HCl hydrolysis caused an important DM loss in CEN leaving a level corresponding to the average DNA DM content. In this material, Con A binding was restricted to the nuclear envelope, which reinforces the idea of the absence of a nuclear matrix in these cells. The other cell types exhibited a correspondence of Feulgen-positive and Con A-reactive areas. The Con A reaction was highly positive in the condensed chromatin areas and heterochromatin. This fact led us to speculate that Con A-positive proteins may play a role in the chromatin condensation mechanism, endowing this structure with physico-chemical stability towards acid hydrolysis and contributing to its rheological properties.

INTRODUCTION

It would be relevant to demonstrate the presence of concanavalin A (Con A) binding to glycoprotein topologically located in condensed euchromatin as well as in heterochromatin. Strong acid hydrolytic treatment, as used to perform the Feulgen reaction, can be assumed to leave some nuclear matrix protein components, at least hypothetically.

The nuclear matrix has been defined mainly operationally using relatively mild methods. It has been described as an acidic group of proteins that make up a cell nucleus inner framework structure and is associated with a granular and fibrous internal matrix structure (Berezney and Coffey, 1974, 1977). Part of these proteins have been identified as glycoproteins (GLP). Some of them have lectin-binding capacity, and are especially reactive to Con A. A GLP of the nuclear matrix reactive to Con A has been identified biochemically, and is sometimes reported to link preferentially to euchromatin or to condensed chromatin. Conflicting reports have been published (Rizzo and Bustin, 1977; Stein *et al.*, 1981; Kan and Silva, 1986; Vannier-Santos *et al.*, 1991; Ferraro *et al.*, 1994).

The controversial results appear to be due to different methodological approaches and confusion in understanding the differences between heterochromatin and merely condensed chromatin. Heterochromatin has biochemical (molecular), structural and functional characteristics that differentiate it from a plain condensed state of euchromatin.

Heterochromatin, especially of the constitutive type, has been properly conceptualized in terms of morphological chromosomal parts, molecular composition (repetitive DNA sequences, satellite DNA, C-band response, higher physico-chemical stability towards acid hydrolysis, and H4 histone acetylation)

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and relative gene silencing ((Mello, 1978, 1979, 1983; John and Miklos, 1979; Mello and Vidal, 1980; Turner *et al.*, 1992; Kellum and Alberts, 1995; Kellum *et al.*, 1995; Murphy and Karpen, 1995).

Observations that BHK-21 culture cells show Con A binding to condensed chromatin especially in apoptotic condensed nuclei (Vidal and Maria, 1994) raise the question of the participation of some GLP in the recognition of repetitive sequences, in the localization and physico-chemical stabilization of chromatin, and in preferential binding to merely condensed chromatin or heterochromatin. Considering that heterochromatin has a higher physico-chemical stability toward HCl hydrolysis and that this procedure removes RNP, histones, and probably some other nuclear proteins from the cell nuclei (Mello *et al.*, 1976; Mello, 1978, 1979, 1983; Mello and Vidal, 1980; Mello and Cordeiro, 1985), it would be worthwhile to search for Con A-binding sites to chromatin after a harsh HCl treatment similar to that used in the Feulgen reaction.

In this type of research, chicken erythrocyte nuclei are used as a control for the methodological approach, as a model of progressive chromatin condensation, and for nuclei in which there is no nuclear matrix (LaFond and Woodcock, 1983; Verheijen *et al.*, 1988). Mouse liver nuclei, *Zea mays mays* cells, and polytene chromosomes were used as models for the study of Con A-binding ability after 4 N HCl hydrolysis, since they are known to contain typical heterochromatic clumps and/or band/interband structures.

The purpose of the present study was to determine the possible capacity of different Con A bindings to euchromatin and/or heterochromatin, as well as, other types of condensed states of chromatin supra-organization under conditions used to characterize the remaining Con A-reactive proteins as being 4 N HCl stable from a physico-chemical point of view. Biochemical attempts to characterize the remaining proteins were outside the scope of the present research.

MATERIAL AND METHODS

Chicken erythrocyte smears were obtained from venous blood. Imprints of mouse liver cells were prepared on the surface of clean, fat-free slides.

Root tips of *Z. m. mays* were obtained from seedlings germinated under constant humidity and room temperature. When the roots were 4 mm long, they were cut, fixed, and squashed in a drop of 45% acetic acid solution. In addition, the coverslips were removed by freezing in liquid nitrogen.

Polytene chromosomes were obtained from the salivary glands of *Drosophila melanogaster*'s third instar larvae. The glands were squashed in 45% acetic acid similarly to *Z. m. mays*.

All materials were fixed with acetic acid: ethanol (1:3 v/v) for 3-5 min. Only the root tips were fixed for 15 min, because of their cellulose walls.

Chicken erythrocyte smears, liver cell nuclei and polytene chromosomes were subjected to the Feulgen reaction, using 4 N HCl at 28°C for 90 min in a hydrolytic bath (Mello and Vidal, 1978, 1980; Mello, 1979, 1983).

The Con A reaction for glucose/mannose was performed according to Kiernan (1981), and was preceded by 4 N HCl hydrolysis like that used in the Feulgen reaction, also for 30 min.

Dry mass (DM) values were obtained for the unstained material using a Zeiss Pol interferential photomicroscope (10/II objective, optovar II and appropriate condenser). Optical path differences (opd) were measured by Senarmont's method DM (in picograms) was calculated using the formula DM = opd . A/χ ($\chi = 100\ \alpha$) (Vidal, 1977). High performance image analysis cytometry was carried out using Global Lab Image, Data Translation Inc. System. For interferometric conditions, 1 squared-pixel corresponds to 0.1232 μm^2.

The stained material was submitted to image analysis with a Zeiss photomicroscope equipped with a Pol-Neofluar 25/0.60 objective, 1.25 optovar, and 1.4 condenser. A 12 volt 60 watt lamp connected to a stabilizer was employed as a light source. Conversion of pixels to μm was done using a micrometer slide as standard. The conversion of gray levels into absorbance or transmittances was calculated using the Minitab program. Average gray level values were converted into average absorbances (OD), and total gray level values were converted into IOD, i.e., total or integrated absorbances.

RESULTS

The interferometric measurements of chicken erythrocyte nuclei revealed that 4 N HCl hydrolysis caused a reduction in area, and a loss in DM probably as a result of histone and some non-histone protein removal. Table I depicts these data and those for DNA DM calculated using values published by Altman and Katz (1976).

ANOVA of interferometric measurements compared to Feulgen data revealed that HCl hydrolysis

Table I.A - DNA dry mass (pg) for chicken erythrocyte nuclei calculated by interferometry.

Before 4 N HCl (pg)	After 4 N HCl (pg)	Loss (%)
5.52	2.957	53.57

Table I.B - DNA dry mass (pg) for chicken erythrocyte nuclei calculated from the data published by Altman and Katz, 1976

x	n	s	C.I.
2.55	11	0.425	0.896

caused a highly significant reduction in nuclear area (Table II). On the other hand, area averages in squared micrometers measured after the Feulgen reaction and by image analysis using interferometry, as expected, did not differ statistically (Table II-C) despite the fact that the values for Feulgen-stained nuclei were slightly higher and less dispersed than those for unstained nuclei treated with 4 N HCl. Dry mass loss in total grey level measurements corresponding to DM was highly significant (F = 884, 76, d.f. = 259).

Erythrocyte nuclei showed weak Con A binding, which was more evident close to the nuclear envelope. This finding was more clearly visible in preparations subjected to acid hydrolysis. It should be mentioned that chicken erythrocyte nuclei subjected to HCl and TCA extractions, and then treated with a fast green solution at pH 2.7, also stained as a thin layer close to the nuclear envelope.

Image analysis of Feulgen-stained liver cell nuclei was consistent with previously reported data and showed polyploid nuclei and nuclear areas corresponding to 2C, 4C and 8C ploidy levels (Vidal *et al.*, 1973, 1994). Chromocentral constitutive heterochromatin appeared as conspicuously condensed bodies easily segmented by image analysis methods (Figures 1 and 2). The best results of the Con A-binding tests were after 30 min 4 N HCl hydrolysis, afterward, the heterochromatic areas bound Con A conspicuously, whereas the other nuclear structures stained slightly, and sometimes showed a frame aspect (network) (Figure 3).

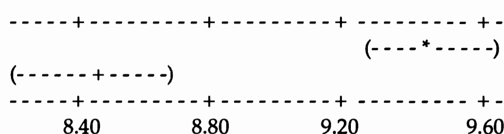
The heterogeneity of chromatin density, expressed as standard deviation (SD) in total pixels of the histograms per nuclear area, was higher for Feulgen-stained material than for Con A-positive areas (Table III). This is due to the fact that the condensed chromatin areas in Con A-stained nuclei were smaller than those of the Feulgen-stained ones (Table IV); even though, the total nuclear area did not differ (Table V). Also, no differences in average absorption (OD) were found (Table VI) despite the fact that the two reactions stain completely different biochemical cell nuclei components. This mathematical descriptor supports the inference that both stainings have the same topography.

Table II.A - ANOVA (Minitab) for area data from chicken erythrocyte nuclei.

F = 25.56.

Level	N	Mean	SD
μm^2 ar	151	9.413	1.258
μm^2 HCl	109	8.508	1.629

Individual 95% CIs for mean based on pooled SD



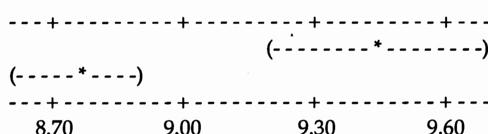
Pooled SD = 1.425
μm^2 ar = Area obtained by interferometry for chicken erythrocyte nuclei before 4 N HCl hydrolysis; μm^2 HCl = area obtained by image analysis of interferometry for nuclei after 4 N HCl hydrolysis.

Table II.B - ANOVA for area data from chicken erythrocyte nuclei.

F = 17.47

Level	N	Mean	SD
μm^2 ar	151	9.413	1.258
μm^2	354	8.780	1.670

Individual 95% CIs for mean based on pooled SD



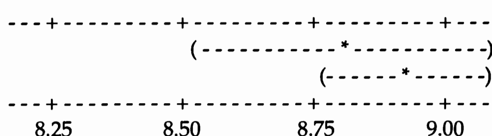
Pooled SD = 1.559
μm^2 ar = Area obtained by interferometry in the absence of hydrolysis; μm^2 = area obtained after the Feulgen reaction.

Table II.C - ANOVA calculated for area data obtained after 4 N HCl hydrolysis compared to data obtained after the Feulgen reaction.

F = 2.24.

Level	N	Mean	SD
μm^2 HCl	109	8.508	1.629
μm^2	354	8.780	1.670

Individual 95% CIs for mean based on pooled SD



Pooled SD = 1.661.

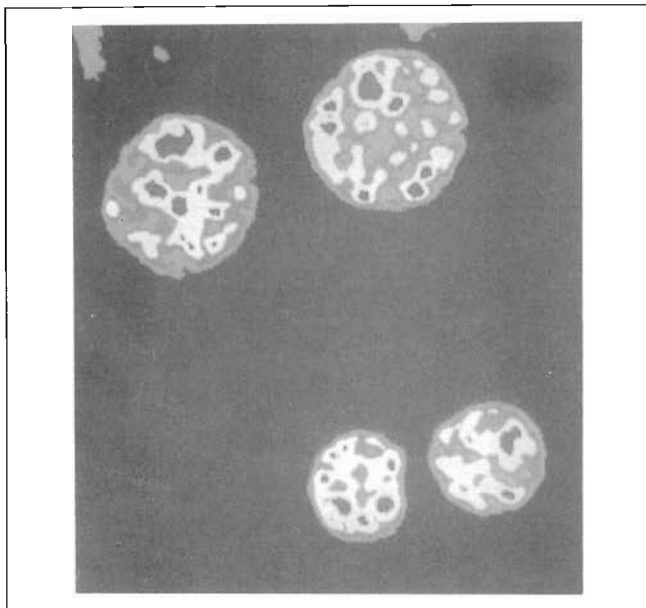


Figure 1 - Feulgen-stained liver cell nuclei photographed with a color monitor. Pseudocolors were used to depict the chromocentral condensed chromatin in red, and less condensed chromatin areas in yellow and light blue. Neofluar 25/0.60 objective, 1.25 optovar; image analysis using the GLI-Data Translation Inc. System (GLI-DT).

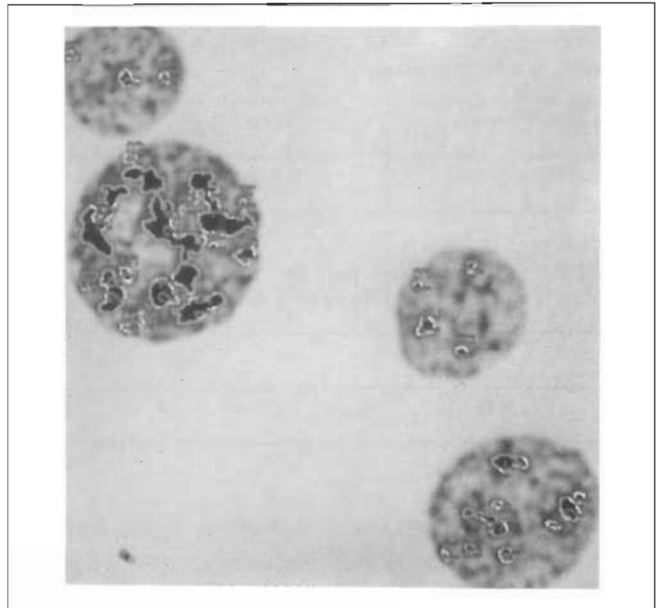


Figure 2 - Feulgen-stained liver cell nuclei photographed with a color monitor, depicting the chromocenter image after segmentation and measurement with GLI-DT.

Similar results were obtained for root tips of *Zea mays mays*. Con A-stained areas were detected in condensed telomere areas and in C-constitutive heterochromatin (Figure 4).

Polytene chromosomes also showed Con A-binding capacity. The characteristic band and interband structure was detected using both the Feulgen reaction and the Con A method (Figure 5a,b).

A ring structure was found to be stained peripherally at some bands after the Con A assay (Figure 5a,b). This agrees with reported immunofluorescence and *in situ* hybridization (Urata *et al.*, 1995) and atomic force microscopy data (Jondle *et al.*, 1995). In fact, the bands showed a distinct peripheral concentration of Con A-positive protein depicting a variable morphology (Figure 5a,b). Image analysis permitted accurate band and interband segmentation of Feulgen-stained and Con A-positive areas of the polytene chromosomes. Figures 5a and 5b depict the staining profile of these chromosomes after Con A reactions. Bands and chromocenters showed the lowest gray level, i.e., the highest average absorbance (OD).

In Feulgen-stained samples, there was no difference in the degree of condensation among bands, which are herein denominated condensed chromatin areas, and chromocenters, as expressed in terms of OD. The difference in OD averages was 0.066 ($F = 2.02$, $P = 0.195$), despite the fact that chromocenter sample weight was small, due to difficulties in segmentation and subsequent measurements. In contrast, as expected, interbands, as noncondensed chromatin areas, showed the lowest statistically significant OD value, 0.31 ($F = 33.50$) compared to the OD of the condensed areas. Nevertheless, a nonhomogeneous absorption distribution was detected in the bands, a situation which was also verified in the Con A-stained chromosomes (Figure 5). No significant differences in OD values were detected between condensed areas (bands) and chromocenters. The average difference was 0.0051 ($F = 0.07$).

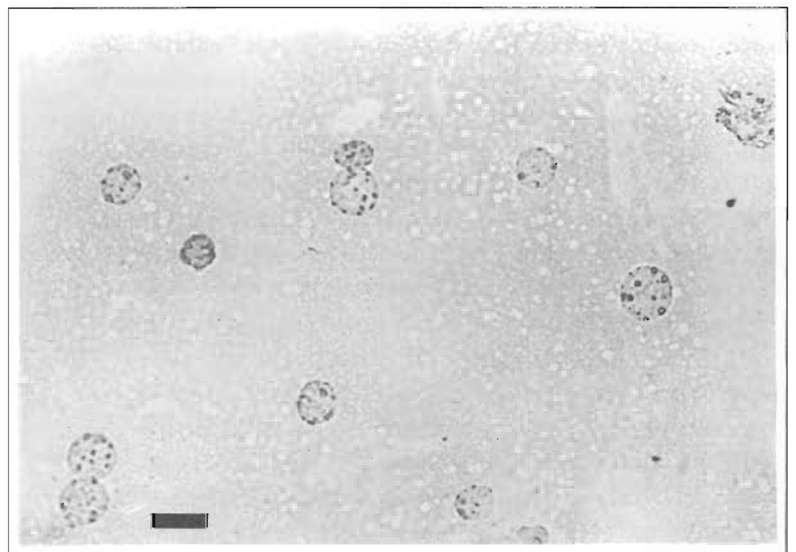


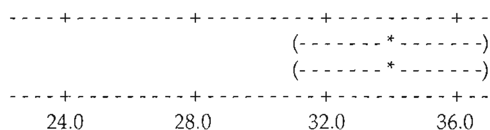
Figure 3 - Con A binding to condensed chromatin in liver cell nuclei. After 4 N HCl hydrolysis, the chromocenters exhibited conspicuous Con A-binding capacity. The bar corresponds to 15 μm.

Table III - ANOVA of the standard deviation of average gray values in pixels from total pixel histograms obtained in the global image analysis system.

F = 9.93

Level	N	Mean	SD
(A) Feulg SD	14	32.444	5.932
(B) Con A SD	14	25.701	5.381

Individual 95% CIs for the mean based on pooled SD



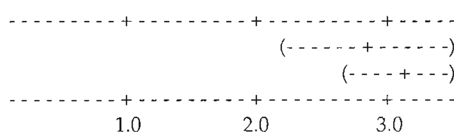
(A) = Values from liver cell nuclei after the Feulgen reaction; (B) = Values after the Con A reaction in liver cell nuclei.

Table IV - ANOVA of condensed chromatin in liver cell nuclei.

F = 26.79

Level	N	Mean	SD
μm^2 cond	138	0.793	0.647
ar 1 μm^2	266	2.964	4.90

Individual 95% CIs for mean based pooled SD



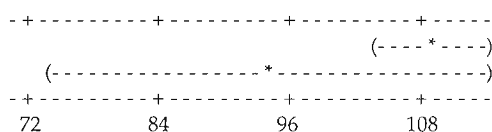
μm^2 cond = Area covered by the Con A reaction in condensed chromatin; ar 1 μm^2 = are covered by condensed chromatin after the Feulgen reaction.

Table V - ANOVA of liver cell nuclear areas.

F = 0.18

Level	N	Mean	SD
ar μm^2	182	88.63	42.02
μm^2tl	14	93.47	32.50

Individual 95% CIs for mean based on pooled SD.



Pooled SD = 41.45

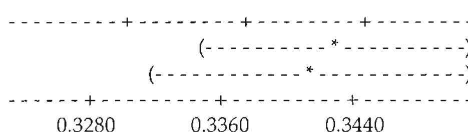
ar μm^2 = Area corresponding to Con A. μm^2tl = Total areas corresponding to the Feulgen reaction.

Table VI - ANOVA of optical density for Feulgen-DNA and for Con A-reactive chromative chromatin. Both reactions carried out in liver cell nuclei.

F = 1.74

Level	N	Mean	SD
ODga	182	0.34177	0.05107
ODcnd	138	0.33234	0.07671

Individual 95% CIs for mean based on pooled SD



ODga = Optical density (calculated from average gray level) for Feulgen-DNA; ODcnd = optical density (calculated from average gray level) for Con A-reactive chromatin.

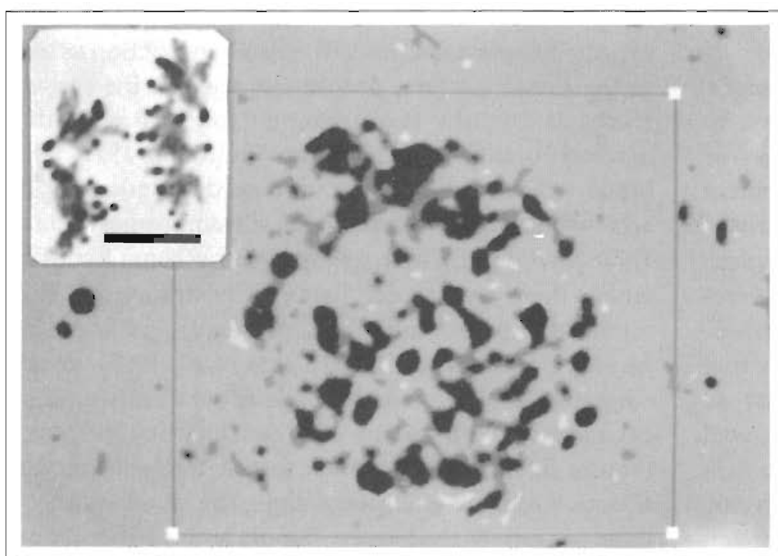


Figure 4 - Con A-binding reaction to *Zea mays mays* root tip cell nuclei. Pseudo-colorized image taken from the monitor. The chromocenters bind Con A deeply, and exhibit darkly stained bodies, which correspond to the telomeric constitutive heterochromatin (TCH). The inset depicts TCH after a C-banding method (Figure 8 in Vidal *et al.*, 1984). The bar corresponds to 10 µ.

OD values were higher for Feulgen-stained bands (F = 94.25) than for Con A-positive bands. This difference was confirmed by the Mann-Whitney confidence interval and test, which showed that the median difference was significant at P = 0.00000 and W = 21289. If the absolute values of the nuclear gray levels' SD are considered to be a measure of the heterogeneity/variability of chromatin condensation-decondensation in Feulgen-stained and Con A-positive polytene chromosomes, Feulgen staining showed the highest average SD ($F = 23.99$; $P < 0.001$).

DISCUSSION

The interferometric results for erythrocyte nuclei revealed that the areas after 4 N HCl hydrolysis and the results after image analysis for the Feulgen reaction were

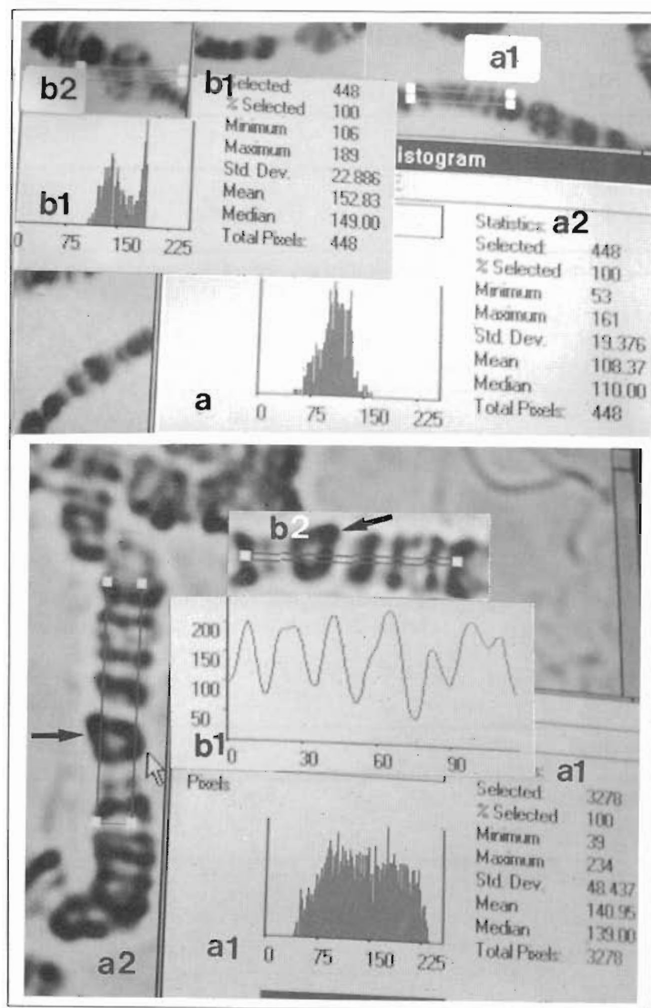


Figure 5 - Con A-binding reaction in polytene chromosomes. Histogram and gray level profile of bands, interbands and ring image. Histogram in pixels of gray levels and statistics, a1, of the respective delimited area at left, a2. Profile of gray levels in pixels, b1, for the respective area, b. Statistics data of 5b1: selected: 3278; % selected: 100; minimum: 39; maximum: 234; Std. dev. 48.437; mean: 140.95; median: 139.00 and total pixels: 3278.

similar. Slightly higher area values after the Feulgen reaction may result from dye molecules banding to apurinic acid. Dry mass loss after acid hydrolysis was confirmed by gray level differences in measurement comparisons before and after acid hydrolysis. Thus, the method's accuracy was demonstrated. Furthermore, average DM remaining after hydrolysis closely corresponded to the reported DNA DM for chicken erythrocyte nuclei (Altman and Katz, 1976). This shows that hydrolysis, as expected, removed all or almost all nuclear protein or as in this case, histones. It is well documented that erythrocyte nuclei are very poor in matrix proteins (see Figure 8 in Lafond and Woodcock, 1983; Verheijen *et al.*, 1988). This may be one explanation for Con A binding only in the nuclear envelope (Ferraro *et al.*, 1994).

The fact that the Con A-stained nuclear area in liver cells did not differ significantly from the nuclear area in Feulgen-stained preparations supports the reasoning that the areas containing Con A-positive proteins follow the known ploidy variability rate occurring in this organ. Con A binding in the chromocentral heterochromatin was more discrete and showed less variability than the Feulgen response. This fact rules out the possibility that a more diffuse staining of Con A would cause intermediate gray level (absorbances) values in contrast to the Feulgen-stained nuclei.

The constitutive heterochromatin of *Z. m. mays* demonstrated higher Con A-binding capacity than noncondensed chromatin and may be telomeric material. Telomeres are important chromosome components. They are characterized by their repetitive DNA composition, specific events of telomerase-dependent replication, and possible functions in keeping chromosome structural integrity and stability (Palladino and Gasser, 1994; Brandes *et al.*, 1995; Salvadori *et al.*, 1995). A few telomeres are recognized to be C-band positive and correspond to knobs, for example, the interphase nuclei of maize (Vidal *et al.*, 1984). These C-banding response characteristics sometimes also occur for acrocentric G-band heterochromatin, as in the case of mouse liver cells (Vidal *et al.*, 1973).

Under this study's conditions, Con A-positive proteins revealed physico-chemical stability to 4 N HCl hydrolysis. It is possible that part of these proteins, revealed in noncondensed chromatin, do not have the same stabilizing behavior and were removed by HCl, and therefore, were not demonstrated by the Con A reaction. Actually, the hydrolytic conditions used do remove almost all histones and some nonhistone proteins (Mello *et al.*, 1976; Mello and Cordeiro, 1985); however, even if these were present, they would not be expected to react to Con A. The Feulgen reaction as well as the image analysis procedures used in the present study satisfy the requirements for distinguishing heterochromatin from euchromatin (Mello, 1979, 1983; Mello *et al.*, 1994). The same considerations may be accepted as valid for polytene chromosomes (Vidal, 1977; Mello and Vidal, 1980). Finding some bands in which there was a peripheral concentration of chromatin, a ring of similar morphology, and a central furrow (Jondle *et al.*, 1995; Urata *et al.*, 1995) closely corresponds to localization of detected Con A-binding capacity as well as the Feulgen staining response (Figure 5). This corresponds to a heterogeneous distribution of DNP complexes along the chromosome. If it is reasoned that overall polytene chromosome architecture must reflect the structures and interactions of chromatin at a molecular level (Urata *et al.*, 1995), one could infer some structural band specificity in which Con A proteins take part in DNA organization and

contribute to the architectural properties of polytene chromosomes.

Heterochromatin has been the subject of extensive research in general and in polytene chromosomes in particular. Although they contain many components, constitutive heterochromatin types share the presence of repetitive DNA. On the other hand, centromeres consist largely of highly repeated DNA sequences. In interphase nuclei, heterochromatin is associated with the nucleoli as well as the nuclear envelope (Kellum *et al*., 1995; Kellum and Alberts, 1995; Murphy and Karpen, 1995). Heterochromatin and telomeres were demonstrated to be Con A reactive in the material studied, which raises a question about the phenomena of chromatin packing states and gene expression-repression. According to Urata *et al.* (1995), repression is a matter of sequestering or masking DNA accessibility. Proof comes from the fact that silent loci and telomeres are resistant to DNA methylating enzymes. Even the accessibility of linker DNA to endonucleolytic attack depends on the conformation of the chromosome, i.e., a condensed state or higher order structured fibers protect DNA against Mnase (Urata *et al*., 1995). Con A-binding glycoproteins in heterochromatin, telomeres and ubiquitous condensed chromatin in polytene chromosomes may play a role in the biomechanical properties of DNA (elasticity and torsion), which are considered relevant for polymerase action and chromosome condensation (Strick *et al*., 1996).

In polytene chromosomes, some non-histone proteins have been reported to increase during formation of DNA puffs and to remain in the puff after its regression. They also may be relevant for the regression and mechanical properties of this part of the chromosome (Vidal, 1977). It would therefore be important to determine whether Con A-reactive proteins exist in this type of biological model and their role in it.

HP1 is a protein associated with centromeric heterochromatin in polytene chromosomes of *Drosophila*. One of its proposed functions is to provide activity that helps maintain the chromatin compact state (Kellum and Alberts, 1995; Kellum *et al*., 1995). As part of the compositional complexity of heterochromatin, possible interactions among proteins such as HP1, topoisomerase II and Con A-binding protein may be further demonstrated.

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RESUMO

A capacidade de ligação da concanavalina A (Con A) a regiões condensadas de eucromatina e heterocromatina foi investigada em núcleos de eritrócito de frango (CEN), hepatócitos de rato, células meristemáticas de *Zea mays mays* e em cromossomos politênicos de *Drosophila melanogaster* após hidrólise com HCl 4 N para determinar a ocorrência de ligação preferencial em bandas e heterocromatina. A variação da massa seca foi investigada em CEN por microscopia de interferência, e reações de Feulgen e Con A foram empregadas para todos os materiais para correlacionar os *loci* de ambas reações. As quantificações e verificações topológicas foram levadas a efeito por análise de imagens (citometria de alta performance). Foi observado que a hidrólise por HCl 4 N causou uma importante perda de massa seca em CEN, permanecendo um nível correspondente ao conteúdo médio de massa seca de DNA. Neste material a ligação com Con A foi restrita ao envelope nuclear, reforçando a idéia da ausência da matriz nuclear nessas células. Os demais tipos celulares exibiram áreas reativas de cromatina condensada e heterocromatina. Este fato permite especular o papel desempenhado por proteínas Con A-positivas no mecanismo de condensação cromatínica. Esta estrutura glicoprotéica contribuiria para uma maior estabilidade físico-química da cromatina condensada, especificamente da heterocromatina, e também para suas propriedades reológicas.

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