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DNA recombinant molecules carrying different allels of human preproinsulin gene as mutagens in Chinese hamster cells

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In our earlier investigations it was discovered the mutagenic activity of two recombinant plasmids (pBR322ins and pAins) containing the human preproinsulin gene in cultivated somatic mammalian cells. The preproinsulin gene (allel-1) was able to function under its own promoter in different cell types since it lacked a regulatory element providing tissue-specific expression. Introduction of a frameshift mutation into the region of initiation of the translation of the transgene (allel-2) diminished the mutagenic effect of a corresponding recombinant plasmid on gene level. In this work the mutagenic activity of the recombinant plasmids carrying allel-1 (pBR322ins) and allel-2 (pBR322insN) of the preproinsulin gene was studied using cytogenetic test. It was shown that the presence of allel-2 (that probably had altered expression of the transgene) in the recombinant plasmid structure decreased the level of induced chromosome breaks per cell. It was made a conclusion that not only viruses and their genes but also recombinant DNAs of nonviral origin are able to induce chromosome and gene mutations in mammalian cells. The mutagenic activity of studied recombinant plasmids depends on the level of expression of the transgenes in the cells.

Introduction. The mutagenicity of some viruses and recombinant DNAs carrying viral genes in the somatic mammalian cells in culture was demonstrated by us earlier [1—7]. Recombinant plasmids containing human preproinsulin gene (*pBR322ins* and *pAins*) has been shown to induce gene mutations and chromosomal aberrations in the same cells as well [8—11]. The preproinsulin gene (allel-1) was able to function in different cell types under its own promoter since it lacked a regulatory element providing a tissue-specific expression [12, 13]. Introduction of a frameshift mutation into the region of initiation of the translation of the transgene (allel-2) diminished the mutagenic effect of the corresponding recombinant plasmid (*pBR322insN*) on gene level [10].

Here we present the results of our experiments which aimed: 1) to study the ability of the recombinant plasmids *pBR322ins*, *pAins* (allel-1 of the preproinsulin gene codes a protein of normal structure) and *pBR322insN* (allel-2 probably codes a changed protein) to induce chromosome aberrations in subsequent generations of transfected cells and 2) using chromosome mutation assay to study the role of the expression of the transgene in genome instability induced in the Chinese hamster cells.

Materials and methods *Cell line* The Blld-ii-FAF 28 Chinese hamster cell line was described in detail previously [1, 2, 4, 8]. It contains 18 chromosomes

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in the modal class (65—80 % of cells examined). Cells were grown in standard Eagle's medium supplemented with 10 % fetal bovine serum and 50 μg/ml kanamycin at 37 °C in an atmosphere of 5 % CO_2 and 95 % air. Stocks of cells were stored in liquid nitrogene in medium containing 10 % DMSO.

Recombinant plasmid DNAs. The human preproinsulin gene containing its own promoter but lacking a regulatory element providing tissue specificity of the expression (*BglII-TaqI*-fragment) was cloned into the recombinant plasmids *pBR322ins* and *pAins* in our institute [12]. (Fig. 1 and 2). Intracellular expression of the human preproinsulin gene included into the plasmid vectors was demonstrated after introduction into fibroblasts of different origin in our earlier investigations [13]. The *pBR322insN* plasmid carrying the frameshift mutation in the region of initiation of the translation of the human preproinsulin gene was also constructed in our Institute (Fig. 3 and 4).

Transfection with the recombinant plassmid DNAs. Chinese hamster cell populations were treated by DEAE-dextrane ($2 \cdot 10^6$ mm) at the final concentration of 100 μg/ml with and without DNA as previously described [4, 8]. The intracellular presence of the plasmid DNAs was proved by a dot-hybridization [14]. Plasmid DNAs in the decreasing concentrations were observed up to the 3rd recultivation of the cells transfected (Fig. 5).

Chromosome aberrations assay. The method was described previously [1, 4, 8]. Chromosome aberrations were scored for 100 and more cells for each variant. We repeated all experiments three times (the average data of three

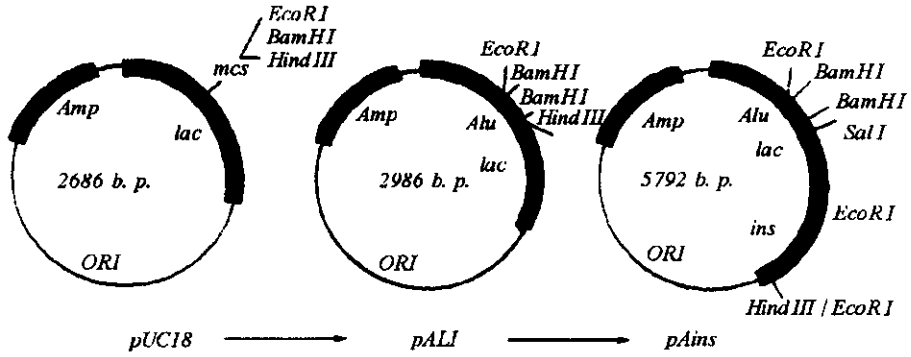

Fig. 1. Map of the *pAins* recombinant plasmid DNA

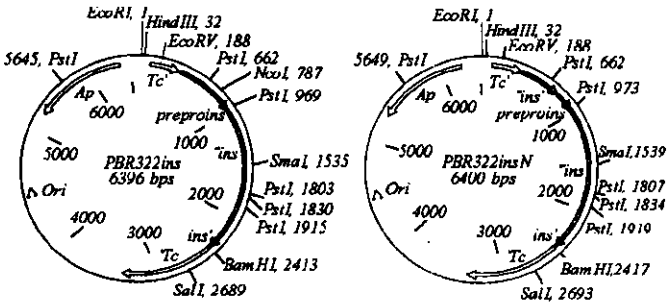

Fig. 2. Map of the *pBR322ins* recombinant plasmid DNA
Fig. 3. Map of the *pBR322insN* recombinant plasmid DNA

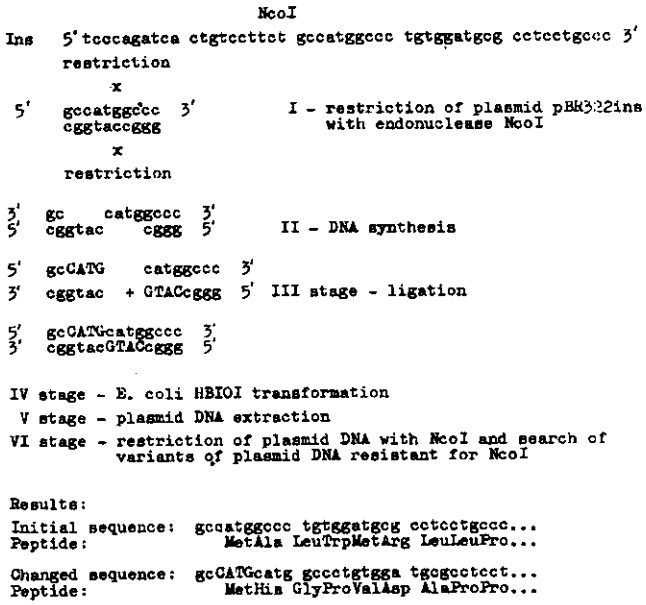

Fig. 4. The frameshift mutation in human preproinsulin gene

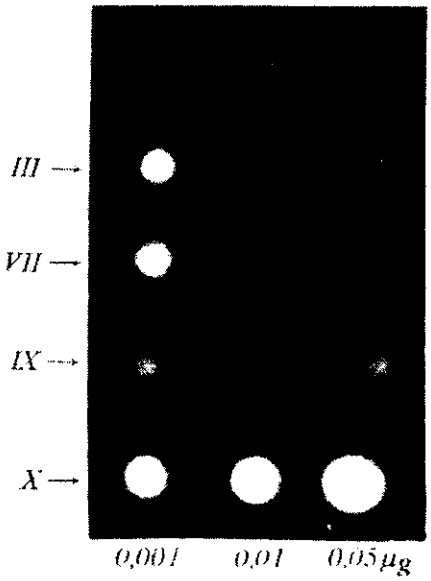

Fig. 5. Intracellular presence of the *pBR322* bacterial DNA and the *pBR322ins* and *pBR322insN* recombinant plasmid DNAs: *III* — *pBR322*; *VII* — *pBR322ins*; *IX* — *pBR322insN*; *X* — positive control *pBR322*

experiments have been performed in the tables). Observed aberrations were classified into breaks (chromatid and chromosome breaks, acentric fragments) and exchanges (translocations, rings, dicentrics) according to the criteria of ISCN [15]. We used two indices: percentage of cells with aberration and number of breaks per cell.

Criteria for judgement whether the recombinant plasmid DNAs induced chromosome aberrations and whether the frameshift mutation within human preproinsulin gene effected an ability of the corresponding recombinant plasmid to induce chromosome mutations in the transfected cells were as follows: 1) significant increase in the portion of the cells with chromosome aberrations and 2) significant increase in the number of breaks per cell.

The following scheme of experiments was used (Fig. 6). The Chinese hamster cell populations were transfected by plasmid DNAs (*pAins, pBR322ins* and *pBR322insN*). Every cell population was divided into three parts. One part of the cell population was recultivated in 24 hrs post-transfection procedure. Another part of the cell population was used for karyotype analysis. The third part was used in dot-hybridization procedure in order 1) to estimate the effectivity of the plasmid DNAs penetration into the cells, 2) to study the presence of introduced DNAs in succeeding cell generations and 3) to establish the relationship between mutation events and presence of the exogeneous DNAs in succeeded generations of transfected cells.

Statistical analysis. Statistical comparisons were made with the help of Fisher's criterium. The confidential intervals were determined as it was described [16].

Results and discussion *Induction of chromosome aberrations by plasmid DNAs.* This part of our investigations was devoted to the study of the mutagenic activity of recombinant DNAs (*pAins, pBR322ins* and *pBR322insN*) in different time after transfection (Table 1). A total number of 6653 metaphases has been examined.

All recombinant DNAs studied at both concentrations 5 and 10 μg/ml have been found to induce chromosome aberrations in Chinese hamster cells. There was no difference in maximum mutagenic effect on chromosome level induced by these agents. Chromosome aberrations including chromosome and chromatid breaks as well as pair-acentric fragments were observed in 24 hrs and 72 hrs post-transfection time. It was shown that the level of breaks sharply declined at 120 hrs post-transfection.

The chromosome exchanges appeared in 24 hrs (for *pBR322insN* it is evident) and mostly in 72 hrs (all other plasmids) after transfection.

Thus, the recombinant DNAs carrying allel-1 and allel-2 of human preproinsulin gene and *pBR322* bacterial plasmid were shown to induce both types of chromosome aberrations in transfected Chinese hamster cells. The breaks of chromosome are the dominant type of aberrations.

The effect of structure of the recombinant DNAs on the occurance of chromosome aberrations. There are two criteria being used in cytogenetic

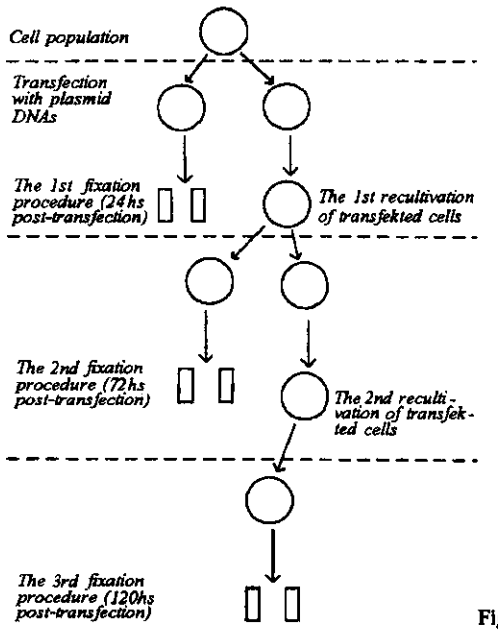

Fig. 6. The design of the experiments

Table 1
Chromosome aberration induced by different plasmids carrying human preproinsulin gene in Chinese hamster cells

Variant	DNA, μg/ml	Number of cell examined	Number of metaphases plates with different types of chromosome aberration, %			P*
			Total	With breaks	With exchanges	
			24 hrs post-transfection			
Control	—	275	0.73 (0.069+2.078	0.73	0	—
pBR322	5	242	10.33 (6.819+14.469	10.33	0	< 0.001
pBR322	10	244	13.52 (6.526+18.086	13.52	0	< 0.001
pBR322ins	5	202	9.90 (6.175+14.386)	9.90	0	< 0.001
pBR322insN	5	300	12.33 (8.858+16.284)	11.00	1.33	< 0.001
pAins	5	340	9.12 (6.296+12.405)	8.82	0.29	< 0.001
			72 hrs post-transfection			
Control	—	300	0.67 (0.064+1.907)	0.67	0	—
pBR322	5	300	7.67 (4.936+10.946)	7.33	0.33	< 0.001
pBR322	10	250	9.20 (5.939+13.087)	8.40	0.80	< 0.001
pBR322ins	5	250	12.40 (8.613+17.764)	11.20	1.20	< 0.001
pBR322ins	10	300	14.00 (10.312+18.148)	13.67	1.00	< 0.001
pBR322insN	5	300	8.00 (5.205+11.332)	7.67	0.33	< 0.001
pAins	5	300	11.00 (7.716+14.783)	9.00	1.67	< 0.001
pAins	10	300	10.67 (7.435+14.408)	8.33	2.33	< 0.001
			120 hrs post-transfection			
Control	—	350	1.14 (0.298+2.518)	1.14	0	> 0.05
pBR322	5	300	3.00 (1.374+5.227)	3.00	0	> 0.05
pBR322	10	300	3.67 (1.843+6.089)	3.00	0.67	> 0.05
pBR322ins	5	300	4.33 (2.324+6.920)	3.33	1.00	> 0.05
pBR322ins	10	300	4.33 (2.324+6.920)	3.33	1.00	> 0.05
pBR322insN	5	300	3.00 (1.374+5.277)	2.67	0.33	> 0.05
pBR322insN	10	300	2.00 (0.725+3.888)	2.00	0	> 0.05
pAins	5	300	3.67 (1.843+6.089)	2.67	1.00	> 0.05
pAins	10	300	2.67 (1.152+4.793)	2.33	0.33	> 0.05

The average data of three experiments. The confidential intervals are given in brackets
*Relationship to the control level.

research. The first of them is the per cent of metaphase plates with chromosome aberrations, it reflects the efficiency of treatment. The second one is the number of breaks per cell, it describes the degree of alteration of the genetic apparatus of treated cells. We used the last one for comparing the mutagenic effect of two recombinant plasmids *pBR322ins* and *pBR322insN*. The data obtained are presented in Table 2.

Two experimental variants (72 and 120 hrs post-transfection) out of the three ones showed the decreased aberration level for *pBR322insN* as compared to the *pBR322ins*. The statistical analysis of these data by the Fisher's test illustrated that the level of chromosome aberrations in Chinese hamster cells transfected with the *pBR322insN* recombinant plasmid was significantly lower ($P < 0.01$, $P<0.001$). Thus, we demonstrated a specific frameshift mutation effect on the ability of recombinant DNA to induce mutations at gene [10] and chromosome level as well.

Table 2
Effect of the frameshift mutation in the human preproinsulin gene inserted into the pBR322insN plasmid on an ability of the recombinant DNA to induce chromosome aberrations

Variant	DNA, μg/ml	Number of cells examined	Number of breaks (breaks per cell)	P
		24 hrs post-transfection		
Control	—	275	0.007 (0.001÷0.020)	—
pBR322ins	5	202	0.149 (0.103÷0.201)	< 0.001
pBR322insN	5	300	0.247 (0.200÷0.297)	< 0.001
		72 hrs post-transfection		
Control	—	300	0.007 (0.001÷0.020)	—
pBR322ins	5	250	0.432 (0.371÷0.494)	< 0.001
pBR322insN	5	300	0.147 (0.109÷0.189)**	< 0.001
		120 hrs post-transfection		
Control	—	350	0.011 (0.003÷0.025)	—
pBR322ins	5	300	0.153 (0.116÷0.196)	< 0.001
pBR322insN	5	300	0.050 (0.028÷0.077)***	< 0.05
pBR322ins	10	300	0.167 (0.127÷0.211)	< 0.001
pBR322insN	10	300	0.023 (0.009÷0.043)***	> 0.05

The average data of three experiments. The confidential intervals are given in brackets.
**, ***The significant differences between the *pBR322ins* level and the *pBR322insN* level ($P < 0.01$ and $P < 0.001$ respectively)

These data are in agreement with the results of our earlier investigations demonstrating the mutagenic activity of the DNA-containing viruses [1—3, 7] and the recombinant DNAs in the same cell system [2, 4, 7—11]. Inactivation of exogeneous DNAs of different origin with a help of UV-light [6, 17] and methylation [11] decreased their mutagenic effect.

We studied the ability of the recombinant molecules constructed on the basis of *pBR325* plasmid and carrying the oncogene of the Bovine Adenovirus of the 3rd type (*pBR325-D*) and the tk-gene of the Herpes Simplex Virus of the 1st type (*pBR325tk*) to induce chromosome and gene mutations in Chinese hamster cells [2—5]. The mutagenic activity of those recombinant plasmids might be due to the presence of the early viral genes since parental plasmid *pBR325* was not mutagenic. It was confirmed by the results that demonstrated a dependence of mutagenic effect on the functional activity of SV40 A gene [19, 20] and the dNTP content as the result of the *tk*-gene activity in the cells transfected by HSV-2 virus [21, 22].

It was shown that bacterial plasmid *pBR322* behaves as week mutagen by itself [11]. This property was probably connected with a toxic nucleotide sequence in the plasmid DNA. The presence of the allel-1 of the preproinsulin gene in the structure of the recombinant plasmid *pBR322ins* showed just a tendency of increasing a mutagenic activity [10, 11]. And the presence of the allel-2 in the same vector decreased the mutagenic activity. So we have shown a possibility to modulate the mutagenic effect of the bacterial plasmid with a help of different allels of the transgene. It is opening a way to influence on the mutagenic process in the system exogeneous recombinant DNA — cell if changing the structure of the transgene. Presumably an alteration in the expression of the gene-mutator prevents the induction of a part of the premutagenic lesions or provides better conditions for repair of these lesions in cultivated mammalian cells.

Similar results were obtained when studying the mutagenic and the

transforming effect of the recombinant plasmid *pEJ6.6* [23]. This one also derives from *pBR322* plasmid and contains the ras oncogene from human bladder carcinoma cells. The recombinant plasmid *pEJ6.6* induced gene mutations (*hprt* locus) only if *c-ras* gene was activated. And it lacked transforming and mutagenic properties if *c-ras* gene was inactivated by deletion. These results confirm the idea that the functional activity of recombinant DNAs and particularly transgenes plays an important role in induction of mutagenesis.

The expression of exogenous preproinsulin gene (allel-1) included into the *pBR322ins* recombinant plasmid in cultivated fibroblasts of different origin have been shown [12, 13]. At the present time we have no comparative data on the expression of two studied allels of the human preproinsulin gene in Chinese hamster cells. However, a decrease of frequences of chromosome breaks and gene mutations [10] if using the recombinant plasmid *pBR322insN* (allel-2) is evident.

The results presented here permit us to suggest a dependence of mutagenic effect of the recombinant DNAs in transfected cells on the expression of the transgene. The alteration in the transgene structure would lead to a changing of a propriate protein (if transcription and translation are realized in the cell system). Comparison of the molecular structures of normal preproinsulin and the speculated protein may reveal that the last one consists of 20 amino acid residues (a stop-codon is located in the position 21), and only two of them are homologous to the preproinsulin. It is not ruled out that the translation system «pays no attention» to the CATG insertion which is introduced into the start-codon region. In this case a normal protein would be synthesized. Then there is a possibility that the insertion of four nucleotides upsets the translation and the level of synthesized protein is reduced.

The differences in the functioning between two allels of preproinsulin gene may be determined by immunologic methods using antibodies to the human preproinsulin and/or to the hypothetical oligopeptide. Only special studies will permit us to estimate the effect of the insertion into the region of initiation of the translation on the expression of the mutant preproinsulin gene. Such an approach may allow to study a regulation of translation and mutagenesis on the level of the primary structure of the transgene.

In [9, 11] we have described the *pALI* and *pAins* recombinant plasmids that had induced the gene mutations with high efficiency. Those plasmids carried *Alu*-repeat from human genome (Fig. 1). It was found that *Alu*-repeat enhanced the mutagenic activity of the recombinant plasmids on gene level. But we did not obtain significant difference between *pBR322ins* and *pAins* (both contain allel-1 of the preproinsulin gene) in their mutagenic action on chromosomes. Probably percentage of aberrant cells reflects the portion of cells in the population which are the most sensitive to mutagenic action of different exogeneous DNAs.

It is obvious that transgenesis may be used as a good approach for investigation of mutator properties of different genes and estimation of the risk of the gene therapy manipulation with recombinant viruses and DNA molecules. We focus our attention on a mutagenic potential of recombinant DNAs and influence of introduced genes on a variety of methabolic pathways involving activation of proto-oncogenes as well [2]. This concerns especially anti-diabet strategy using recombinant adenovirus [24, 25] and plasmids containing the preproinsulin gene [13].

Insulin exerts profound effects on human physiology by controlling the activities of many enzymes, especially those involved in energy metabolism [26, 27]. The list of insulin-regulated genesis is extensive and rapidly growing [28]. It contains the following groups of enzymes: 1) integral membrane proteins (insulin and growth hormone receptors, etc.); 2) proteins involved in energy

methabolism (pyruvate kinase, ATP cytrate lyase, etc.); 3) proteins involved in reproduction (casein, ovalbumin); 4) secreted proteins/hormones (IGF-1 and IGF-2, prolactin, glucagon, etc.); 5) miscellaneous proteins (p53, thyroglobulin) and 6) transcription factors (proteins coded by *c-fos* and *c-jun*, etc.). Insulin may have either positive or negative effects on expression of many cellular genes [28—30] depending on the tissue specificity and cause the cascade of reactions.

So changing of the expression of the normal preproinsulin gene or functioning of a mutant one would lead to different consequenses. The most dangerous are destabilization of cellular genome and malignant transformation. And we have shown that the recombinant plasmids carrying the human preproinsulin gene reveal mutagenic properties depending on the presence of different regulatory elements and mutations in the structure of the transgene. That is why we emphasize the gene therapy experiments should be followed by testing of recombinant molecules on mutagenic and transforming abilities.

The main conclusion of this investigation is that not only viruses and their genes but also recombinant DNAs of nonviral origin are able to induce chromosome and gene mutations in somatic mammalian cells. The mutagenic activity of studied recombinant DNAs depends on the expression of the transgenes in transfected cells.

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Рекомбінантні ДНК, що містять різні алелі гена препроінсуліну людини, як мутагени в клітинах китайського хом'ячка

Резюме

У наших попередніх дослідженнях було відкрито мутагенну активність двох рекомбінантних плазмід (pBR322ins та pAins), які несуть ген препроінсуліну людини, у соматичних клітинах ссавців, що культивуються. Ген препроінсуліну (алель-1) має здатність функціонувати під своїм власним промотором у різних типах клітин, оскільки втратив регуляторний елемент, який забезпечує тканинну специфічність експресії. Введення мутації зсуву рамки зчитування в район ініціації трансляції трансгена (алель-2) призводило до зменшення мутагенного ефекту відповідної рекомбінантної плазміди на генному рівні. У цій роботі вивчалась мутагенна активність рекомбінантних плазмід, що несуть алель-1 (pBR322ins) та алель-2 (pBR322insN) гена препроінсуліну, з використанням цитогенетичного тесту. Показано, що присутність алеля-2 (який, можливо, має пошкодження експресії трансгена) у структурі рекомбінантної плазміди призводить до зниження рівня хромосомних розривів на клітину. Зроблено висновок стосовно того, що не тільки віруси та їх гени, а й рекомбінантні ДНК невірусної природи здатні індукувати хромосомні та генні мутації у клітинах ссавців. Мутагенна активність досліджуваних рекомбінантних плазмід залежить від експресії трансгенів у клітинах.

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Рекомбинантные ДНК, несущие разные аллели гена препроинсулина человека, как мутагены в клетках китайского хомячка

Резюме

В наших предыдущих исследованиях была обнаружена мутагенная активность двух рекомбинантных плазмид (pBR322ins и pAins), содержащих ген инсулина человека, в культивируемых соматических клетках млекопитающих. Ген инсулина (аллель-1) способен функционировать под своим собственным промотором в различных типах клеток, поскольку утратил регуляторный

элемент, обеспечивающий тканеспецифичность экспрессии. Введение мутации сдвига рамки считывания в район инициации трансляции трансгена (аллель-2) приводило к снижению мутагенного эффекта соответствующей рекомбинантной плазмиды на генном уровне. В этой работе изучена мутагенная активность рекомбинантных плазмид, несущих аллель-1 (pBR322ins) и аллель-2 (pBR322insN) гена препроинсулина, с использованием цитогенетического теста. Показано, что присутствие аллеля-2 (имеющего, вероятно, поврежденную экспрессию трансгена) в структуре рекомбинантной плазмиды, вызывало снижение уровня хромосомных разрывов на клетку. Сделан вывод о том, что не только вирусы и их гены, а также рекомбинантные ДНК невирусной природы способны индуцировать хромосомные и генные мутации в клетках млекопитающих. Мутагенная активность исследуемых рекомбинантных плазмид зависит от экспрессии трансгенов в клетках.

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