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ULTRASTRUCTURE OF INTERPHASE CHROMOSOMES

By

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Electron microscopy of thin sectioned material has been of very limited value in unravelling the organisational pattern of chromosomes. The difficulty of interpreting the complex three dimensional structure of chromosomes from the essentially two dimensional pictures has been the bane of this technique. A partial solution to this difficulty was provided by the ingenious water spreading technique of Kleinschmidt (10,11,12, 13), originally devised to study the fine structure of bacterial and viral genophore. Gall (9) greatly extended the usefulness of this technique by modifying it for the study of spread nuclei and chromosomes of eukaryotes. Several investigators have used Gall's technique to study the fine structure of mitotic (6,17, 19, 20) and meiotic chromosomes (21).

In this paper, an attempt has been made to analyse the chemical composition and molecular organisation of microfibrils of interphase nuclei by combing the surface spreading technique with various enzymatic and other extraction procedures.

MATERIAL AND METHODS

Cultured human leucocytes and erythrocytes of frog were used in the present study. In either case, the material was spread on the cleaned surface of double distilled water contained in a Langmuir's trough. The thin film formed at the air-water interphase was picked up on formvar coated copper grids, fixed

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for 2 hours in 4% neutral formaldehyde, stained for 3 hours in 5% aqueous solution of uranyl acetate (pH 3.5), dehydrated in ethanol, passed through 2 baths of amyl acetate and finally air dried.

Enzymatic and other extractions

The following solutions were used for extraction processes:

i) 0.01 N HCl at room temperature for 30 minutes.

ii) 0.3% tyrpsin (Sigma, 2x Crystallized) aqueous solution, pH adjusted to with 0.01 (N) Na OH for 30 minutes, 1 hour and $1\frac{1}{2}$ hours.

iii) 0.3% DNAse (Sigma) with 0.003 M magnesium sulphate for 30 minutes at 37°C.

iv) 1M ammonium acetate, pH 8 at room temperature.

v) 0.1 N sodium hydroxide for 7 minutes at room temperature.

Treatments were given to the fixed material by floating the grids with the specimen side down, on these solutions. Extraction with 1M ammonium acetate pH 8, was effected by spreading the material on a substrate of this solution contained in a Langmuir's trough.

Electron micrographs were taken on Gevaert Scientia Films (35 mm) using a Philips EM100 electron microscope.

RESULTS

Erythrocytes of Rana tigrina and human leucocytes spread on an air-water interphase showed nuclei at various stages of disruption from which emerged a plethora of microfibrils. The microfibrils were irregularly coiled and showed very few free ends. The following two distinct categories of microfibrils were observed both in frog erythrocytes and human leucocytes: (i) 500-400Å fibrils (Figs. 2 and 3) which were continuous with the more ubiquitous (ii) 300-250Å fibrils (fig. 1).

The intact 500 Å fibrils, did not show any substructure because of their great electron opacity after Uranyl staining. However, mechanically stretched preparations revealed that the 500 Å fibrils were supercoils of 250 Å fibrils with some associated sheath material (Fig. 10). Upon stretching, the sheath got stripped off at places giving a 'beads on the string' appearance.

The helically twisted 250 Å fibrils did not show the existence of 100 Å subunits as reported by Ris (16) and Ris and Chandler (17). Our observations support the contention of Dupraw (6) that the helical twists cannot be regarded as unequivocal evidence for the existence of two intercoiled 100 Å subunits because a very similar configuration would result from the coiling of a single 250 Å strand upon itself.

Digestion of the microfibrils with DNAse followed by Uranyl staining showed a considerable loss of contrast in comparison with the control preparations. DNAse digestion, however, did not disrupt the continuity of both 500 Å and 250 Å microfibrils.

Digestion of the material with trypsin resulted in uncoiling of the microfibrils. After 1½ hours digestion with this enzyme, the microfibrils could be traced out in straight lines upto distances of several micra. Besides uncoiling, the microfibrils showed loss of material resulting in the reduction of their diameters to 100 Å - 40 Å. Trypsin digestion, however, did not disrupt the continuity of the fibrils (Fig. 6, 7, 8).

Extraction with 0.1N HCl resulted in coagulation of the fibrils, In well-spread material, however, 40 Å fibrils were visible.

Treatment with 0.1 sodium hydroxide caused fragmentation of the 500 Å and 250 Å fibrils into small bits (Fig. 9).

2M sodium chloride caused partial dissociation of the fibrils. The 500 Å and 250 Å fibrils showed loss of material resulting in smoother and thinner fibrils of diameter 100 Å - 40 Å (Fig.5).

Erythrocytes of Rana tigrina spread over a subtrate of 1M ammonium acetate, pH 8, showed considerable uncoiling of the 500 Å and 250 Å fibrils (Fig.4).

DISCUSSION

The results of this study indicate the existence of two categories of microfibrils in both frog erythrocytes and human leucocytes: (i) 500 Å fibrils and (ii) 250 Å fibrils. The thicker fibrils appear to be supercoils of 250 Å fibrils with associated sheath material. This interpretation is based on a study of both mechanically stretched fibrils and those treated with 1M ammonium acetate, pH 8. Ammonium acetate under these conditions is expected to remove histones. The 500 Å fibril was reported earlier by several workers (9,15, 22), in a variety of materials. There were, however, differences of interpretation about its molecular organisation. Yasuzumi (22) discovered in Drosophila salivary gland chromosomes 500 Å fibrils which broke down to 200 Å fibrils after ultrasonic treatment.

Ris (15) suggested that the 500 Å fibril is bipartite in nature consisting of two 250 Å submits. Gall (9), however, observed in erythrocytes of Triturus spread on an airwater interphase, 500 Å microfibrils with a 150 Å Uranyl staining core. Our observations are in accord with Gall's (9) interpretation.

The plethora of helically coiled 200-250 Å microfibrils with very few free ends, observed in the course of this investigation is in accord with other recent reports(6,17, 19, 20). Ris (16) and Ris and Chandler (17) have reported the existence of two 100 Å submits in the 250 Å fibrils. Our observations alongwith those of

others (6,19, 20), however, do not support this interpretation. Digestion with trypsin brings about uncoiling of the 500-250 Å microfibrils along with a progressive reduction in their diameters to 100-40 Å. This observation would suggest that the 500Å, 250Å and 100 Å microfibrils represent different orders of coiling of a basic 60 Å-40Å microfibril (2,3,14). This interpretation is at variance with that of Ris (16), who regards the 100 Å fibrils, on the basis of his 0.2M HCl digestion experiments, as a bipartite structure with two 40 Å subunits. Digestion of spread erythrocytes of frog with 0.1N HCl does show 40 Å subunits. However, a precise splitting of 100 Å fibrils into 40 Å fibrils has not been observed by us.

Digestion of microfibrils with 0.1 M sodium hydroxide was attempted with a view to investigate the possibility of the existence of linkers of acidic proteins. The extensive fragmentation of microfibrils caused by this treatment appears to be due to the hydrolysis of nucleoprotein complex rather than due to the removal of linkers.

The following observations suggest that the 500 Å-250Å microfibrils are nucleohistone in nature: (i) The reduction of uranyl binding resulting in a loss of contrast after DNAse digestion, (ii) dissociation of microfibrils after 2M sodium chloride treatment, and (iii) loss of material from the microfibrils after digestion with trypsin, which is more specific to histones and (14). The observations that DNAse does not disrupt the continuity of the microfibrils is at variance with the reported disruption of the loops of lampbrush chromosomes by the same enzyme. The loops of lampbrush chromosomes are known to be active sites of RNA synthesis. Frenster (7,8) has shown that a chromatin

actively engaged in RNA synthesis has a 'loosely' associated DNA histone complex, while the chromatin repressed with respect to RNA synthesis has a more tightly bound DNA-histone complex. It is known that erythrocytes are largely inert with respect to RNA synthesis and, therefore, would be expected to have a more tightly bound DNA-histone complex. It is expected that DNA in a loosely bound DNA-histone complex would be more susceptible to fragmentation by DNAse rather than in a more tightly bound complex.

Mechanism of Coiling of the Microfibrils

Zubay (23) has proposed a sheet like model of oriented nucleohistone in the gel state in which histone molecules are disposed parallel to the large grooves of and with their long axes subtending an angle of 60° to the long axes of the DNA molecules. The model looks like a lattice in which histone molecules link up the alternate large grooves of the DNA molecules. Zubay's model was based primarily on X-ray diffraction and infra red studies of nucleo-histones. One particularly attractive feature of the model is that it would promote super coiling of DNA - histone complex, if the histones are disposed only on one side of DNA.

The uncoiling of microfibrils brought about, presumably by the removal of histones, by 1M ammonium acetate and trypsin digestion, along with similar observations of Du Praw (6) and the infra red studies of Bradbury et al. (4) tends to support Zubay's proposition regarding the role of histones in the supercoiling of DNA. The observations of Trosko and Wolff (18) regarding the stretching and uncoiling of formalin fixed, isolated metaphase chromosomes of Vicia faba after trypsin digestion, also suggest that histones are probably involved in chromosome coiling.

Legends

Fig.1. Electron micrograph of interphase chromosomes of Rana tigrina showing helically twisted 250 Å microfibrils with very few free ends. x 45,520.

Fig.2. Interphase chromosomes of Rana showing 500 Å microfibrils x 17,236.

Fig.3. Human interphase chromosomes showing 400-500 Å microfibrils x 27,972.

Fig.4. Electron micrograph showing the uncoiling of 500-250 Å fibrils of Rana brought about by spreading on 1M ammonium acetate, pH8. x 24,130.

Fig.5. Dissolution of microfibrils of Rana after 2M sodium chloride extraction x 24,130.

Fig.6,7, and 8 : Effect of trypsin digestion on microfibrils of Rana.

Fig.6. Showing stretching and uncoiling of 250 Å fibrils x 51,210.

Fig.7. Showing Reduction in diameter of the fibrils to 100 Å x 56,900.

Fig.8. Showing uncoiled 60 ± 20 Å fibrils x 44,382.

Fig.9. Fragmentation of microfibrils of Rana caused by 0.1M sodium hydroxide digestion. x 20,680.

Fig.10. A mechanically stretched 500 Å fibre showing a 150 Å Core and sheath x 12,000.

Summary

A study of the ultrastructure of interphase chromosomes of frog erythrocytes and human leucocytes suggested that the 500, 250 and 100 Å microfibrils observed in different preparations may represent different orders of coiling of a basic 60-40 Å microfibril in lampbrush chromosomes. Unlike the situation DNAse digestion did not result in the disruption of microfibril continuity. It seems likely that DNA in a loosely bound DNA-histone complex may be more prone to fragmentation than in a more tightly bound complex. The uncoiling of microfibrils brought about by treatments with 1 M ammonium acetate and trypsin supports Zubay's (23) model involving histones in the supercoiling of DNA.

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Induced Variability and Selection Advance for Branching in Autotetraploids of *Brassica campestris* var. *Toria*

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Induced Variability and Selection Advance for Branching in Autotetraploids of *Brassica campestris* var. *Toria*

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Toria (*B. campestris* var. *toria*) is an important oilseed crop in India and autotetraploids (2n = 4x = 40) were produced at the Indian Agricultural Research Institute in 1941 through colchicine treatment in the strain T.22. The seed fertility of the autoploid was initially very low but was brought to the level of the diploid through a cycle of mass pedigree selection. In spite of the improved fertility and also larger seed size, the yield of the autoploid did not exceed that of the diploid, owing to a reduction in its ability to produce secondary and tertiary branches. Hence, seeds of an 'evolved' autoploid culture belonging to the C_{19} generation were exposed to 96 and 128 Kr of X-rays in 1960. A biometrical study of progeny means and variances was conducted in the M_2 and M_3 generations. Selection norm for secondary branch number was fixed at a minimum of 20 per plant and the selected plants were allowed to inter-cross. Recurrent selection was practised in the M_4, M_5 and M_6 generations. An yield trial comparing the selected bulks from the treated and control families was conducted at two locations during 1965-66. The results indicated that (a) the variance for secondary branch number

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was enlarged in the M_2 and M_3 populations, while the means were not affected, (b) there was a positive selection advance for branch number during the M_3, M_4 and M_5 generations and (c) true-breeding tetraploid cultures with a significantly higher number of secondary branches could be established. Thus, the poor branching ability of the autoploid population could be remedied and promising tetraploid cultures developed.

Introduction

Toria (*B. campestris* var. *toria* Duth & Full.,2n=20) is an important oilseed crop in India and its oil is widely used as a cooking medium. Out of a total area of 319,000 hectares for rapes and mustards, nearly 25% is under *toria*. The average yield of this crop is about 900 Kilos per hectare. Polyploidy was induced in the strain T.22 in 1941 at the Indian Agricultural Research Institute, New Delhi by RAMANUJAM and DESHMUKH[19]. The raw tetraploids (2n = 4x = 40) had a low seed fertility but through the adoption of the mass pedigree system of selection, the fertility of the autotetraploids was brought almost to the level of the diploids[17,18]. Coincident with the increase in fertility, the multivalent frequency registered a drop[23]. In spite of the great improvement in the number of seeds per siliqua in the 'evolved' autotetraploids and also the larger size of seed in them, they yielded less than the diploids in yield trials. An analysis of the causes for the lower yield potential of the autotetraploids showed that the tetraploids had a

significantly lower number of secondary and tertiary branches per plant[2]. Thus, while gaining in seed fertility, there has been a loss in branching ability. The evolved autotetraploids in the C_{19} generation showed very little variability for the number of the primary and secondary branches per plant and hence offered little scope for selection. Since radiations can induce variations in polygenically determined traits, it was considered worthwhile to irradiate the evolved tetraploids, screen the population for variability for the number of secondary branches and assess the scope for making selection advance for this trait. The results obtained during the years 1960-66 are summarised in this paper.

Material and Methods

Seeds from a single open pollinated plant of tetraploid toria were taken and were irradiated with 96 Kr and 128 Kr of X-rays. For these treatments, a Phillips X-ray machine was used, operating at 50 KV, 2ma, without filter, at a dose rate of 16 Kr per minute at a distance of 2 cm. Seeds arranged in a single layer were exposed for different durations of time depending upon the dose. Control and treated seeds were allowed to germinate in pans and three week-old seedlings were transplanted in the field in rows 1.5 ft. apart with one foot inter-plant distance. For raising the M_2 generation, M_1 plants from each treatment were selected at random and their progenies were grown in a Randomised Complete Block Design with three replications in 12 ft. long rows, 1.5 ft. apart with one row per progeny in each replication.

A similar layout was adopted for comparing M_3 progenies. Data on the number of primary and secondary branches were taken from 10 plants chosen at random in each progeny both in the M_2 and M_3 generations. The progeny mean and variance (plot variance) were calculated and statistical analysis was done on these means and variances. Means were analysed according to the randomised block design analysis and F-values were calculated. For the plot variances of each progeny, the following transformation was used:

$$(n_i-1) \log_e s_i^2$$ where s_i^2 = plot variance

and n_i = number of plants on which s_i^2 is calculated.

These transformed values were then subjected to an analysis of variance and F-values were calculated. The plant progenies which were significantly superior to the control in plot mean and plot variance were selected for being carried forward to the next generation. This procedure was adopted upto M_3 generation. Average means and variances in control and irradiated plant progenies of M_2 and M_3 generations (Table 1) were compared against C.D. values. As the number of progenies in each treatment was variable, the standard error of the averages was calculated by using the harmonic mean of the number of observations in each treatment. In M_4 generation, a selection pressure of about 10-15 per cent was applied in the control and treated population, keeping twenty secondary branches as the selection mean. These genotypes

with high branch number in each treatment group, were sown in isolation and allowed to intercross. This seed was carried to the next generation and this procedure was also followed in the M_5 generation. Finally at M_6, the selected bulks from each treatment were tested for branching ability at two locations. A randomised block design with four replications was adopted for these trials.

Results

The observations on population mean and variances in the M_2 and M_3 generations are summarised in Table 1. Mean values did not change while variance was enlarged for primary and secondary branches. The increase in variance was more pronounced in the population derived from 96 Kr treatment. The analysis of variance carried out in plot means and plot variances in each generation revealed significant differences between control and irradiated progenies. F-values for secondary branch number were significant at 1% level both for plot means and plot variances (Table II). Since the variance values from the two doses were not significantly different, the data for the two doses have been pooled in Table II and Fig. 1. Following selection, the mean number of primary and secondary branches increased (Fig. 1). The increase was marked in the M_5 population.

The relative performance of the elite bulks in the M_6 generation at three different locations revealed that a faimly derived from 96 Kr treatment was superior in secondary branch number at both the locations (Table III). The number of silliquae per main shoot and the number of seeds per silliqua were either the same as in the control population, or a little

higher.

Discussion

Following the reports of CLAYTON and ROBERTSON[8] and BUZZATI-TRAVERSO and SCOSSIROLI[7] many studies have been conducted on mutagen-induced enlargement of variability for polygenically determined characters (see review by SCOSSIROLI[20]). In most of these studies it has been observed that the mean of the irradiated population either remains unchanged or is altered in the minus direction. The variance, in contrast, is significantly increased in comparison with the control for the particular character studied. The present findings are in agreement with this general trend. While the population means did not change significantly after irradiation, the variances were significantly increased. In the M_3 generation, the variance was of a higher order than in the M_2 generation, particularly in the 96 Kr treatment. BHATIA and SWAMINATHAN[4] in hexaploid wheat and KOO[13] in hexaploid oats have also reported that the increase in variance is more in the M_3 generation in comparison with M_2. KAO *et al.*[11] observed in rice a greater increase in variance in the M_3 generation and constructed formulae to calculate the radiation-induced genetic variance expected in M_2 and M_3 generations. Their studies reveal that the fixable part of the variance is more in the M_3 generation. Similar results have also been reported by KONDO[12]. LAWRENCE[14] found a significant increase in the additive and dominant components in irradiated populations of *Arabidopsis thaliana* for flowering time.

Increase in the genetic variability is caused by the occurrence of extreme types, both on the positive and negative

sides of the mean value. Therefore, it should be possible to shift the mean in any direction through artificial selection. OKA et al.[16] observed in irradiated material of rice that selections for plant height and heading date were equally effective in both directions. However, BATEMAN[3] in a re-interpretation of OKA's data concluded that the altered mean and variance arise from mutations in one direction. He further remarked that there is excess of mutations in the positive direction for most of the quantitative characters. BHATIA and VAN DER VEEN[5] also reported that selection led to a shift in the mean value for maturity period only in the positive direction, in EMS-treated Arabidopsis thaliana. BROCK[6] proposed the hypothesis that in species which have previously been subjected to breeding and selection, random mutations would result in an increase in variance and a shift in the mean in a direction opposite to that of the previous selection history. GAUL and AASTVEIT[9] did not agree with this hypothesis of BROCK and suggested that random mutations result in a change of the mean in a direction associated with reduced vitality. Thus, the alteration of the mean would be largely independent of the genotype used for the mutagenic treatment.

In the present investigation it was possible to shift the mean for secondary branch number towards the positive side in tetraploid toria, after recurrent selection in the 96 Kr treated population. Induction of variability must be followed by selection in order to shift the mean value of a quantitative

character. BROCK[6] pointed out that the mean value for a character also moves away from the direction of previous selection; hence, selection must be applied to stabilise the mean at a desired level. The present data provide evidence in support of the effectiveness of promoting cycles of recombinations within the selected lines. Mutagenic efficiency as applied to polygenic traits should be measured not by the induced variance alone but by the selection advance rendered possible.

Polyploidy, by virtue of the buffering effect caused by gene and segmental duplications, has been a favourable feature in mutation breeding [15,21,22]. In autoploids, recessive mutations are expressed more readily in M_3 and later generations than in the M_2[21]. So far, no data on the induction of mutations for polygenic traits in autoploids are available. The present study suggests that autoploids are equally suitable for the recovery of mutations in qualitative and quantitative traits.

Mutation breeding has until now been largely confined to the improvement of self-pollinated crops. This is due to the difficulties inherent in allogamous plants in distinguishing between induced mutations and the release of the variability already present[10]. Nevertheless, some improvement has been made through mutation breeding in cross-pollinated crops like Sinapis alba. Toria is a self-incompatible crop and diploid and auto-tetraploid toria are cross incompatible. The tetraploid population, derived from a few plants of single original diploid strain could obviously undergo mating only within a restricted

genotypic spectrum. Cross-pollination in such instances is not synonmous for heterozygosity. This could be one factor responsible for the striking selection advance made in irradiated cultures, in contrast to the lack of response to selection met with in the control families. The failure of extensive attempts to make advance in branching ability through recurrent cycles of selection in the autoploid population (Rajan, unpublished) suggests that induced mutations may constitute the predominant cause of the selection advance made in the irradiated cultures.

The improvement of autotetraploid toria for the number of secondary branches through induced polygenic variation is hence another example of the value of the mutation breeding technique for special purposes in plant breeding.

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Table I

Average Means and Variances in Control and Irradiated M_2 and M_3 progenies of tetraploid *toria*.

Treatment	Total no. of progenies	Primary Branches		Secondary Branches	
		Average mean	Average variance	Average mean	Average variance
M_2 generation					
Control	21	6.54	4.02(9.29*)	9.24	14.93(23.99*)
X-ray, 96 Kr	78	6.48	3.86(10.09)	9.24	26.47(26.24)
X-ray,128 Kr	72	6.38	3.28(9.36)	8.77	23.95(26.46)
C.D.		0.26	(1.48)	0.84	(0.56)
M_3 generation					
Control	15	6.89	5.88(11.53)	6.49	24.37(25.37)
X-ray, 96Kr	66	6.71	6.73(13.65)	6.64	31.03(25.13)
X-ray,128Kr	60	6.55	5.98(12.14)	6.38	29.73(24.92)
C.D.		0.60	(2.38)	0.78	(3.52)

* Values in the brackets are averages of transformed variances and the C.D. is also from the transformed data.

Table 2

Analysis of plot means and plot variances in M_2 to M_6 generations of tetraploid toria

Character	Generation	F-value (Means)	F-value (Variance
Primary Branches	M_2	2.61**	1.88**
" "	M_3	1.59	1.27
" "	M_4	1.73*	1.18
" "	M_5	2.89*	4.63**
" "	M_6	6.55**	22.17**
Secondary branches	M_2	1.79**	1.59**
" "	M_3	1.94**	2.09**
" "	M_4	1.61*	1.81**
" "	M_5	2.21*	2.12**
" "	M_6	2.53**	0.05

* Significant at 5% level.
** Significant at 1% level.