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Regulation of karyotype stability in tobacco tissue cultures of normal and tumorous genotypes

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Summary. Callus cultures of *Nicotiana glauca, N. langsdorffii* and of their tumor-forming hybrid plants contained a high frequency of cells with irregular chromosome numbers and chromosome aberrations (hypo-, hyper-, polyploid, aneuploid cells; bridges, polytene, broken, fragmented chromosomes, megachromosomes, etc.). Meristematic cells of shoot tips regenerated from the same cultures contained only regular chromosome numbers with normal chromosome structures. Variability in chromosome numbers is a consequence of abnormal mitoses. The data suggest genome segregation in the cultures. Cytological instability appears to be independent of genome segregation composition, genotype, tumorous condition, hormonal requirement and level of ploidy. The karyotype stability of the cultures is only dependent on the degree of organization of tissues and is regulated by factors involved in the control mechanisms of organizational processes.

Key words: *Nicotiana* – Chromosomal instability – Abnormal mitoses – Polyteny – Tumor – Organization – Regulation

Introduction

Genetic uniformity and stability of cell and tissue cultures are prerequisites for their use in somatic cell genetical research and in practical breeding work.

Nevertheless, a number of researchers have provided evidence for genetic instability in cell, tissue, anther and protoplast cultures (Street 1973; Smith 1974; Kao et al. 1970; Guo 1972; Collins et al. 1972). Thus, research on the genetic stability of cultures is important in furthering our knowledge.

The instability of morphogenesis, and the biochemical and cytological characteristics of tissue cultures of *Nicotiana* plants and their hybrids have been studied earlier (Kovács 1967a, b, 1974, 1977, 1983).

It has also been established that tobacco plants of a tumorous genotype are characterized by an increased shoot forming capacity which appears in both intact plants and their unstable tissue cultures. Plants and tissue cultures of non-tumorous, normal genotypes have no spontaneous increased organ formation under the same experimental conditions (Kovács 1967, 1968). On the basis of the spontaneous shoot forming capacity of plants and their tissue cultures the tumorous (or the normal) genotype of them can be predicted in the experimental system used (Kovács 1967, 1968).

In the present experiments the karyotype stability of the above mentioned tumorous and normal cultures, at different degrees of organization, was studied.

Materials and methods

The tissue cultures were initiated from leaf pieces of diploid and tetraploid *Nicotiana glauca* (2x=24, *GG*; 4x=48, *GGGG*), diploid *N. langsdorffii* (2x=18; *LL*) and of their F_1 allotriploid tumor-forming hybrid plants (3x=33; *GGL*). The chromosome numbers of the plants were verified in the cells of root tips and young leaf tips.

The explants and tissues were grown under the same experimental conditions as described previously (Kovács 1971). The composition of the culture medium (mg/l) was as follows: NH_4NO_3 1,000; KNO_3 1,000; $Ca(NO_3)_2 \cdot 4\,H_2O$ 250; $MgSO_4 \cdot 7\,H_2O$ 150; KH_2PO_4 125; KCl 25; Na_2EDTA 17.5; $FeSO_4 \cdot 7\,H_2O$ 6.25; $ZnSO_4 \cdot 7\,H_2O$ 3.75; $MnSO_4 \cdot H_2O$ 2.5; H_3BO_3 2.5; KI 0.4; glycine 3, hydrolized casein 50, thiamine 0.1, pyridoxin 0.5, nicotinic acid 0.5; growth substances: indole-3-acetic acid 2.0; kinetin 0.2; sucrose 20,000; pH 5.8–5.9; agar 0.8%. (Tumorous cultures were grown on hormoneless medium. They were studied in more detail because the effects of IAA and kinetin on stability were excluded.)

Small pieces of calli and the shoot tips were pretreated with 3 mM 8-hydroxyquinoline for 4 h and then fixed in ethyl alcohol : acetic acid 3 : 1 (v/v), stained in aceto-carmine, then squashed. To distinguish the large *glauca* and the small

langsdorffii chromosomes materials without pretreatment were also used. At high chromosome numbers, the determination of the absolute chromosome number of individual cells was generally more difficult. For this reason each metaphase plate was counted ten times and the mean count recorded. (Counts for one plate generally varied by approximately ±3 chromosomes. Hence, at high chromosome numbers characterization of aneuploidy is limited by this value.)

The experimental results were subjected to chi-square (χ^2) analysis (Mather 1964).

Results

Characterization of the primary explants

On the cut surface of the primary leaf explants of the *N. glauca* (*GGGG*) and the hybrid (*GGL*) plants cell division was observed on the second day of cultivation. After 4–5 days, small calli appeared. Primary calli of *N. langsdorffii* (*LL*) explants appeared later.

After two weeks the primary callus of the *GGL* leaf explants spontaneously produced buds and shoots surrounded by significantly large green areas. These chlorophyll rich zones suggested that a high level of cytokinins were being produced by the organized tissues (Kovács 1977 a, b).

In the primary calli of the 4-day-old *GG, GGGG, GGL* and the 14-day-old *LL* explants, cells with irregular chromosome number were found (Table 1, Figs. 1–3) in similar frequencies (Table 2, $0.80 > P > 0.70$). The appearance of the abnormal chromosome numbers was accompanied by some irregular mitoses (e.g. bridges). Cells of buds, shoots induced in *LL* primary explants contain regular chromosome numbers (2x = 18).

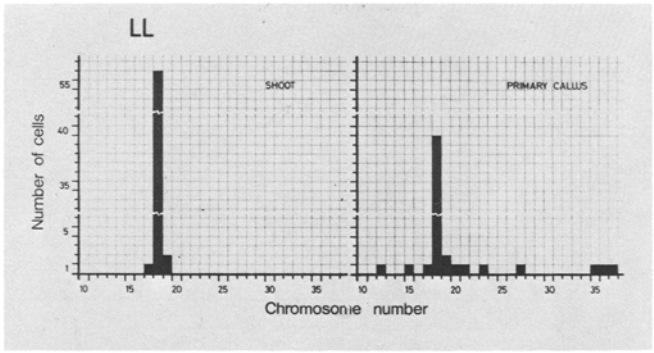

Fig. 1. Frequency distribution of chromosome numbers of cells from shoot tips and calli of *N. langsdorffii* origin

Irregular chromosome numbers were only rarely found (Table 1, Fig. 1).

Characterization of cultures of GGGG and GG origin

Callus cultures of *GGGG* plant origin contain cells with the expected chromosome number (48) and, at a high frequency, cells with irregular numbers (Table 1). The lowest chromosome number is 25 and the highest ones are above 90. The frequency distribution of the chromosome counts is shown in Fig. 2. In cells of these calli abnormal mitoses can be detected (e.g. bridges, broken and sticky chromosomes). Irregular chromosome numbers were rarely found in cells of the shoot tips induced in the same cultures. The shoot-tip squashes showed metaphase plates having 48 chromosomes and containing only normal mitoses (Tables 1 and 2, Fig. 2).

Table 1. Frequency of cells with regular and irregular chromosome numbers in organized and unorganized tissues of normal as well as tumorous genotypes

Genotype	Genome composition	Tissues	No. of cells with a chromosome count:		Total cells obs.	Cells with irregular counts (%)
			Regular	Irregular		
Tumorous	*GGL*	Shoot	123	11	134	8.20
		Primary callus	41	14	55	25.45
		Callus	74	51	125	40.80
Normal	*GGGG*	Shoot	83	6	89	6.74
		Primary callus	35	15	50	30.00
		Callus	71	40	111	36.03
Normal	*LL*	Shoot	56	3	59	5.09
		Primary callus	39	12	51	23.52
–	–	Total shoot	269	20	282	7.09
–	–	Total callus	260	132	392	33.67
–	–	Total	522	152	674	22.55

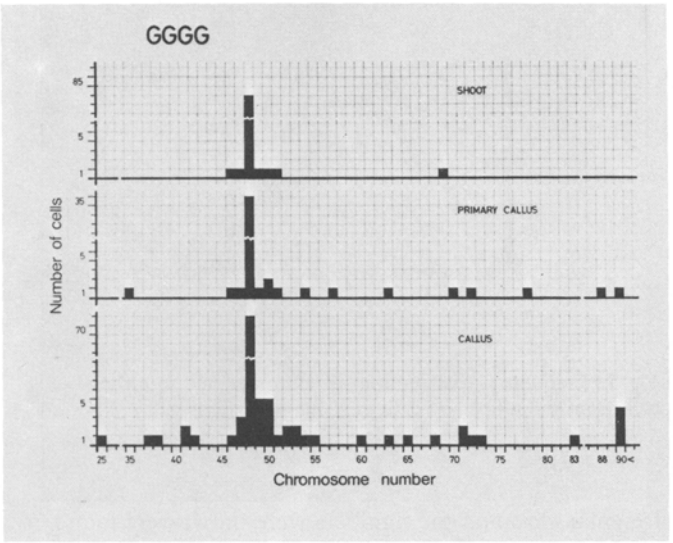

Fig. 2. Frequency distribution of chromosome counts of cells from shoot tips and calli of *N. glauca* origin

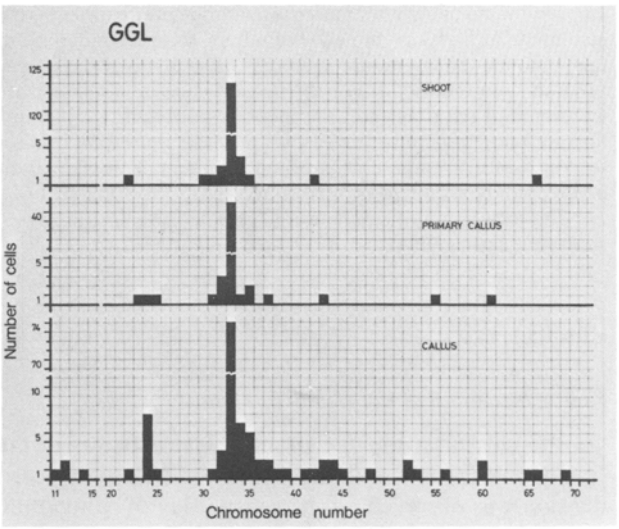

Fig. 3. Frequency distribution of chromosome numbers of cells derived from shoot tips and calli of tumor forming F_1 hybrid of *N. glauca* × *N. langsdorffii*

Table 2. Analysis of dependence of karyotype stability on organization, tumorous condition, hormone requirement and genome composition

Item	χ^2	d.f.	P
Primary callus/shoot (unorganized, organized)	29.274	1	<0.001
Subcultured callus/shoot	73.701	1	<0.001
Total callus/shoot	64.835	1	<0.001
Primary callus/ subcultured callus	5.801	1	0.02 – 0.01
Normal/tumorous condition	0.478	1	≈ 0.50
With/without hormones	0.478	1	≈ 0.50
Primary calli (genome composition): *GGGG/GGL/LL*	0.575	2	0.80 – 0.70
Subcultured calli: *GGGG/GGL*	0.380	1	>0.50
Shoots: *GGGG/GGL/LL*	0.631	2	0.80 – 0.70

Callus cells of the *GG* diploid plants also show higher chromosomal variability (range of 17–56) than cells of the shoot tips induced in the same cultures ($\chi^2 = 19.38$, d.f. = 1, $P < 0.01$). The callus contained numerous abnormal mitoses, such as tissues of *GGGG* origin.

Characterization of the tumorous cultures

Tissue cultures of the tumor prone *GGL* hybrids spontaneously produce buds and shoots. From these cultures a slow growing and two fast growing callus clones were isolated. The callus tissues consisted of cells with variable chromosome numbers. About forty percent of the callus cells contained irregular chromosome counts between 11–69. The expected chromosome number of the tumorous (*GGL*) cells was 33 (24 from *glauca* + 9 from *langsdorffii*). These are illustrated by Table 1 and by the frequency distribution (Fig. 3). Cells with 24 chromosomes appeared to be more frequent than other irregular numbers (Fig. 3). This number corresponds to the *GG* (2x = 24) genome. Since some of these cells contained only large chromosomes (probably of *glauca*) elimination of the *L* chromosomes, or the complete genome, could possible lead to *genome segregation.*

In the calli of *GGL* origin, mitotic and chromosomal abnormalities were found. Figure 4 a–d show abnormal anaphases with chromosome bridges. The abnormalities were brought about by sticky, lagging, delaying (Fig. 4 a, c) and interlocked (Fig. 4 b) chromosomes. The cultures also contained chromosome aberrations, breaks, fragments (Fig. 5 a), abnormally long, mega-chromosomes (Fig. 5 a, b), and probably dicentrics.

There is a variability in *size* of chromosomes. It is interesting that the chromosomes of the cells with a higher level of ploidy are usually smaller than those of cells with a lower level of ploidy. Figure 6 shows that cell A of the callus contains larger (longer, thicker) chromosomes in lower numbers than cell B, which contains smaller (shorter, thiner) chromosomes in higher numbers. The frequency distributions of hypo-, hyper-, poly- and aneuploid cells of the tumorous calli is shown by Fig. 3.

Cytological characteristics of the fast growing tumorous callus clone were similar to those of the

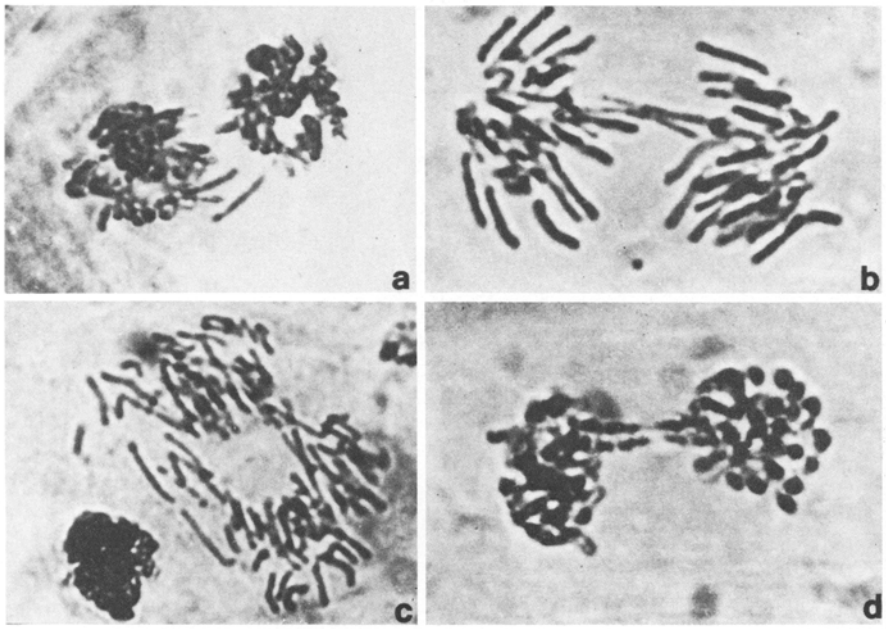

Fig. 4a–d. Mitotic, chromosomal aberrations and bridges in callus cells of *N. glauca*×*N. langsdorffii* origin: **a** lagging, delayed chromosomes, **b**, interlocked chromosomes (in the middle); **c** breaks, fragments, delaying chromosomes, **d** bridge in late anaphase

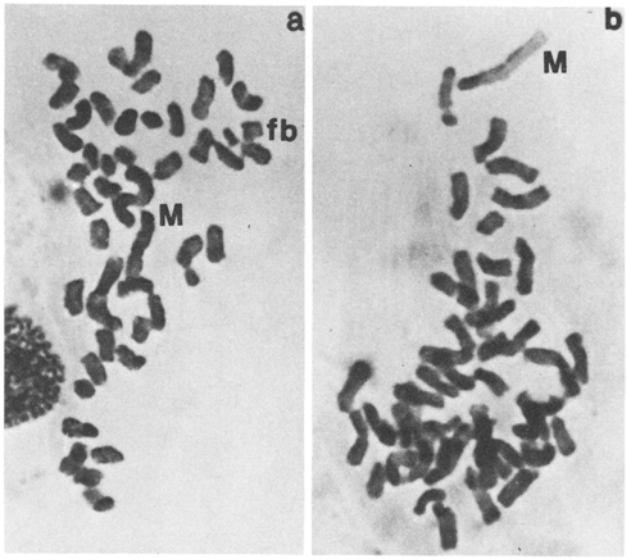

Fig. 5 a, b. Chromosome aberrations in tumorous callus cells. **a** fragments (f), breaks (b), megachromosome (M); **b** megachromosome (M).

slowly growing one. In addition, the fast growing callus contained cells with "giant" nuclei, at a lower frequency (0.9%, Fig. 7a). The diameter of the "giant" nuclei were double the normal size and their origin may be a consequence of the spontaneous fusion of nuclei and cells, (Kovács 1977) or of endoreduplication. This explanation is supported by the microscopic observations where nuclei were very close to each other (Fig. 7b). In both callus lines binucleated cells were also detected (Fig. 7c).

Cells of shoot and bud tips of the organ forming tumorous cultures generally contained 33 chromosomes – as expected. At a low frequency, cells with irregular chromosome numbers also occurred (Table 1). The frequency distribution of chromosome counts of both these and normal cells is shown by Fig. 3. Only rarely were binucleated cells also found. However, in shoot tips of the tumorous cultures abnormal mitoses could not be detected.

Dependence of karyotype stability

The observations presented clearly show that variability of chromosome numbers of the unorganized subcultured callus tissues is significantly higher than that of the organized tissues (shoots, buds). According to the χ^2-test the chromosomal variations of cultures depend on the organ forming ability of tissues ($P < 0.001$, Table 2). The variability of chromosome numbers of primary callus is also higher than that of shoots ($P < 0.001$). Generally, it is evident that in shoots from cultures of the parent and hybrid plants the karyotype is much more stable than in calli of the same plants (Table 2; total callus/total shoots: $P < 0.001$).

The chromosomal instability is independent of the genetic tumorous condition and it is similar in the tumorous and normal cultures ($0.80 > P > 0.70$; Table 2).

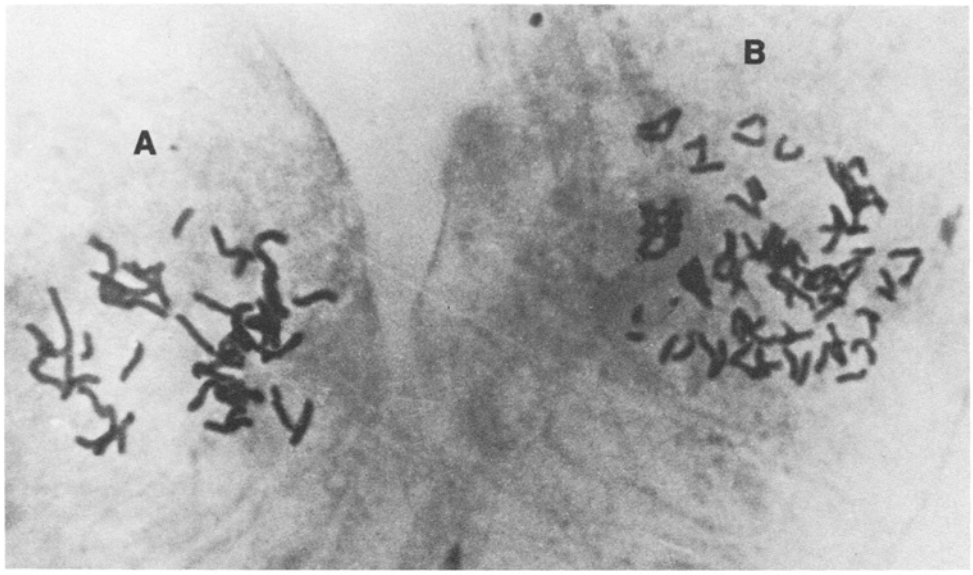

Fig. 6. Differences in chromosome size in cells with different level of ploidy

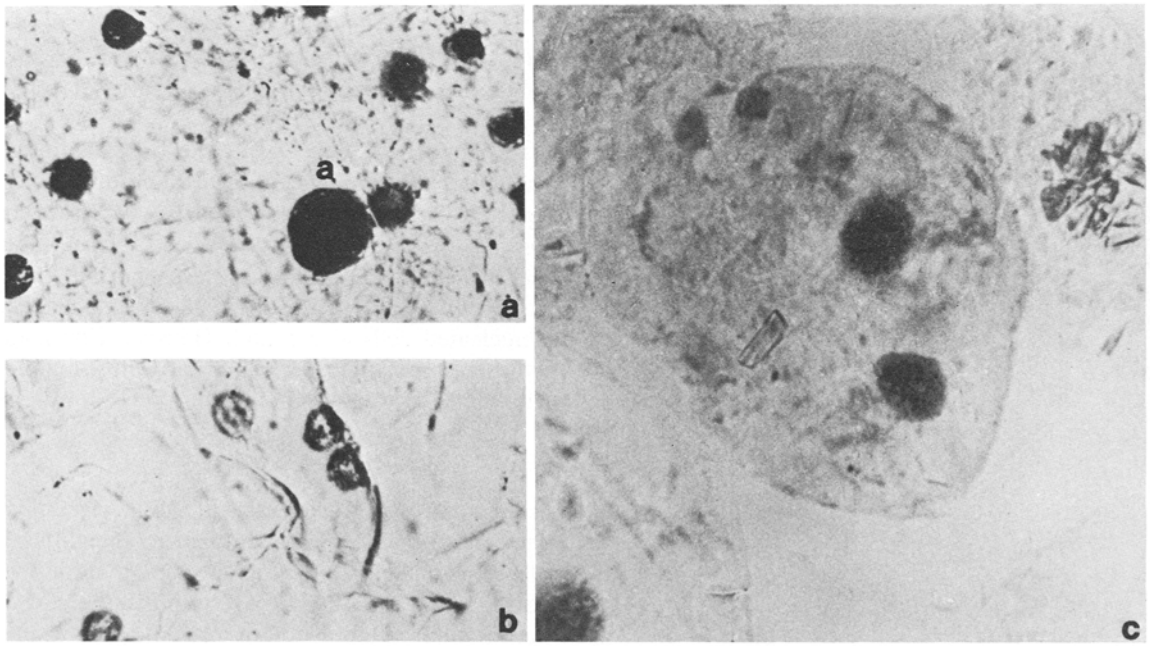

Fig. 7. "Giant" nucleus (**a**) and binucleated cells (**b, c**) in callus cultures of tumorous hybrid origin

The karyotype instability is independent of genome composition and chromosomal variability is similar in calli of *GGGG, GGL, LL* origin and in shoots of different genomes (Table 2; P values are above 0.50).

The karyotype stability is also independent of level of polyploidy (see callus of *GG, GGL, GGGG* origin, $P > 0.50$) and of hormonal requirement (normal and tumorous cultures have different growth factor requirements; $P \approx 0.50$; Table 2).

The above data provide strong evidence that karyotype stability depends only upon the degree of organization of the tissues (see Tables 1 and 2).

Discussion

On the basis of present day data, the cytological and genetic instability of cultures are induced either by components of the media used (e.g. growth substances, etc.; Torrey 1958; Street 1973) or by pre-existing genetic changes in some cells of the primary explants (e.g. endoploidy etc.; Vig 1979; Bayliss and Gould 1974; D'Amato 1975).

The results presented here demonstrate unequivocally that cytological instability depends upon only the degree of organization: that is, the lower the degree of organization (e.g. calli) the higher the instability of the karyotype, and vice versa. Hence, in shoots the karyotype is more stable (see Table 1). The cytological instability studied here appears to be independent of genome composition, genotype (chromosomal variability is similar in calli of tumorous and nontumorous *mutants* of *GGLL* plants), ploidy, tumorous or non tumorous condition and hormonal requirement. The experiments presented here supply evidence that in tissue cultures chromosomal variability, (the appearance of aneuploid, hyper-, hypoploid and polyploid cells) is produced by nondisjunction, lagging, displacement and stickiness of chromosomes; interlocked chromosomes; endomitotic polyploidy and by spontaneous fusion of cells (Kovács 1977). The genetic instability of cultures is increased by chromosome aberrations (e.g. dicentrics, breaks, acentric fragments, rings). The present results are supported by others (Singh et al. 1972; Nuti-Ronchi et al. 1976).

The size of chromosomes appears to be dependent on the level of ploidy (Fig. 6): the higher the level of ploidy, the smaller the chromosomes. This is odd in plants and it could be explained with polyteny. A similar phenomenon has been described by Frankhauser (1934): chromosomes of haploid *Triton* embryos were larger than those of the diploid ones. Studies on polyteny in plant tissues (Buiatti et al. 1977) support the author's explanation.

According to the chromosome theory of plant tumors the malignant growth originates in those cells which have acquired an irregular chromosome content as a result of abnormal mitoses (Partanen 1956; Torok and White 1960; Burk and Tso 1960). In the light of the present experiments chromosomal instability is *not a preliminary condition* for tumor formation but this phenomenon is a consequence of tumors changing processes of differentiation and organization. In tumors, the proportions of organized and less organized tissues having different karyotype stability lead to the chromosomal variability which occurs.

According to the present experiments the karyotype instability can lead to somatic genome segregation by eliminating specific chromosome sets (experiments are in progress).

The results of long term cultivation suggest that cell selection plays an important role in establishing the dominant karyotype in the cell populations of normal and tumorous origin. The author's results are supported by findings of others (Kao et al. 1970; Singh et al. 1972).

On the basis of the experiments presented it is clear that karyotype stability of tissues maintained by regular morphogenetic events is controlled by specific processes of organization and differentiation. Different growth factors (e.g. auxins, cytokinins, vitamins, etc.) influence the karyotype stability indirectly by modifying the organizational processes. Hence, karyotype stability is only a part of the problems of differentiation and organization. Consequently, the effect of medium is one of the most important factors in the induction of genetic instability disturbing the regular processes of organization (Kovács 1983).

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