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Histone Acetylation in *Zea mays* I

ACTIVITIES OF HISTONE ACETYLTRANSFERASES AND HISTONE DEACETYLASES*

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DEAE-Sepharose chromatography of extracts from *Zea mays* meristematic cells revealed multiple histone acetyltransferase and histone deacetylase enzyme forms. An improved method for nuclear isolation allowed us to discriminate nuclear and cytoplasmic enzymes. Two nuclear histone acetyltransferases, A1 and A2, a cytoplasmic B-enzyme and two nuclear histone deacetylases, HD1 and HD2, have been identified. The histone specificity of the different enzyme forms has been studied in an *in vitro* system, using chicken erythrocyte histones as substrate. The cytoplasmic histone acetyltransferase B is the predominant enzyme, which acetylates mainly histone H4 and to a lesser extent H2A. The nuclear histone acetyltransferase A1 preferentially acetylates H3 and also H4, whereas enzyme A2 is specific for H3. This substrate specificity was confirmed with homologous *Z. mays* histones. The two histone deacetylases differ from each other with respect to ionic strength dependence, inhibition by acetate and butyrate, and substrate specificity. The strong inhibitory effect of acetate on histone deacetylases was exploited to distinguish different histone acetyltransferase forms.

The reversible postsynthetic acetylation of ϵ-amino groups of specific lysine residues within the highly basic N-terminal domains of core histones was discovered by Allfrey and coworkers (Allfrey *et al.*, 1964; Allfrey, 1970). 26 N-terminal lysine moieties per nucleosome are subject to reversible acetylation, thus giving rise to a remarkable heterogeneity with respect to the degree of modification. Since the sequential acetylation of lysine residues does not occur randomly, but in a highly ordered, site-specific way, as shown for H4 (Chicoine *et al.*, 1986; Couppez *et al.*, 1987), it is unlikely that acetylation exerts its biological effect by simply neutralizing positive charges of N-terminal histone domains (Loidl, 1988).

The dynamic state of core histone acetylation is maintained by two enzyme activities, histone acetyltransferase and histone deacetylase. Soon after the discovery of reversible histone acetylation the first histone acetyltransferase activity was described in pigeon liver (Nohara *et al.*, 1966, 1968). These enzymatic activities have then been investigated in mammalian cells (*e.g.* Libby, 1978; Belikoff *et al.*, 1980; Garcea and Alberts, 1980), in *Drosophila* (Wiegand and Brutlag, 1981); in *Artemia salina* (Cano and Pestana, 1979), in trout testis (Candido, 1975), in plants (Salvador *et al.*, 1985; Sendra *et al.*, 1986), and yeast (Travis *et al.*, 1984; López-Rodas *et al.*, 1985, 1989, 1991). The studies led to a classic grouping of histone acetyltransferases based on subcellular localization, chromatographic behavior, and histone specificity; according to this, histone acetyltransferase A was characterized as a nuclear enzyme form which acetylates histones in nucleosomes and all core histones *in vitro*, whereas histone acetyltransferase B was defined as a cytoplasmic enzyme form not active with nucleosomes, which only acetylates histone H4.

In the past years a more complex picture of histone acetyltransferase activities has evolved. Böhm *et al.* (1980) discovered an enzyme (termed DB) in bovine lymphocytes which acetylates free core histones and nucleosomes; in *Artemia* two cytoplasmic and one nuclear enzyme were reported (Estepa and Pestana, 1983); Yukioka *et al.*, (1984) separated two cytoplasmic and two nuclear enzyme forms in rat liver, whereas in yeast the situation is even more complex, since three nuclear acetyltransferases apart from a cytoplasmic could be discriminated (López-Rodas *et al.*, 1989).

Histone deacetylases have been investigated in cells of animals and fungi (Inoue and Fujimoto, 1969, 1970; Fujimoto, 1972; Vidali *et al.*, 1972; Waterborg and Matthews, 1982a, 1982b; Alonso and Nelson, 1986). Ion-exchange chromatography indicated the presence of multiple enzymes or enzyme forms (Kikuchi and Fujimoto, 1973; Cousens *et al.*, 1979). In pea three enzyme fractions with deacetylase activity have been characterized (Sendra *et al.*, 1988). A basic difference between deacetylases from animals and plants exists with respect to the inhibitory effect of sodium *n*-butyrate. Butyrate inhibits mammalian deacetylases at millimolar concentrations (2–5 mM) in a noncompetitive manner (Riggs *et al.*, 1977; Cousens *et al.*, 1979), whereas in plant cells butyrate exerts a less inhibitory effect on deacetylase enzymes even at 50–100 mM (Arfmann and Haase, 1981; Pederson and Minocha, 1988; Sendra *et al.*, 1988; Waterborg *et al.*, 1990). Numerous experiments have been performed with respect to the biological function of histone acetylation, using *in vivo* butyrate treatment for hyperacetylation of core histones (Kruh, 1982). Recently a novel, more specific inhibitor of histone deacetylase, trichostatin, has been reported (Yoshida *et al.*, 1990).

In contrast to the huge body of data on the possible corre-

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Histone Acetylation in *Zea mays* II

BIOLOGICAL SIGNIFICANCE OF POST-TRANSLATIONAL HISTONE ACETYLATION DURING EMBRYO GERMINATION*

(Received for publication, March 12, 1991)

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Multiple forms of histone acetyltransferases and histone deacetylases, which have been separated and characterized in the accompanying manuscript (López-Rodas, G., Georgieva, E. I., Sendra, R., and Loidl, P. (1991) *J. Biol. Chem.* 266, 18745–18750), together with *in vivo* acetate incorporation, were studied during the germination of *Zea mays* embryos. Total histone acetyltransferase activity increases during germination with two maxima at 40 and 72 h after start of germination. This fluctuation is mainly due to the cytoplasmic B-enzyme which predominantly acetylates histone H4 up to the diacetylated form. The nuclear histone acetyltransferase A2, specific for H3, is low throughout germination, except at 24 h, when it transiently becomes the main activity. Both enzymes are also present in the dry embryo, whereas the second nuclear enzyme A1, specific for H3 and H4, is absent in the initial stage of differentiation. The two histone deacetylases, HD1 and HD2, exhibit entirely different patterns. Whereas HD1 activity is low in the dry embryo and increases during germination, HD2 is the predominant enzyme at the start of differentiation, but almost disappears at later stages. Analysis of the *in vivo* acetate incorporation reveals that H4 is present in up to tetraacetylated subspecies. The pattern of acetate incorporation into core histones closely resembles the fluctuations of histone acetyltransferase B. Based on the analysis of thymidine kinase activity a close correlation was established between histone acetyltransferase B and DNA replication, whereas the A2 enzyme is associated with transcriptional activity. Histone deacetylase HD1 obviously serves a specific function in the dry embryo and could be a prerequisite for DNA repair processes. The study confirms the idea of multiple functions of histone acetylation and assigns distinct enzymes, involved in this modification, to certain nuclear processes.

Post-translational acetylation of core histones is an active metabolic process whose precise biological functions remain controversial. Studies of this type of modification in the ciliate protozoan *Tetrahymena* indicated that the biological function may not be a unique one, but rather variable; in macronuclei of this organism core histone acetylation is involved in the regulation of transcriptional activity, whereas in micronuclei the modification plays a role in the deposition of histones onto chromatin during DNA replication (Chicoine *et al.*, 1986; Lin *et al.*, 1989).

Multiple functions of core histone acetylation are adequate to explain the different and often controversial results in the literature: in a variety of organisms and cell types histone acetylation has been linked to transcription (Doenecke and Gallwitz, 1982; Reeves, 1984; for reviews), DNA replication (*e.g.* Loidl *et al.*, 1983; Loidl and Gröbner, 1987; Weiss and Puschendorf, 1988; Talasz *et al.*, 1990), DNA repair (*e.g.* Smerdon *et al.*, 1982; Ramanathan and Smerdon, 1989), histone replacement during differentiation processes (*e.g.* Christensen and Dixon, 1982; Christensen *et al.*, 1984; Grimes and Henderson, 1984; Loidl and Gröbner, 1986), and recombination events (Schaffhausen and Benjamin, 1976). The common denominator of these nuclear events is a transient destabilization of nucleosome structure and subtle rearrangement reactions occurring within distinct chromatin domains. Based on the numerous results in the literature and on experimental data of the myxomycete *Physarum polycephalum* our laboratory has proposed a model (Loidl, 1988) which explains the biological role of histone acetylation as a general mechanism to destabilize chromatin structure in a distinct way during different nuclear processes.

Recently, Megee *et al.* (1990) have pointed out the essential importance of a correct "physiological" acetylation of H4 in yeast; by introducing directed point mutations it was demonstrated that exchange of single lysine residues in the N-terminal histone domains resulted in mating sterility or cell cycle delay.

The effect of histone acetylation on structural properties of nucleosomes or chromatin is not completely clear at present. Although no dramatic changes of the physicochemical properties of nucleosomal structure are observed following hyperacetylation of chromatin, subtle changes within the nucleosome core as well as alterations of higher order structures occur; recently it was shown that butyrate-induced hyperacetylation causes an unfolding of the nucleosome core particle

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Communication

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Specificity of *Zea mays* Histone Deacetylase Is Regulated by Phosphorylation*

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Mono Q ion exchange high performance liquid chromatography (HPLC) reveals that the main histone deacetylase activity (HD1) of germinating *Zea mays* embryos consists of multiple enzyme forms. Chromatography of HD1 after treatment with alkaline phosphatase yields two distinct histone deacetylase forms (HD1-A, HD1-B). The same is true for chromatography after phosphatase treatment of a total cell extract. One of these enzyme forms (HD1-A) is subject to phosphorylation, which causes a change in the substrate specificity of the enzyme, as shown with HPLC-purified individual core histone species; the substrate specificity for H2A increases more than 2-fold after phosphorylation, whereas the specificity for H3 decreases to about 60%. The total histone deacetylase activity is quantitatively released from isolated nuclei after extraction with moderate ionic strength buffers; no significant residual enzyme activity could be detected in the nuclear matrix.

Core histones can be reversibly modified by post-translational acetylation at distinct lysine residues within the N-terminal protein domains. The biological function is still unclear for this modification, but it is assumed to be involved in changes of chromatin structure and function during different nuclear processes (Doenecke and Gallwitz, 1982; Loidl, 1988; Turner, 1991).

The dynamic equilibrium of core histone acetylation is maintained by two enzyme activities, histone acetyltransferase and histone deacetylase. Histone acetyltransferases are generally grouped in two categories, nuclear A-type enzymes, acetylating all core histones in nucleosomes as well as *in vitro*, and cytoplasmic B-type enzymes, acetylating H4 *in vivo* and *in vitro* but inactive with nucleosomes. Histone deacetylases have been investigated in fungi, plants, and animal cells (Hay and Candido, 1983; Alonso and Nelson, 1986; Mold and McCarty, 1987; Sendra *et al.*, 1991). In pea, three enzyme forms with deacetylase activity have been described (Sendra *et al.*, 1988). Our laboratory has recently separated and characterized two histone deacetylases in germinating *Zea mays* embryos (López-Rodas *et al.*, 1991); apart from the predominant enzyme form HD1, there was another histone deacetylase (HD2), specific for the very early stage of embryo differentiation (Georgieva *et al.*, 1991). For *Physarum polycephalum* we could demonstrate that the two histone deacetylases differ considerably in their substrate specificities (López-Rodas *et al.*, 1992).

In this communication we present evidence that the main histone deacetylase activity HD1 of germinating *Z. mays* embryos consists of two distinct enzymes. One of these two deacetylases is phosphorylated. A change in the substrate specificity results from this reversible phosphorylation. In contrast to recently published data that demonstrate histone deacetylase to be a constituent of the internal nuclear matrix of chicken (Hendzel and Davie, 1992), the histone deacetylases of maize are chromatin bound enzymes, eluting from nuclei at moderate ionic strength.

EXPERIMENTAL PROCEDURES

Materials—These were purchased from the following suppliers: Mono Q ion exchange HPLC[1] column HR 5/5 from Pharmacia-LKB (Uppsala, Sweden), Nucleosil 300-5 C_4 from Macherey-Nagel (Düren, Germany), alkaline phosphatase from bovine intestinal mucosa, type VII-N (2250 units/mg protein) from Sigma (St. Louis, MO), [^{3}H] acetic acid from Amersham International plc (Amersham, Buckinghamshire, United Kingdom).

Preparation of Cellular Extracts and Ion Exchange HPLC—Maize seeds (*Z. mays* M320) were germinated in the dark for 4 h at 28 °C on cotton layers soaked with water. Whole embryos (3 g) were harvested and used for the subsequent experiments. Preparation of extracts was done essentially as described (López-Rodas *et al.*, 1991). Before loading to the Mono Q column the dialyzed extract was made 15 mM $MgCl_2$ and centrifuged for 10 min at 2500 × *g*. The supernatant was filtered through a Millipore filter, and the equivalent of 1.5 g of tissue was loaded onto the column. Proteins were eluted with 80 ml of a linear gradient of NH_4Cl (0.01–0.5 M) in buffer B (15 mM Tris/HCl, pH 7.9, 0.25 mM EDTA, 10 mM NH_4Cl, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol). Fractions of 1.2 ml were collected and assayed for histone deacetylase activity.

Treatment with Alkaline Phosphatase—Crude cellular extracts or chromatographic eluates (collected from Mono Q chromatography) after dialysis against buffer B and centrifugation were incubated with alkaline phosphatase (130 units/ml) for 1 h at 28 °C in the presence of 15 mM $MgCl_2$. After centrifugation (2500 × *g*) and filtration through Millipore filters the samples were loaded onto the Mono Q column.

Separation and Purification of Chicken Erythrocyte Histones by HPLC—Chicken erythrocyte histones were acetylated *in vivo* by incubating a reticulocyte-enriched red blood cell population with [^{3}H] acetic acid (5.0 Ci/mmol) according to Ferenz and Nelson (1985) and then fractionated as described (Helliger *et al.*, 1988) with modifica-

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[1] The abbreviation used is: HPLC, high performance liquid chromatography.

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Hypothesis

Histone deacetylase

A key enzyme for the binding of regulatory proteins to chromatin

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Core histones can be modified by reversible, posttranslational acetylation of specific lysine residues within the N-terminal protein domains. The dynamic equilibrium of acetylation is maintained by two enzyme activities, histone acetyltransferase and histone deacetylase. Recent data on histone deacetylases and on anionic motifs in chromatin- or DNA-binding regulatory proteins (e.g. transcription factors, nuclear proto-oncogenes) are summarized and united into a hypothesis which attributes a key function to histone deacetylation for the binding of regulatory proteins to chromatin by a transient, specific local increase of the positive charge in the N-terminal domains of nucleosomal core histones. According to our model, the rapid deacetylation of distinct lysines in especially H2A and H2B would facilitate the association of anionic protein domains of regulatory proteins to specific nucleosomes. Therefore histone deacetylation (histone deacetylases) may represent a unique regulatory mechanism in the early steps of gene activation, in contrast to the more structural role of histone acetylation (histone acetyltransferases) for nucleosomal transitions during the actual transcription process.

Chromatin; Histones; Histone acetylation; Transcription factor; Oncogene protein; Histone deacetylase; Transcription

1. INTRODUCTION

The DNA of the eukaryotic cell nucleus is associated with histones to form nucleosomes, which represent the basic structural subunit of chromatin [1]. Histones can be reversibly modified by a number of posttranslational reactions, such as phosphorylation, acetylation, ADP-ribosylation and ubiquitination (see [2] for a recent review). The posttranslational acetylation of lysine residues within the N-terminal domains of core histones was first discovered by Allfrey and co-workers [3] and is assumed to play a critical role in the modulation of structural transitions of chromatin during different nuclear processes [2,4–8], although the precise mechanisms are still far from clear.

The dynamic state of histone acetylation is maintained by two enzyme activities, histone acetyltransferase and histone deacetylase. Histone acetyltransferases link the acetyl-moiety of acetyl-CoA to the ε-amino group of specific lysine residues. The presence of such an acetyl-group neutralizes a positive charge within the N-terminal part of the histone molecule (Fig. 1). This modification can be reversed by the action of deacetylases. The amino acid sequence of the N-terminal histone tails has been conserved during evolution; therefore the lysine positions which are accessible to this enzymatic acetylation are identical in a wide variety of organisms ranging from yeast to human. There are 26–28 possible acetylation sites within a nucleosome [4]; it has to be considered that these sites are concentrated on relatively small, but flexible protein domains of the nucleosome (Fig.1). The usage of these lysine residues for acetyltransferases has been shown to occur in a non-random fashion, thus generating a further level of complexity, although the extent of site specificity may be different among various organisms [9–14].

It was proposed that histone acetylation represents a general mechanism for the destabilization of nucleosomes [5,15]. Such a transient destabilization has to occur during nuclear processes that are accompanied by structural transitions of defined chromatin areas, i.e. DNA replication, transcription, DNA repair, recombination or differentiation specific exchange of histone variants. The site specificity, the high variability in the extent of acetylation, and the multiplicity of enzymes involved in this modification, would finally lead to highly specific acetylation patterns which serve as distinct signals for nucleosome destabilization in different

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Planta

Maize embryo germination

I. Cell cycle analysis

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Abstract. The cell-cycle progression of germinating embryos of maize (*Zea mays* L.) was studied from 0 to 72 h after the start of imbibition using DNA flow cytometry on isolated nuclei, and analyses of thymidine kinase activity, histone biosynthesis and levels of proliferating cell nulcear antigen (PCNA). At the start of germination, 75% of the cells were in G1, but this population had decreased to 25% by 72 h. The concomitant increase of cells in S-phase did not occur continuously, but stepwise, indicating that during germination most of the cells enter S-phase as a partially synchronized population. Within the initial 60 h of embryo germination the cells passed through one S-phase; the start and duration of this period of replicative DNA synthesis was further substantiated by the analysis of S-phase-associated events, the biosynthesis of core histones and the enzyme activity of thymidine kinase, which both began to increase at about 12 h after the start of differentiation. Thymidine kinase fluctuated periodically during germination with a transient maximum at 30 h and a second peak at 72 h; histone biosynthesis was not detectable until 12 h after the start of germination. The levels of PCNA protein closely resembled the pattern of thymidine kinase during germination. Together with the cytometric data this allows a clear assignment of cell cycle events to different times of embryo differentiation.

Key words: Cell cycle – Differentiation – Flow cytometry – Histone – Proliferating cell nuclear antigen – *Zea*

Dedicated to Prof. Walter Larcher on the occasion of his 65th birthday

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Abbreviation: PCNA = proliferating-cell nuclear antigen

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Introduction

In the last decade there has been a considerable improvement in our knowledge of gene expression during plant differentiation processes at both the cellular and the molecular level (Goldberg 1988; Goldberg et al. 1989; Sussex 1989; Poethig 1990; Dudits et al. 1991; for reviews). Among the various developmental pathways the germination of plant embryos represents a differentiation process in which the cell nucleus undergoes changes from an extremely low metabolism to a state of fully restored activity. In quiescent embryos, macromolecular syntheses and nuclear division have stopped, but these activities are resumed sequentially during the initial hours after the start of seed imbibition (Rejman and Buchowicz 1971; Dobrzanska et al. 1973; Deltour and Jacqmard 1974). Embryo germination therefore represents a sequence of molecular events that transform the heterotrophic embryo into a complex autotrophic organism.

Studies of cellular and molecular events during plant embryo germination require a detailed analysis of nuclear processes and cell cycle parameters. Most of the available data on maize embryo germination indicate that during seed maturation the majority of embryo cells are blocked in the G_1-period of the cell cycle (Deltour and Jacqmard 1974; Conger and Carabia 1976; Deltour 1985), although an early report claimed most cells to be captured in the G_2-period (Stein and Quastler 1963). However, it has to be considered that environmental conditions during seed maturation may strongly influence the percentage of cells accumulating in the G_1-period. The arrest of cells in G_1 or in a G_1-like phase (G_0) as a starting point for differentiation is also common to mammalian cells, and it has been demonstrated that cells in G_1/G_0 maintain a high viability for extended periods of time (Yanishevsky and Stein 1981). In contrast to the high percentage of G_1-cells in germinating plant embryos the degree of synchrony of cell cycle progression is poor (Deltour and Jacqmard 1974). However, for *Vicia faba* radicless, it was reported that 96% of the cells were dividing during the early stages of germination, as shown

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Maize embryo germination

II. Proteins related to nuclear proto-oncogene- and tumor suppressor gene-products

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Abstract. The existence of proteins related to nuclear proto-oncogenes (c-MYC, N-MYC, c-FOS, c-JUN) and to a mammalian tumor suppressor gene (*p53*) is shown at the protein and mRNA level in embryos of *Zea mays* L. The encoded proteins fluctuated individually during maize embryo differentiation, indicating specific regulatory functions during the differentiation program. A detailed description of cell-cycle progression during germination (see accompanying manuscript) enabled the expression pattern of these proteins to be correlated with nuclear events. We suggest that the fluctuations of the c-FOS- and c-JUN-related proteins reflect a functional role as transcription activators. The patterns of the c-MYC, N-MYC and *p53* homologues are rather related to a function during DNA replication and growth control. The level of the *p53*-related protein in maize embryos may represent a cellular marker for quiescence.

Key words: Cell-cycle – Differentiation – Proto-oncogene – Tumor supressor gene (*p53*) – *Zea*

Introduction

Proto-oncogenes, the cellular cognates of retroviral transforming genes, have been highly conserved during evolution, indicating a basic function of these genes and their protein products in the regulation of cell proliferation and differentiation. In contrast to the progress in understanding the function of proto-oncogenes in the regulation of cellular processes in vertebrates, very little is known about these entities in plants. Nuclear proto-oncogenes encode proteins which are involved in intranuclear signal transduction (Pierce 1989; Bishop 1990; Gutman and Wasylyk 1991), although their precise function is far from clear in most cases. At least for some nuclear proto-oncogene proteins, it has been demonstrated that they function as transcription factors, e.g. c-FOS and c-JUN, and therefore have a crucial role as nuclear targets that mediate mitogenic signals into distinct changes of gene expression (Ransone and Verma 1990; Gutman and Wasylyk 1991). Since proto-oncogenes have been highly conserved throughout vertebrate evolution, investigation of these genes and the corresponding proteins in lower eucaryotes (Waitz and Loidl 1991; López-Rodas et al. 1992) and plants is warranted.

Using specific antibodies against mammalian nuclear proto-oncogene proteins and heterologous cDNA probes, we could detect *c-myc, N-myc, p53, c-fos* and *c-jun* in maize embryo tissue. We were able to show that these proteins fluctuate individually during embryo germination and are involved in the regulation of growth and gene expression in plant cells.

Materials and methods

Immunodetection of proteins. Maize (*Zea mays* L., M320; Institute of Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria) seeds were germinated in darkness at 27° C on cotton layers soaked with water as described by Georgieva et al. (1991). At different times after the start of germination (0, 4, 12, 24, 40 h), embryos and the meristematic part of the root (72 h) were harvested into liquid nitrogen, and nuclei were isolated according to the procedure outlined in the accompanying manuscript.

Isolated nuclei were lysed in sodium dodecyl sulfate (SDS) sample buffer (Laemmli 1970); equal amounts of total nuclear protein from each time point (checked colorimetrically as well as on test gels) were subjected to SDS-polyacrylamide gel electrophoresis (Laemmli 1970); 10% gels were run for c-FOS and c-JUN; 15% gels for N-MYC, c-MYC and p53. Gels were blotted onto nitrocellulose membranes, blocked and processed as described by Waitz and Loidl (1991). Proteins were immunodetected using the following anti-

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Subcellular Location of Enzymes Involved in Core Histone Acetylation†

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ABSTRACT: Multiple enzyme forms of histone deacetylase and histone acetyltransferase exist in germinating maize embryos. We analyzed the association of the different enzymes to chromatin by ion exchange chromatography of subcellular fractions from different time points of embryo germination. The vast majority of histone deacetylase HD-1A was not bound to chromatin, since it was solubilized during chromatin isolation, regardless of its phosphorylation state and the phase of embryo germination. In contrast, HD-2 was chromatin bound during the entire germination pathway. Histone deacetylase HD-1B was present in a chromatin-bound and a soluble form; the ratio between these two forms changed during germination. Both nuclear histone acetyltransferases, HAT-A1 and HAT-A2, were tightly chromatin-bound and could only be released from chromatin by salt extraction. To test whether histone acetyltransferases or deacetylases are associated with the nuclear matrix, we analyzed nuclear matrix preparations from yeast, *Physarum*, and maize step by step for both enzyme activities. This analysis confirmed that part of the activity is chromatin bound, but no significant enzyme activity could be found in the final nuclear matrix, regardless of the preparation protocol. This result was further substantiated by detailed analysis of histone deacetylases and acetyltransferases during cellular fractionation and nuclear matrix preparation of chicken erythrocytes. Altogether our results suggest that the participation of these enzymes in different nuclear processes may partly be regulated by a distinct location to intranuclear components.

Core histones can be reversibly modified by a number of posttranslational reactions, such as phosphorylation, acetylation, ADP-ribosylation, and ubiquitination (Bradbury, 1992). The posttranslational acetylation of the ϵ-amino group of lysine residues within the N-terminal histone domains was first discovered by Allfrey and co-workers (Allfrey et al., 1964) and is assumed to play an important role in the modulation of structural transitions of chromatin during different nuclear processes, although the precise mechanisms are still far from clear (Loidl, 1988, 1994; Turner, 1991; López-Rodas et al., 1993; Tordera et al., 1993).

The dynamic state of histone acetylation is maintained by two enzyme activities, histone acetyltransferase and histone deacetylase. Histone acetyltransferases link the acetyl moiety of acetyl-CoA to the ϵ-amino group of specific lysine residues. This modification can be reversed by the action of deacetylases. There are 26−28 possible acetylation sites within a nucleosome (Doenecke & Gallwitz, 1982). The usage of these lysine residues by histone acetyltransferases has been shown to occur in a nonrandom fashion (Turner & Fellows, 1989; Thorne et al., 1990; Clarke et al., 1993), although recent *in vitro* studies have shown that histone acetyltransferase B from pea does not display a strict order of lysine site usage (Mingarro et al., 1993). Histone acetyltransferases can be classified with respect to their intracellular location and substrate specificity into nuclear A-type and cytoplasmic B-type enzymes. Extensive investigations of the substrate specificity of histone acetyltransferases in yeast, pea, maize, and *Physarum* have revealed that H3 and H4 are the predominant substrate molecules for these enzymes (Sendra et al., 1986; López-Rodas et al., 1989; López-Rodas et al., 1991a,b; López-Rodas et al., 1992), whereas H2A and H2B only represent minor substrates, although multiple enzymes or enzyme forms exist.

Histone deacetylases have been most extensively studied in plant cells (Sendra et al., 1988; López-Rodas et al., 1991b; Brosch et al., 1992). In maize embryos three distinct enzymes are present, HD-1A, HD-1B, and HD-2, which differ in their substrate specificity. The deacetylases follow a distinct individual activity pattern during maize embryo germination, indicating distinct and individual functions during the differentiation program (Georgieva et al., 1991). The substrate specificity of HD-1A is furthermore regulated by reversible phosphorylation (Brosch et al., 1992); the fact that histone deacetylase HD-1A is phosphorylated and as a consequence exhibits a shift in its substrate specificity provide evidence that this enzyme could be involved in cellular signal transduction pathways.

It has been recently claimed that histone deacetylases are specifically associated with the nuclear matrix, representing

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Matrix Attachment Regions and Transcribed Sequences within a Long Chromosomal Continuum Containing Maize *Adh1*

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We provide evidence for the location of matrix attachment sites along a contiguous region of 280 kb on maize chromosome 1. We define nine potential loops that vary in length from 6 kb to >75 kb. The distribution of the different classes of DNA within this continuum with respect to the predicted structural loops reveals an interesting correlation: the long stretches of mixed classes of highly repetitive DNAs are often segregated into topologically sequestered units, whereas low-copy-number DNAs (including the *alcohol dehydrogenase1* [*adh1*] gene) are positioned in separate loops. Contrary to expectations, several classes of highly repeated elements with representatives in this region were found to be transcribed, and some of these exhibited tissue-specific patterns of expression.

INTRODUCTION

The linear chromosomes of eukaryotes are compacted into a tiny organelle, the nucleus, in a manner that supports the biological functions of DNA with high fidelity, despite its enormous concentration. Different levels of DNA compaction exist, including the folding of the nucleosomal fiber into loops of different sizes. The bases of these loops are attached to a protein structure called the matrix or scaffold, and specific DNA sequences are responsible for the attachment of the chromatin to this structure (for reviews, see Gasser and Laemmli, 1987; Jackson, 1991). These loops might act not only as compaction units but also may define a separately regulated unit (for reviews, see Gross and Garrard, 1987; Bodnar, 1988; Goldman, 1988). Different experimental approaches have provided evidence that many biological activities are associated with the nuclear matrix; matrix attachment regions (MARs), beyond acting as anchoring sites, may bear positional information required for controlling transcription, may act as origins of replication, and may serve as chromatin border elements (Gasser, 1988; Gasser et al., 1989; Garrard, 1990; Bonifer et al., 1991; Eissenberg and Elgin, 1991).

Most of the data on MARs are in relation to known genes and their immediate surroundings; as a whole, information on domain organization and chromosome folding at a supragenic level is very limited. All of the current information on the relationship between loop organization, positioning of transcribed units, and potential origins of replication on a larger DNA continuum comes from two regions in Drosophila: a 320-kb continuum in the *rosy-Ace* region (Mirkovitch et al., 1988) and an 800-kb portion of chromosome 1 (Brun et al., 1990; Surdej et al., 1990). In addition, the 240-kb amplicon of the dihydrofolate reductase (DHFR) gene (Dijkwell and Hamlin, 1988), 200 kb around the mouse heavy chain IgH locus (Cockerill, 1990), and 90 kb containing the human globin loci (Jarman and Higgs, 1988) provide data on the relative distribution of MARs and transcribed units in relatively long genomic regions. In these latter cases, the genomic regions represent known loci containing several genes belonging to the same family and expressed in a developmentally controlled manner. Therefore, we still know very little about the structural and functional organization of large regions containing single genes or extensive genomic regions with no identified function. In plants, information on the loop organization of genomes is limited to the identification of matrix binding sites flanking known genes (Hall et al., 1991; Slatter et al., 1991; Breyne et al., 1992; Avramova and Bennetzen, 1993; Paul and Ferl, 1993; van der Geest et al., 1994).

An important aspect of the organization of most higher eukaryotic genomes is that the gene-containing component of the genome composes only a small fraction of the total DNA; the rest represents mainly repeated DNA. In maize, for example, the repetitive DNA makes up ~80% of the total nuclear genome (Flavell et al., 1974; Hake and Walbot, 1980). We still have little understanding of the possible roles repetitive DNA might serve, and we have only a limited knowledge of its structural organization within the maize genome.

A detailed analysis of a yeast artificial chromosome (YAC) containing the maize *alcohol dehydrogenase1* (*adh1*) locus has been published by Springer et al. (1994). They found the

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Gcn5p, a Transcription-related Histone Acetyltransferase, Acetylates Nucleosomes and Folded Nucleosomal Arrays in the Absence of Other Protein Subunits*

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Gcn5p is the catalytic subunit of several type A histone acetyltransferases (HATs). Previous studies performed under a limited range of solution conditions have found that nucleosome core particles and nucleosomal arrays can be acetylated by Gcn5p only when it is complexed with other proteins, *e.g.* Gcn5-Ada, HAT-A2, and SAGA. Here we demonstrate that when assayed in buffer containing optimum concentrations of either NaCl or $MgCl_2$, purified yeast recombinant Gcn5p (rGcn5p) efficiently acetylates both nucleosome core particles and nucleosomal arrays. Furthermore, under conditions where nucleosomal arrays are extensively folded, rGcn5p acetylates folded arrays ~40% faster than nucleosome core particles. Finally, rGcn5p polyacetylates the N termini of free histone H3 but only monoacetylates H3 in nucleosomes and nucleosomal arrays. These results demonstrate both that rGcn5p in and of itself is catalytically active when assayed under optimal solution conditions and that this enzyme prefers folded nucleosomal arrays as a substrate. They further suggest that the structure of the histone H3 N terminus, and concomitantly the accessibility of the H3 acetylation sites, changes upon assembly into nucleosomes and nucleosomal arrays.

Histone acetylation is a reversible dynamic process that occurs at specific lysine residues in the N termini of all the core histone proteins and has been correlated with several key biological processes, including nucleosome assembly and modulation of gene expression (1–4). The recent discoveries of the specific histone acetyltransferases (HATs),[1] Hat1p (5, 6) and Gcn5p (7), have directly linked histone acetylation with nucleosome assembly and transcriptional activation, respectively. Hat1p and Gcn5p are specific examples of the two general families of HATs, termed HAT-A and HAT-B. The HAT-A enzymes reside in the nucleus and acetylate the core histone N termini following their assembly into nucleosomes and chromatin. HAT-B type enzymes are primarily cytoplasmic and acetylate only free histones prior to nucleosome assembly (6), although Hat1p has recently been localized in the nucleus as well (8).

Hat1p is the catalytic subunit of both the major cytoplasmic HAT-B complex and the nuclear HAT-A3 complex (8). Consistent with their roles in nucleosome assembly, recombinant Hat1p, the HAT-B complex, and the HAT A-3 complex all acetylate free histone H4 *in vitro* but do not acetylate H4 after its incorporation into nucleosomes (5, 6, 8, 9). However, seemingly at odds with its role as a HAT-A enzyme, Gcn5p has been reported to acetylate free histones H3 and H4 but not nucleosomal H3 or H4 (10–13). This result has been reconciled by a model in which Gcn5p acetylates nucleosomal substrates only when a component of specific multiprotein complexes and is supported by the recent identification of several Gcn5-Ada complexes that are capable of acetylating histone H3 and to a lesser extent H4 in nucleosomes (12, 14–16). Nevertheless, because all previous studies of rGcn5p activity were performed using a very limited range of buffer and substrate conditions, it remains to be determined whether other proteins are absolutely required for the HAT activity of Gcn5p or whether these proteins coincidentally accentuate Gcn5p activity *in vitro* under the conditions tested previously.

To address this issue, we have assayed the HAT activity of rGcn5p over a wide range of NaCl or $MgCl_2$ concentrations, using an excess of free core histones, nucleosome core particles, and differentially folded nucleosomal arrays as substrates. Under optimal salt conditions, we find that both nucleosome core particles and nucleosomal arrays are efficiently acetylated by rGcn5p. Moreover, relative to core particles, highly condensed nucleosomal arrays are acetylated much more rapidly. Finally, whereas free histone H3 is polyacetylated by rGcn5p, nucleosomal H3 is only monoacetylated. These results demonstrate that when studied under appropriate solution conditions, rGcn5p both exhibits pronounced catalytic activity in the absence of other protein subunits and prefers folded nucleosomal arrays as a substrate. They further suggest that the structure of the H3 N termini changes upon assembly of free histones into nucleosomes and nucleosomal arrays.

EXPERIMENTAL PROCEDURES

Materials—Whole chicken blood was purchased from Pel-Freeze. BL21 cells containing plasmid pRSET-yGCN5 were obtained from Dr. David Allis (University of Virginia). DNA templates consisting of 12 tandem repeats of a 208-base pair sequence derived from the *Lytechinus* 5 S rRNA gene (208–12 DNA) were grown and purified from plasmid using pPOL208–12 as described previously (17). All chemicals were of reagent grade.

Assembly of 208–12 Nucleosomal Arrays and Purification of Core Particles—Core histones were purified from chicken erythrocyte oligo-

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[1] The abbreviations used are: HAT, histone acetyltransferase; AUT, acetic acid/urea/Triton.

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№10

Mobility of Acetylated Histones in Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis[1]

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We describe an altered mobility for acetylated histone isoforms in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Isoforms of histones H3 and H4 with a higher acetylation degree have a slightly faster electrophoretic mobility. Since acetylation neutralizes the positive charge of the ε-amino group of lysine, without significantly changing the molecular mass of the protein, the acetylation-dependent mobility shift could be explained by the increase of the net negative charge of the SDS–histone complexes. A possible consequence of this differential mobility for the acetylation site determination by protein microsequencing from SDS gels is discussed. © 1999 Academic Press

Nuclear eukaryotic DNA is packaged into chromatin, of which the basic repeating structural unit is the nucleosome. The nucleosomal core particle consists of 146-bp DNA organized around a histone octamer of two each of the core histones H2A, H2B, H3, and H4. These small, basic proteins are susceptible to a variety of postsynthetic modifications, e.g., acetylation, methylation, phosphorylation, ubiquitination, and/or poly-ADP-ribosylation (1), resulting in a high heterogeneity of histone isoforms.

Reversible acetylation occurs on ε-amino groups of specific and highly conserved lysine residues within the N-terminal tails of the core histones. It has long been known that histone acetylation is involved in diverse cellular processes, including nucleosomal assembly, transcription, and the maintenance of heterochromatin (2). Moreover, the recent findings that several transcriptional regulatory proteins are histone acetyltransferases (HATs)[4] or histone deacetylases (HDACs) have directly linked this histone modification to gene expression (3). The distinct HATs display different specificities on histones and on lysine residues that are targets for acetylation (3). Although the underlying mechanisms are still unclear, it seems likely that these site specificities reflect differential functions in the regulation of gene expression.

Acetylation site patterns have been determined by means of antibodies specific for particular acetylated lysine residues in histones H3 and H4 (e.g., 4), by using short synthetic peptides, corresponding to the N-terminal tails of histones as substrates for HATs in *in vitro* assays (4, 5), or more frequently by protein microsequencing after Western blotting from SDS–polyacrylamide gels (6, 7). In this report, we show that histones with different levels of acetylation, as determined by acetic acid/urea/Triton X-100 (AUT)–polyacrylamide gel electrophoresis, have distinct mobilities in SDS–PAGE. A higher degree of acetylation causes a faster migration in SDS–PAGE. This should be considered when excised bands of H3 or H4 are used for acetylation site determination by protein microsequencing, since sites in highly acetylated isoforms may be preferentially lost by improper excision.

MATERIALS AND METHODS

Preparation of histones. Core histones were isolated from nuclei of chicken erythrocytes by 0.25 M HCl extraction after removal of H1/H5 histones with perchloric acid (8). Individual histones were purified by reversed-phase chromatography on a C18 column as described Gurley *et al.* (9). Histones H3 and H4 were fractionated, according to the acetylation level, on a

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[4] Abbreviations used: AUT, acetic acid–urea–Triton X-100; HAT, histone acetyltransferase; HDAC, histone deacetylase; PVDF, polyvinylidene fluoride.

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ORIGINAL

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Optimized polymerase chain reaction-based single-strand conformation polymorphism analysis of p53 gene applied to Bulgarian patients with invasive breast cancer

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Abstract During the last few decades a substantial amount of evidence has accumulated proving that the abrogation of the normal p53 pathway is a critical step in the initiation and progression of tumors. Decoding the genetic mechanisms involved in carcinogenesis requires screening for consistent genetic tumor alterations, including those concerning the p53 gene. Thus, practical, efficient, and inexpensive techniques for accurate determination of p53 mutational status are needed. Polymerase chain reaction/single-strand conformation polymorphism (PCR-SSCP) analysis is considered to be a useful tool to investigate the role of the p53 gene in the development and progression of human cancers. The sensitivity of the method can be increased considerably by varying the experimental conditions. Here we demonstrate a scheme of PCR-SSCP optimization for detection of p53 gene mutations of patients with various cancers. Optimal conditions for PCR-SSCP of p53 exons 4–9 are reported. Such PCR-SSCP optimization could allow an increase in the sensitivity and reproducibility of the technique and facilitates screening of large series of patients to assess the clinical significance of p53 mutations in human cancers. Using the optimized PCR-SSCP analysis we screened Bulgarian patients with invasive breast cancer for p53 gene mutations and registered a 33.33% frequency of mutations. To date, there are no data concerning the p53 status of Bulgarian breast cancer patients. Screening for p53 gene mutations enables an accurate and routine determination of the p53 status of patients with cancer and may be applied in clinical oncology to cancer diagnosis, prediction of prognosis and response to treatment.

Key words p53 · Mutation · Polymerase chain reaction · Single-strand conformation polymorphism · Cancer

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Introduction

Mutations in the p53 tumor suppressor gene, affecting its overall function, are the most-common genetic alteration in human cancers. Approximately 50% of adult colon, stomach, liver, lung, breast, brain, and other cancers contain a mutant p53 gene [1–6]. Compared with other tumor suppressor genes, p53 has an unusual spectrum of mutations, since more than 85% of the p53 mutations reported to date are missense mutations (base substitutions) within the coding sequence of the gene that give rise to an altered protein synthesis [7, 8]. More than 90% of the substitution mutations are clustered within the conserved regions of exons 5–8, among which, at least four mutation hot spots, located at the amino acid residues 175, 248, 273, and 282, have been identified. Although a p53 mutation may occur at the earliest clinically detectable stages of the neoplastic process in some types of cancer, the proportion of tumors with an altered p53 gene is generally higher in the late-stage of disease [9, 10].

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A Short-range Gradient of Histone H3 Acetylation and Tup1p Redistribution at the Promoter of the *Saccharomyces cerevisiae SUC2* Gene*

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Chromatin immunoprecipitation assays are used to map H3 and H4 acetylation over the promoter nucleosomes and the coding region of the *Saccharomyces cerevisiae SUC2* gene, under repressed and derepressed conditions, using wild type and mutant strains. In wild type cells, a high level of H3 acetylation at the distal end of the promoter drops sharply toward the proximal nucleosome that covers the TATA box, a gradient that become even steeper on derepression. In contrast, substantial H4 acetylation shows no such gradient and extends into the coding region. Overall levels of both H3 and H4 acetylation rise on derepression. Mutation of *GCN5* or *SNF2* lead to substantially reduced *SUC2* expression; in *gnc5* there is no reduction in basal H3 acetylation, but large reductions occur on derepression. *SNF2* mutation has little effect on H3 acetylation, so SAGA and SWI/SNF recruitment seem to be independent events. H4 acetylation is little affected by either *GCN5* or *SNF2* mutation. In a double *snf2/gcn5* mutant (very low *SUC2* expression), H3 acetylation is at the minimal level, but H4 acetylation remains largely unaffected. Transcription is thus linked to H3 but not H4 acetylation. Chromatin immunoprecipitation assays show that Tup1p is evenly distributed over the four promoter nucleosomes in repressed wild type cells but redistributes upstream on derepression, a movement probably linked to its conversion from a repressor to an activator.

The *Saccharomyces cerevisiae SUC2* gene codes for invertase, an enzyme that catalyzes the hydrolysis of sucrose and raffinose to provide the cell with glucose in the absence of this essential fuel. It has been widely used to study the mechanisms underlying glucose regulation in yeast. Because these mechanisms result in changes in chromatin structure, this has been a long-lasting field of research. We first analyzed the DNase I sensitivity of the *SUC2* gene (1) as well as nucleosome positioning under repressing and derepressing conditions, in both wild type cells (2) and in regulatory mutants (3). These initial data, obtained by indirect end labeling, showed that four nucleosomes are positioned on the promoter in such a way that certain crucial elements, including the TATA box, are occluded, whereas other regulatory sequences are nucleosome-free (2). These results were refined by other workers (4, 5) who mapped the four nucleosomes at high resolution by primer extension analysis.

The *SUC2* gene is repressed in the presence of glucose by the Ssn6-Tup1 corepressor complex (6, 7), which is tethered to the promoter by Mig1p (8). Tup1-mediated repression of *SUC2* results in deacetylation of histone H3 at the promoter, as shown by an increase in H3 acetylation in *tup1* mutants, but a lack of Tup1p does not change the acetylation level of H4 (9). This result contrasts with findings for other Tup1-regulated genes in which the repression also results in deacetylation of H4 (9).

The *SUC2* gene is effectively derepressed by lowering the concentration of glucose (10), and several genes required for derepression were identified early through genetic screening (10, 11). These genes were collectively named *SNF* (sucrose non-fermenting). Further studies revealed that the *SNF* genes play two clearly different roles. The first is accomplished by the *SNF1* and *SNF4* genes. The former encodes a protein kinase (12), which acts in the pathway leading to *SUC2* derepression (13), whereas Snf4p regulates the kinase activity of Snf1p (14). The Snf1p kinase itself is rapidly phosphorylated in response to low glucose (15) and then, in turn, phosphorylates several proteins, including Mig1p (16). This seems to be a signal for the nucleus to cytoplasm translocation of Mig1p (17), and recent results seem to indicate that protein kinase C is also involved in this process (18). Importantly, translocation of Mig1p does not result in the release of Ssn6-Tup1, which remains bound to the *SUC2* promoter (19). The remaining *SNF* genes are involved in chromatin remodeling, as part of the SWI/SNF[1] complex as revealed by the pioneering studies of Hirschhorn *et al.* (20).

Derepression of *SUC2* also requires the action of the HAT

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[1] The abbreviations used are: SWI/SNF, mating-type switching/sucrose nonfermenting; SAGA, Spt-Ada-Gcn5-acetyltransferase; YPD, yeast extract-peptone-glucose; X-ChiP, cross-linking/chromatin immunoprecipitation; wt, wild type; HAT, histone acetyltransferase.

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Germline *p53* single-base changes associated with Balkan endemic nephropathy

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Abstract

The Balkan endemic nephropathy (BEN) is a significant clinical and scientific problem in need of novel effective therapies. Though many genetic and environmental factors have been investigated the basis, cause, and predisposition to BEN are still unclear. In this study, based on the hypothesis that the genetic pathways leading to BEN might be associated with p53 dysfunction, we screened for *p53* gene mutations 90 Bulgarian BEN patients using optimized PCR-SSCP-sequencing analysis. Germline *p53* single-base changes were found in blood samples in 10% of BEN cases. Three of them caused amino acid substitutions (p.Arg283Cys, p.Gln317His, and p.Lys321-Glu); the other six were either synonymous amino acid substitutions (p.Arg213Arg) or intron polymorphisms (T14766C). To the best of our knowledge, these are the first data investigating tumor suppressor gene mutations in patients with BEN. The obtained results are in support of our hypothesis that *p53* gene alterations are possibly involved in BEN genetic pathways.

Keywords: Balkan endemic nephropathy; Tumor suppressor genes; *p53*; Mutation; Cancer; PCR-SSCP

The Balkan endemic nephropathy (BEN) is a chronic, slowly evolving kidney disease affecting some well-defined rural areas of Bulgaria, Romania, Croatia, Serbia, and Montenegro, and Bosnia and Herzegovina. The disease has no acute onset and may be asymptomatic for many years [1]. It is characterized by progressive renal failure and by rule has a fatal outcome. Uroepithelial cancers appear as a frequent complication of BEN [2]. Approximately 30–48% of BEN patients develop urinary tract tumors localized mainly in the renal pelvis and rarely in the ureter and bladder [3]. BEN is a familial disease without distinct Mendelian pattern of inheritance [4]. The etiology of BEN is still a mystery. The distinctive BEN characteristics (mosaic-like area distribution and familial clustering) have led to the concept that it arises as a multifactorial phenomenon in genetically susceptible individuals subjected to exposure to specific environmental agents. Several hypotheses on the etiology of BEN have been proposed. Thus, it has been assumed that the disease may result from long-term exposure to environmental agents, such as, mycotoxins like ochratoxin A (the mycotoxin hypothesis) [5], polycyclic aromatic hydrocarbons, and other toxic organic compounds leaching into the drinking water from the coal deposits (the Pliocene lignite hypothesis) [6], herbs containing aristolochic acid [7], heavy metals [8], and others. The screening for chromosomal aberrations has led to the indication of a putative chromosomal region 3q24–3q26, which may contain a BEN candidate gene [9]. A recent cytogenetic study on three tumors of Bulgarian BEN patients found multiple chromosomal abnormalities including loss of heterozygosity at 3q24, genetic loss at 4q, and genetic gains at 17 other chromosomal regions [10]. An inherited metabolic susceptibility [11] and lecithin-cholesterol-acyltransferase partial deficiency [12] were supposed to be involved in the pathogenesis of the disease. Recently, a study on 54 blood samples of Bulgarian BEN patients suggested that polymorphic glutathione *S*-transferase

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Transcription of the *MAT2A* gene, coding for methionine adenosyltransferase, is up-regulated by E2F and Sp1 at a chromatin level during proliferation of liver cells

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Abstract

Methionine adenosyltransferase (MAT) is an essential enzyme because it catalyzes the formation of *S*-adenosylmethionine, the main methyl donor. Two MAT-encoding genes (*MAT1A*, *MAT2A*) are found in mammals. The latter is expressed in proliferating liver, dedifferentiation and cancer, whereas *MAT1A* is expressed in adult quiescent hepatocytes. Here, we report studies on the molecular mechanisms controlling the induction of *MAT2A* in regenerating rat liver and in proliferating hepatocytes. The *MAT2A* is up-regulated at two discrete moments during liver regeneration, as confirmed by RNApol-ChIP analysis. The first one coincides with hepatocyte priming (i.e. G_0–G_1 transition), while the second one takes place at the G_1–S interface. Electrophoretic mobility shift assays showed that a putative E2F sequence present in *MAT2A* promoter binds this factor and ChIP assays confirmed that E2F1, E2F3 and E2F4, as well as the pocket protein p130, are bound to the promoter in quiescent liver. *MAT2A* activation is accompanied by changes in the binding of histone-modifying enzymes to the promoter. Interestingly, p130 is not displaced from *MAT2A* promoter during hepatocyte priming, but it is in the late expression of the gene at the G_1–S transition. Finally, the transcription factor Sp1 seems to play a decisive role in *MAT2A* induction, as it binds the promoter when the gene is being actively transcribed. In summary, the present work shows that the molecular mechanism of *MAT2A* expression is different during G_0–G_1 or G_1–S transition and this may be related to the distinct requirements of *S*-adenosylmethionine during liver regeneration.

Keywords: Histone acetylation; Chromatin immunoprecipitation; ChIP; RNApol ChIP; Liver regeneration

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Analysis of the K-ras/B-raf/Erk signal cascade, p53 and CMAP as markers for tumor progression in colorectal cancer patients

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Abstract. Colorectal cancer patients may succumb to their disease because of local recurrence or formation of metastasis. To develop a prognostic tool for these fatal types of progression, 23 patients with colorectal carcinoma were included in this study for the detection at the time of surgery of the incidence of K-ras, B-raf and p53 mutations, the phosphorylation status of Erk and the expression of cystatin-like metastasis-associated protein (CMAP) in tumor, mucosa and liver samples. Polymerase chain reaction-restriction fragment length polymorphism and PCR-SSCP were used to detect the respective mutations. The results of these assays were complemented by sequencing the K-ras, B-raf and p53 mutations. A multiplex RT-PCR assay was used to detect the CMAP mRNA levels and the phosphorylation status of Erk in tumor samples was assessed by Western blot using a phospho-specific Erk antibody. The carcinomas were classified as stages T4 (70%), T3 (17%), T2 (9%) and T1 (4%) and thus represent a group of advanced colorectal carcinomas. The carcinomas (8 out of 23, 39.1%) were mutated in K-ras codons 12 or 13 and two patients had a B-raf (V599) mutation in their tumor. Of 22 tumors, 11 (50%) were positive for pErk, indicating the activation of the RAS/RAF/ERK signaling pathway. Of the 23 tumors, 13 (65.5%) showed an increased CMAP RNA level. Notably, 10 of these 13 patients have already died and two developed liver metastasis. Mutations in p53 were found in only 6 patients (26%), with 6 being detected in carcinoma, 1 in mucosa and 1 in liver tissue. These alterations were classified as non-sense (n=1), mis-sense (n=2) and frame-shift mutations (n=1) as well as intron polymorphisms (n=5). There was a significant correlation between Erk activation and K-ras codon 12 mutation (p=0.016), but not between K-ras codon 13 or B-raf mutations and Erk activation. Furthermore, there was a significant correlation of each positive marker with tumor stage (p=0.001).

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Key words: colorectal cancer, K-ras mutations, B-raf mutations, ERK activation, p53 mutation, cystatin-like metastasis-associated protein expression

Introduction

A significant amount of research in the past several years suggests that a number of prognostic markers may be useful in defining the individual risk of colorectal cancer patients after surgery and in determining which patients may benefit the most from adjuvant chemotherapy (1,2). Therefore, it is critical to investigate molecular markers that will identify more aggressive colon cancer phenotypes to tailor patient therapy accordingly.

Colorectal cancers develop as a result of different combinations of genetic alterations, epigenetic changes and post-translational modifications. Such molecular alterations affect oncogenes, tumor-suppressor genes and the genes encoding the enzymes critical for mismatch excision repair (3). Phosphorylation plays a key role in the post-translational modification of proteins that regulate their function and activity (4).

The RAS/RAF/MEK/ERK/MAP signal transduction pathway is a conserved RAS-activated protein kinase cascade that regulates cell growth, proliferation and differentiation in response to growth factors, cytokines and hormones (5). The constitutive activation of this pathway is common to numerous cancers. Of human cancers, ~15% have activating ras mutations (6) and mutations in B-raf were identified in a large-scale screen for genes mutated in human cancer (7). The most frequently activated positions of the K-ras gene in human tumors have been found to be codons 12 and 13. These activating mutations produce an alteration in the transduction of signals in the RAS pathway and ultimately lead to increased mitogenic signaling. The frequency of K-ras codon 12 and 13

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ORIGINAL PAPER

Alterations in *p53*, *BRCA1*, *ATM*, *PIK3CA*, and *HER2* genes and their effect in modifying clinicopathological characteristics and overall survival of Bulgarian patients with breast cancer

Stefan S. Bozhanov · Svetla G. Angelova · Maria E. Krasteva · Tsanko L. Markov · Svetlana L. Christova · Ivan G. Gavrilov · Elena I. Georgieva

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Abstract
Purpose Though *p53*, *BRCA1*, *ATM*, *PIK3CA*, and *HER2* genes are shown to be involved in various aspects of breast carcinogenesis, their functional relationship and clinical value are still disputable. We investigated the genetic status or expression profile of these genes to further elucidate their clinical significance.
Methods PCR-SSCP-Sequencing of *p53*, *BRCA1*, *ATM*, and *PIK3CA* was performed in 145 Bulgarian patients with sporadic breast cancer. Expression profiles of *HER2* were determined by ICH and CISH. Relationship between mutations and clinicopathological characteristics was evaluated by Chi-squared and Fisher's exact tests. Multivariate Cox proportional hazard test and Kaplan–Meier analysis were used to evaluate differences in overall survival between groups.
Results The frequency of *p53* (22.07%), *BRCA1* (0.69%), *ATM* (7.59%), and *PIK3CA* (31.25%) alterations and HER2 (21.21%) overexpression was estimated. Mutated *p53* was associated with tumor size ($P = 0.033$) and grade of malignancy ($P = 0.001$), *ATM*—with grade of malignancy ($P = 0.032$), and *PIK3CA*—with PR-positive tumors ($P = 0.047$). HER2 overexpression correlated with age of diagnosis ($P = 0.009$), tumor size ($P = 0.0004$), and ER expression ($P = 0.011$). Univariate survival analysis showed that mutated *p53* is an indicator for worse outcome ($P = 0.041$). Combination of two genetic abnormalities did not correlate with more aggressive carcinogenesis and worse overall survival.
Conclusions Our data indicated that *p53*, *BRCA1*, *ATM*, *PIK3CA*, and *HER2* alterations specifically correlate with clinicopathological characteristics of Bulgarian patients with breast cancer. Of these genes, only mutated *p53* showed significant, though not independent, negative effect on overall survival.

Keywords Breast cancer · Tumor suppressor genes · Proto-oncogenes · Mutations · Clinicopathological characteristics · Overall survival

Abbreviations

BC	Breast cancer
IHC	Immunohistochemical analysis
CISH	Chromogenic in situ Hybridization
OS	Overall survival
T	Tumor size
N	Nodal status
G	Grade of malignancy
ER	Estrogen receptor
PR	Progesterone receptor
HR	Hazard ratio
CI	Confidence interval

Stefan S. Bozhanov and Svetla G. Angelova have contributed equally to this work.

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Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Factor binding and chromatin modification in the promoter of murine *Egr1* gene upon induction

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Abstract The influence of chromatin on immediate-early gene expression has been studied in a model of *Egr1* induction in intact mouse cells. ChIP analysis of factor and RNA polymerase binding reveals that the gene is constitutively poised for transcription in nonstimulated cells, but a repressing chromatin structure hampers productive transcription. Stimulation with phorbol esters results in a transient activation, which starts at 5 min and peaks at 30 min. Quantitative mapping of promoter occupancy by the different factors shows for the first time that no direct competition between SP1 and EGR1 occurs. The phosphorylation of ELK1 and CREB, which involves both the cascades of MEK1/2 and p38 kinases, is required for gene expression, which ceases following the binding of NAB1 and NAB2 to the promoter. The changes in histone acetylation and the differential recruitment of histone-modifying complexes further show the role of chromatin in the activation of this immediate-early gene.

Keywords Immediate-early genes · Chromatin structure · Factor binding · Histone deacetylases · Histone acetyltransferases

Abbreviations

CBP	CREB binding protein
ChIP	Chromatin immunoprecipitation
CRE	cAMP-response element
CREB	cAMP-response element-binding protein
ELK	Ets-like
ERK	Extracellular signal-regulated kinase
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
IE	Immediate-early
NAB1/2	NGFI-A binding protein 1/2
S.E	Standard error
SP1	Specificity protein 1
SRE	Serum response element
SRF	Serum response factor
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
TSS	Transcription start site

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Introduction

Activation of cellular immediate-early (IE) genes occurs within a few minutes following the reception of an appropriate stimulus, which reaches the nucleus usually via signalling cascades. The roles played by IE gene products are multiple, ranging from transcription factors to secreted proteins to cellular enzymes (see [1] for an early review). The protooncogenes c-*Myc*, c-*Fos*, and c-*Jun* were the founder members of the IE gene class, to which a large number of genes have been added.

The activation of IE genes usually occurs in a transient fashion. For instance, in the course of a study on liver regeneration, we have described that the expression of c-*Myc*, *Id2*, *c-Fos*, and *Mat2A* genes, as measured by RNAPol-ChIP [2], is detected within 30 min after partial hepatectomy [3, 4] and declines afterwards, dropping to the basal values by 6 h. The mechanisms by which expression of IE genes occurs, including the cascades involved in

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PCR-SSCP PATTERN ALONE IS HIGHLY PREDICTIVE OF A SPECIFIC MUTATION WITHIN A DNA SEQUENCE

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(*Submitted by Corresponding Member G. Roussev on October 22, 2007*)

Abstract

Molecular screening for genetic and epigenetic abnormalities in specific cancer related genes needs rapid and efficient techniques. Of the methods available, most widely used for screening and routine diagnosis is the PCR-SSCP – sequencing analysis. Here we demonstrate that different DNA samples with identical aberrant SSCP pattern show identical nucleotide change after sequencing analysis. This finding was established during a massive screening for mutations in two tumour-suppressor genes (*p53* and *ATM*) in a group of 284 Bulgarian patients overall, 147 of which with breast cancer, 114 with Balkan endemic nephropathy and 23 with colorectal carcinoma. The samples with aberrant electrophoretic behaviour were grouped at equal conditions of SSCP gel electrophoresis according to their mutant pattern and were sequenced in both strands. Sequencing analysis revealed that all samples with identical profile harboured identical nucleotide changes. Reproducibility of this observation was confirmed using different PCR and SSCP electrophoretic devices. On the basis of the obtained results we suggest that the SSCP pattern alone can be used as a diagnostic tool to directly identify specific already characterized genetic abnormalities. Introduction of this approach into scientific and clinical practice will increase considerably the speed of molecular screening analysis.

Key words: PCR, SSCP, sequencing, mutation, cancer

Introduction. Many studies validated the concept that molecular-genetic and epigenetic abnormalities in two main groups of functionally related genes – tumour suppressor genes and proto-oncogenes, were proved to be engaged in carcinogenetic

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BIOLOGIE

Génétique

FREQUENCY AND SPECTRUM OF *ATM* MUTATIONS IN BULGARIAN BREAST CANCER PATIENTS

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(*Submitted by Corresponding Member I. Ivanov on October 23, 2008*)

Abstract

Germline mutations in *ATM* gene lead to a rare hereditary syndrome, Ataxia-Telangiectasia (A-T), characterized with oculocutaneous telangiectases, an increased incidence of cancer, hypersensitivity to ionizing radiation and chromosomal instability. Women heterozygous for *ATM* show a relative risk to develop cancer, with breast cancer being most prevalent. Hence, it is critical to investigate whether *ATM* mutations result in breast cancer susceptibility.

PCR-SSCP-sequencing analysis was performed in 17 specific exons of *ATM* gene to estimate the type and frequency of mutations in a group of 145 Bulgarian breast cancer patients. A total of 11 patients with *ATM* mutations were identified. Two mis-sense variants – pSer707Pro (T>C) and pAsp1853Asn (G>A), and four identical intronic variants IVS38-8 T>C were found. Our data showed that *ATM* mutatioual frequency is 7.59%.

Comparisou with clinical characteristics showed that in the group with *ATM* mutation the carcinogenic process has an early age expression, but is less aggressive, confirmed by the grade of malignancy G ($p = 0.032$). Identifying carriers of mutations iu the *ATM* geue will contribute to assessment of its role in breast cancer pathogenesis.

Key words: ATM, PCR-SSCP-sequencing, mutation, breast cancer

Introduction. Of the ten million new cases of invasive cancer world-wide each year in males and females, 10% arise in the breast, which makes it the second most common site of malignant neoplasms after the lung [1]. Most cases are sporadic, but about 7% are due to dominantly inherited predisposition, with germline

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INDUCED ANDROGENESIS IN *Lycopersicon, Medicago, Capsicum* AND *Triticum*

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Methods for producing plants from haploid gametes have been, are and will be great interest to plant geneticist and breeders. They are of great importance especially in studies on the induction of mutations and also for the production of homozygous plants for very short time. Despite the considerable results obtained with anther cultures of tobacco, rice and potatoes research in tomato, alfalfa and pepper is yet insufficient.

The aim of this study was to identify factors influencing the androgenesis in anther cultures of tomato, alfalfa, pepper and wheat as well as to characterize the regenerated plants.

Material and Methods

Licopersicon: One hundred varietes and lines of *L. esculentum* L. and fifteen genotypes of *L. peruvitanum*, *L. chesmanii* var. *minor*, *Solanum penelii* were used as initial material. Two stages of pollen development – late meiosis and uninucleat microspore were tested. More than fifty variants of nutrient media after Nitsch (1969), Murashige and Skoog (1962), Gresshof and Doy (1972) in combination with different concentrations of phytohormones were tired out. Twelve different variants of pretreatment with temperature and gamma rays were tested. MS nutrient medium + IBA were used for rooting.

Medicago: Anthers of 10 alfalfa lines containing pollen in meiosis at uninuclear stage of development have been used as initial material. More than 30 variants of nutrient media after Murashige and Scoog (1962), Gamborg (1968), Blaydes (1966) with various concentrations kinetin, BAP, 2-ip, zeatin, IAA, NAA, 2,4-D were tested. Twenty-five different treatments with low temperatures and gamma rays have been tried in order to find out the best effect on callus induction and organogenesis.

Capsicum: Three basic nutrient media (Murashige and Scoog, 1962; Nitsch, 1969; Sibi *et al.*, 1979) were used for *in vitro* androgenesis in pepper. Different modifications (14 variants), solid and liquid (Dumas de Vaulx, 1981), with phytohormons and carrot extract were tested. Modifications with or without activated charcoal were also used. Pretreatment wit temperature (35°C) and gamma rays (400 and 800 r) were tried out (Pundeva *et al.*, 1990). Anthers in uninucleate stage of 5 pepper varieties and 2 interspecies hybrids were used as donor material.

Triticum: Anthers from 80 varieties and lines of *Triticum aestivum* L. and hybrids between *Triticum aestivum* x *Agropyron intermedium* were used as initial material. Haploid calli were induced from anthers at uninuclear stage of microspore development on Potato II modified medium. All regeneration procedures were after Mentewab *et al.* (1999). Embryos and calli are observed from anthers at least 8 days after inoculation. They are transferred until sterile conditions on the regeneration medium R9 (de Buyser and Henry, 1986).

Results and discussion

Our results suggest that the genotype, growing conditions of the donor plants, stage of microspore development, phytohormonal composition of the nutrient media and pretreatment of anthers with physical agents (temperature and gamma rays), alone or in combination, affected the frequency of organogenesis and regeneration in anther cultures of the studied species. The results showed that the best medium for androgenesis in tomato is MS + zeatin or 2-IP; in alfalfa - Blaydes + 2-IP; in pepper - MS + carrot extract; in wheat - MS + L-glutamine, potato extract, 2,4-D and kinetin. To stimulate androgenesis 25 different pretreatments (temperature and gamma rays) were tested. Hig-

INTRODUCTION OF *Arnica montana* L. TO BULGARIA USING *IN VITRO* TECHNIQUE

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Introduction

Arnica montana L. (*Asteraceae*) is an herbaceous plant spread in the mountains of Europe. In some countries it is law protected. In Bulgaria *A. montana* is not spread as a wild species. The flowers, roots and rhizomes were widely used in traditional medicine. The main active constituents of *Arnica* are sesquiterpene lactones, flavonoids, volatile oil etc. The sesquiterpene lactones of *Arnica* are associated with anti-inflammatory and cytotoxyc effects, the flavonoids possess antimicrobial, antiflogistic and anti-rheumatic properties and the volatile oil is of antiseptic activity (Roki *et al.*, 2001).

The first experiments for *A. montana in vitro* propagation were performed in the early nineties (Daniel and Bomme, 1991). The influence of various plant growth regulators on *A. montana in vitro* propagation was also examined. For bud initiation and proliferation different combinations of BA, kinetin, zeatin, 2- ip, thidiazuron, NAA, IAA etc were used (Lê, 1998, 2000; Butic-Keul and Deliu, 2001; Werememczuk-Jeyna and Wysokinska, 2000).

The purpose of the investigation is to introduce and to cultivate *A. montana* in Bulgaria through *in vitro* techniques.

Material and methods

Seeds of *A. montana* from the German Botanic Garden were used for *in vitro* culture initiation. Sterilization was performed with 70% ethanol and 0.1% $HgCl_2$ applied in combination or independently. The seed samples were rinsed three times for 5, 10 and 15 min in sterile distilled water. *A. montana* germinnation *in vivo* is low. To stimulate it the seeds were treated with 0.03% GA_3 for 24 h and 48 h, then the treated seeds were cultivated on five nutrient medium variants containing different amount of GA_3, 2% sucrose and 0.6% agar. After germination the seedlings were cut into fragments with an apical or axillary bud. The fragments were cultivated on ten nutrient media based on that of MS enriched with different growth regulators (BA, zeatin, 2-ip, IAA, NAA).

To induce root formation the regenerants were transferred to half strength MS medium supplemented with 1% sucrose, 0.4% agar and either IAA, IBA or NAA.

Results and Discussion

Shoot tips of *A. montana* are preferable for *in vitro* micropropagation (Lê, 1998, 2000; Wermemczuk-Jeyna and Wysokiñska, 2000). To initiated *in vitro* culture, we used plant material from seeds germinated *in vitro*. Two patterns of seed sterilization were examined. Disinfecting with 70% ethanol for 2 min and 0.1% $HgCl_2$ for 3 min resulted in 100% seed sterilization. Highest germination (62.00%) resulted from the treatment with 0.03% GA_3 for 24 h followed by cultivation on Murashige and Skoog (1962) nutrient medium enriched with 50 mg l^{-1} GA_3.

Regeneration proceeded on all kinds of nutrient media tested including MS medium without growth regulators. Among the tested nutrient media, containing cytokinins (BA, zeatin

MICROPROPAGATION *IN VITRO* OF *Rhodiola rosea* L.

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Introduction

Rhodiola rosea L. (*Crassulaceae*) is a perennial grass of quite limited distribution. Over the last years *Rh. rosea* area drastically decreases because of over exploitation. Therefore the species in included in the Red Book of Bulgaria. The plant contains biologically active substances: flavonoids: rosavin (2.1%), salidrosid (0.8%), rhodiolin, antraglikosides, β-sitosterin, monoterpenoids and tanins (16-18%) (Satsiperova *et al.*, 1993). Substances isolated from the roots are highly efficient in therapy of nervous system diseases, during rehabilitation after infections, in cancer prevention (Kelly, 2001), etc.

There exists only quite insufficient reference concerning *R. rosea in vitro* propagation. Kaftanat *et al.* (1988) have elaborated a method to root shoots segments. Kirichenko *et al.* (1994) studied callus induction and regenerating ability in the leaves. Ishmuratova (1998) obtained regenerants from stem segments about 10-95% of them survived after acclimatization in soil.

The purpose of our experiments was to elaborate an efficient method for *in vitro* propagation of *Rhodiola rosea* L. and its application in the practice.

Material and Methods

Rh. rosea germination *in vivo* is low. Seeds of *R. rosea* were collected from natural locations in the Rila Mountain and were used for *in vitro* culture initiation. To stimulate seed germination the seeds were treated with 0.03% gibberellic acid (GA_3) for 24 h. Ten variations of Murashige and Skoog (1962) nutrient medium (MS) containing 25, 50, and 100 $mg.l^{-1}$ gibberellic acid respectively and 0.4-0.6% agar-agar were tested.

The plants obtained *in vitro* were cut into 2-3 explants with a stem and an apical bud. The explants were grown on 10 variants of different amount of nutrient medium containing organic supplements and phytohormons - zeatin, 2-ip, BA, IAA, NAA and caseinhydrolysate. Sucrose and agar-agar content was constant, 3.0% and 0.7%, respectively. Regeneration proceeded under 22 ^{0}C and 2500 lx illumination at 16 h photoperiod.

Results and Discussion

R. rosea seed germination reaches 20% in the control variants. Highest germination was obtained at MS nutrient medium containing 50 $mg.l^{-1}$ gibberellic acid and 0.4% agar-agar - 84.38% on the 20th day. Germination lasted from the 8th until the 40th day.

Our data suggest that nutrient medium composition and particularly phytohormone concentration are very important for *in vitro* induction of organogenesis and regeneration.

In vitro Shoot Proliferation

Ten variants of nutrient medium based on that of Murashige and Skoog (1962) with different phytoregulators were tested. MS-Z medium containing 2 $mg.l^{-1}$ zeatin and 0.2 $mg.l^{-1}$ IAA as well as the MS-B medium containing 2 $mg.l^{-1}$ BA and 0.2 $mg.l^{-1}$ IAA proved to be the most efficient for propagation. The mean number of regenerants per explant on MS-Z medium was 3.78 with mean plant height 3.84 cm. In the second passage to stimulate propagation the concentration of growth regulators was reduced. Certain increase up to

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Original Research Paper
Оригинален научен труд

DISTRIBUTION OF (AAT)n MICROSATELLITE DNA REPEAT SEQUENCE IN PEPPER LINES RESISTANT TO ECONOMICALLY IMPORTANT DISEASES COMPARED TO OTHER PLANT FAMILIES

ДИСТРИБУЦИЈА НА (AAT)n МИКРОСАТЕЛИТ ДНА ПОВТОРЕНА СЕКВЕНЦА КАЈ ОТПОРНИТЕ ЛИНИИ ПИПЕРКА НА ЕКОНОМСКИ ЗНАЧАЈНИ БОЛЕСТИ И КОМПАРАЦИЈА СО НЕКОИ ДРУГИ РАСТИТЕЛНИ ФАМИЛИИ

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Summary

Microsatellites, also called sequence tagged microsatellite sites (STMSs), have become important markers for genome analysis but are currently not sufficiently understood in plants. Characterization of the type of (AAT)n distribution is of substantial importance due to its use as a genetic marker in a broad spectrum of gene screening approaches. By the application of molecular genetic approaches, we studied the distribution of (AAT)n simple repeat sequence in several pepper lines resistant to economically important diseases and compared the results to other plant families. We found that (AAT)n repeat is well distributed in some plant genomes, including the genus *Capsicum*, in big clusters in DNA regions lacking sites for restriction endonucleases.

Key words: DNA markers, sequence tagged microsatellite site, *Solanaceae*, complex disease resistance

Извадок

Микросателитите, таканаречени sequence tagged microsatellite sites (STMSs), станаа значаен маркер за анализа на геномите, но се уште се слабо проучени кај растенијата. Карактеризација на типот од (AAT) дистрибуција е од основно значење како резултат на тоа што се користи како генетички маркер со широк спектар на проучување на гените. Со алликација на молекуларната генетика, ја проучуваме дистрибуцијата на (AAT) поединечни повторени секвенци кај отпорните линии пиперка на економски значајни болести и ја споредуваме со резултатите од другите растителни фамилии. Утврдено е дека (AAT) повторувањето е дистрибуирано во некои растителни геноми, вклучително со родот *Capsicum*, во голем кластер во ДНК региони намалувајќи ги местата за рестрикција на ендонуклеидите.

Клучни зборови: ДНК маркери, sequence tagged microsatellite site, *Solanaceae*, отпорност на комплекс болести

Introduction

Tandem arrays of short (2-5bp) nucleotide motifs are present in high numbers in most eukaryotic genomes and have also been found in prokaryotes (Field, 1996, and Van Belkum, 1998). Despite the large number of DNA markers now available, the identification and isolation of additional, novel, highly polymorphic DNA markers remains important. Such markers are needed for the improvement of genetic linkage maps in disease-resistance studies. Polymorphic markers are also required for the construction of genetic maps for economically important plant species. The knowledge of DNA-

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CHARACTERISATION OF RESISTANCE AND VALUABLE AGRICULTURAL QUALITIES OF PEPPER LINES

КАРАКТЕРИСТИКИ НА ОТПОРНИ И СТАНДАРДНИ ЗЕМЈОДЕЛСКИ ЛИНИИ ПИПЕРКА

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Summary

Newly created sweet pepper lines were pyramided with disease resistant genes and genes for taste, quantity of β-carotene (provitamin A), vitamin C, reducing sugars, lycopene, dry matter content and the anthocyaninless trait contributing to improved nutritional quality of the pepper.

Three sweet pepper lines resistant to CMV, TMV, ToMV and *Ph. capsici*, anthocyaninless and two lobed kapiya type fruits were developed. The fruits were of different colour at technical maturity: L14 - dark green, L16 and OKalR– light green. At botanical maturity the fruits of OKalR were orange while those of L14 and L16 were red. The quantity of vitamin C and lycopene in L16 and reducing sugars and lycopene in L14 was higher than that of the standard cultivars. The values of remaining traits were found to be approximately equal. The content of β-carotene in the orange fruits of OKalR and OKal was nearly three times higher than in the red fruits of the standard cultivars.

All lines were found to be suitable for early and middle early field production and may be efficiently used for pepper breeding programs.

Key words: sweet pepper, complex disease resistance, anthocyaninless, nutritional quality, orange and red pepper fruits.

Извадок

Новокреираните линии слатка пиперка се одликуваат со гени на отпорност на болести и гени за добар вкус, висока содржина на β-carotene (провитамин А), витамин С, намалени шеќери, ликопен, суви материи, без антоцијани.

Развиени се три линии слатка пиперка отпорни на CMV, TMV, ToMV и *Ph. capsici*, без антоцијан од типот капија. Плодовите се со поинаква боја во техничката зрелост: L14 – темно зелена, L16 и OKalR– светло зелена. Во ботаничката зрелост плодовите од OKalR се портокалови, а оние од L14 и L16 се црвени. Количеството на витаминот С и ликопен кај L16 и намалените шеќери и ликопен кај L14 се повисоки отколку кај стандардните сорти. Вредностите на преостанатите карактеристики се приближно еднакви. Содржината на β-carotene кај портокаловите плодови од OKalR и OKal е приближно три пати повисока отколку во црвените плодови од стандардните сорти.

Сите линии се погодни за рано и средно рано полско одгледување и може да се користат ефикасно за производство на пиперка.

Клучни зборови: слатка пиперка, отпорност на комплекс болести, без антоцијан, хранлива вредност, портокалови и црвени плодови

Introduction

Bulgarian sweet and hot pepper cultivars are prevalent in Bulgaria due to their preferred flavour. Promoting Bulgarian pepper cultivars provides a valuable opportunity to preserve genetic diversity and evade dangerous genetic erosion in the context of the existing restriction of variation of plant species and varieties. All Bulgarian pepper cultivars are susceptible to CMV, tobamoviruses and *Ph. capsici* which cause the most economically important diseases of pepper crops.

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The p53 tumor suppressor gene in carcinogenesis

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(A review)

ABSTRACT. The p53 tumor suppressor is involved in maintaining of genetic stability of cells via cell cycle arrest or apoptosis. Roles in cell differentiation, morphogenesis and in the control of cell cooperation are also attributed to p53. The p53 suppressor is found to be frequently mutated in a wide variety of tumors. The incidence of point mutations within the coding sequence of the gene is the main reason for loss of the normal p53 function. Mutational p53 status correlates with various clinicopathological parameters and is associated with more aggressive tumor development, early metastasis and a poor clinical outcome. The majority of the data show an association between lack of functional p53 and reduced sensitivity to chemotherapy and/or radiation therapy. This is an overview of recent data concerning the known p53 function and the p53 alterations found in human cancers with a specific focus on the clinical significance of p53 as an independent diagnostic, prognostic and predictive variable.

Key words: tumor suppressor, p53, mutation, cancer.
Abbreviations: LOH — loss of heterozygosity.

Consistent with the present concepts of carcinogenesis malignancy results when cells escape the normal regulatory mechanisms and acquire the ability to ignore the intra- and extra-cellular signals that attenuate cell growth or direct cells into pathways of cell death. Current data indicate that the neoplastic transformation is a multi-step process, in which diverse genetic and epigenetic events are involved. The genetic events fall into two main categories: activation of the growth-promoting proto-oncogenes and inactivation of the growth-attenuating tumor suppressor genes (Harris, 1991). The tumorigenic potential of the proto-oncogenes is due to the fact that their protein products are part of the complex signalling system that controls cell growth and proliferation (Cantley et al., 1991). The activation of oncogenes, generally through point mutations, amplifications and rearrange-

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Effects of retinoic acid on the development of parthenogenetic mouse embryos

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ABSTRACT. The aim of this study was to evaluate the effects of retinoic acid (RA) on the development of diploid parthenogenetic mouse embryos (PE). The embryos were derived from the inbred strains C57BL/6 and CBA, treated with RA for 72 to 96 hours, and after development to blastocyst stage, transplanted in the uterus of pseudopregnant females. It has been shown that at preimplantation stages, the range of RA doses used in this work did not improve the development of parthenogenetic embryos and did not increase the number of blastocysts. At higher doses RA was even detrimental. By contrast, treatment of PE from C57BL/6 for 96h with 0.1 μM or 0.5 μM RA statistically significantly improved embryo implantation in the uterus, reaching 76% and 78% respectively, against 57% in the untreated PE. With the same line of mice (C57BL/6) a higher dose of RA (1.0 μM) was applied for 72h and also significantly improved embryo implantation in the uterus yielding 64% of transplanted PE embryos against 45% in the control. Treatment of CBA PE with RA did not improve implantation and a dose of 2.0 μM was damaging for the embryos. During the postimplantation period, PE from C57BL/6, treated with RA did not develop to somite stages, neither did those from the control experiments. With the other line used in the study (CBA), 45% of the control PE developed to various somite stages and treatment with RA did not increase the number of somitic embryos. In the treatment of CBA strain's PE, no living embryos were detected beyond day E10,5. In another series of experiments, pseudopregnant females with transplanted CBA PE were injected either with 0,1 μM and 0,5 μM RA on days E7.5, E8.5 and E9.5 or with 1.0 μM RA on day E8.5. These experiments of RA administration of to fosters mothers carrying PE did not allow to recover neither embryos beyond day E10,5, nor PE with more than 25 somites. In conclusion, RA significantly improves PE implantation in C57BL/6 mice, but not in CBA

НОВИ ДАННИ ВЪРХУ ЕТИОЛОГИЯТА НА БАЛКАНСКАТА ЕНДЕМИЧНА НЕФРОПАТИЯ

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NEW DATA ON THE ETIOLOGY OF THE BALKAN ENDEMIC NEPHROPATHY

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Abstract

The Balkan Endemic Nephropathy (BEN) is a severe familiar disease that is spread only in restricted areas of the Balkans. Approximately 30-48 % of BEN patients develop uroepithelial tumors. The etiology of the disease has not been disclosed so far. One of the reasons for the inherited cancer predisposition could be the incidence of mutations in p53 tumor suppressor gene. P53 gene mutations have been registered in about half of the studied human neoplasms.

The results here-reported concern screening of 90 Bulgarian BEN patients for the presence of p53 gene mutations by optimized PCR-SSCP analysis. Blood samples of the patients are analyzed. Until now, 18 mutations in four studied p53 exons have been registered. Up to date, no data on the frequency of p53 mutations in BEN patients have been reported. The presence of p53 mutations in blood samples of the patients presumes that they are germline in character. The DNA fragments with p53 mutations were cloned in pMOSBlue vector and will be sequenced for determination of the type of mutations. The results here-obtained give us reasons to presume that the mutational p53 status is one of the factors, which determine the genetic predisposition for the development of uroepithelial tumors in BEN patients and could find implications for prophylaxes, early detection and therapy of the disease.

Key words*: p53, PCR-SSCP, BEN, carcinogenesis*

Резюме

Балканската Ендемична Нефропатия (БЕН) е тежко фамилно заболяване, което се среща в ограничени райони на България, Румъния, Хърватско, Сърбия, Босна и Херцеговина. Около 30-48 % от пациентите с БЕН развиват уроепителни тумори. Етиологията на заболяването до този момент не е изяснена. Една от причините за унаследеното предразположение към туморообразуване би могла да бъде възникването на мутации в p53 тумор-супресорния ген. Мутации в p53 гена (предимно базови субституции) са установени в около 50% от изследваните неоплазии при човека. Мутантният p53 статус корелира с по-агресивно поведение на тумора и лоша прогноза.

Чрез оптимизиран PCR-SSCP анализ, за наличие на p53 мутации, беше скринирана група от 90 пациента с БЕН от три ендемични села във Врачанско. Изследванията са проведени върху кръвни проби. До този момент са установени общо 18 мутации в четири изследвани p53 екзона. Това са първите данни за p53 мутации при пациенти с БЕН. Наличието на p53 мутации в кръвни проби предполага, че те имат герминативен характер. ДНК фрагментите с мутации са клонирани в pMOSBlue вектор и ще бъдат секвенирани за установяване на типа на мутациите. Получените резултати ни дават основание да предположим, че мутантният p53 статус е един от факторите, определящи генетичната предиспозиция за развитието на уроепителни тумори при

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КЛИНИКО-ПАТОЛОГИЧНО ПРОУЧВАНЕ НА ВЛИЯНИЕТО НА МУТАЦИИ В ТУМОРНО СУПРЕСОРНИЯ ГЕН P53 ПРИ ФАМИЛНО ОБРЕМЕНЕНИ ПАЦИЕНТИ С РАК НА МЛЕЧНАТА ЖЛЕЗА

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CLINICO-PATHOLOGICAL STUDY ON THE INFLUENCE OF MUTATIONS IN TUMOR SUPPRESSOR GENE P53 IN PATIENTS WITH BREAST CANCER AND POSITIVE FAMILY HISTORY

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Abstract

Our current research includes 97 patients who went through breast cancer operation. The aim is to find out the relationship between the frequency of mutations in p53 gene, clinico-pathological progress of the disease and its prognosis. By means of high sensitive PCR-SSCP analysis of DNA, isolated from tumor tissues, 6 exons of the p53 gene were screened. Mutations were found in 23 women (23.71%). The patients were divided into two groups: ***A*** *- with mutated p53 and* ***B*** *- with normally functioning p53 gene. We compared morphological and clinical-pathological characteristics such as age, histological variant of the tumor, differentiation rate, nodal status, clinical stage, ER- status and applied adjuvant or nonadjuvant therapy. Statistical differences dependent on the presence or the absence of p53 mutations was observed regarding: average age, positive lymphatic nodules and grade of malignancy for G3 and ER- status. In group* ***A*** *the disease is more aggressive which imposes a complex pre- and post- operative treatment. On the basis of the obtained results we couldn't find consistent data showing some connection between mutations in p53 and histopathological variant.*

Key words*: p53, mutation, PCR_SSCP, breast cancer, tumor suppressor gene*

ВЪВЕДЕНИЕ

Ракът на млечната жлеза (РМЖ) заема важно място в съвременната онкология и представлява сериозен диагностичен, терапевтичен и прогностичен проблем. Той е най-често срещаното злокачествено заболяване при жените в световен мащаб. В България годишно заболяват около 3100 жени и приблизително 1300 умират от рак на млечната жлеза. Според Световната Здравна Организация главна причина за смъртността при жените в средна възраст е това онкологично новообразувание **[1]**. Ракът на млечната жлеза се отнася към скрито протичащите и бързо развиващи се заболявания, имащи свойството бързо да прогресират под влияние на много ендогенни и екзогенни фактори. Многостъпалните модели на канцерогенезата предполагат редица генетични промени, свързани с активиране на протоонкогените, инактивиране на туморно супресорни гени (ТСГ), както и мутации в ключови за регулацията гени и техните белтъчни продукти.

МИКРОРАЗМНОЖАВАНЕ *IN VITRO* НА *SIDERITIS SCARDICA* GRISEB

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MICROPROPAGATION *IN VITRO* OF *SIDERITIS SCARDICA* GRISEB

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Abstract

Sideritis scadica Griseb. is Bolkan endemic plant. The species is included in the Red Book of Bulgaria and in the register of the plants saved by the low. Currently, Sideritis scardica is used in Bulgarian folk medicine as antioxidant, antibacterial and antiinflammatory drug. It has wide ecological plasticity and difficult breeding in the nature by seeds (1). In the natural locations the seeds show very low germination activity approx. 5% (2).

The aim of our study, represented herewith, is to develop an approach for in vitro micropropagation, adaptation and cultivation in natural conditions of the above mentioned medicinal plant.

By examination of different mediums we have established the most advantageous conditions for in vitro micropropagation of Sideritis scadica Griseb.

Key words: *Sideritis scardica Griseb., micropropagation, in vitro*

Използвани съкращения: БАП – 6-бензиламинопурин, 2,4-D-дихлорфеноксиоцетна киселина, ИОК-индолилоцетна киселина, АНО-α-нафтилоцетна киселина, ИМК-индолилмаслена киселина, GA_3-гиберелинова киселина, НОК-нафтилоцетна киселина, MS – основна среда по Murashige and Skoog, G_1-среда за покълване, D_1-среда за калусогенез, R_1 и R_2-среди за регенерация, K_1,K_2,K_3,K_4,K_5-среди за вкореняване

Въведение:

Род *Sideritis* (сем. Lamiaceae) включва около 140 вида и е съставен от едногодишни и многогодишни треви и храсти, широко разпространени предимно в Средиземноморския регион. Те обикновено обитават сухи, скалисти и слънчеви места (3, 4).

Видовете от род *Sideritis* имат антиоксидантно, противовъзпалително, антимикробно и бактерицидно действие и се използват широко в народната медицина на страни като Испания (5. 6), Турция (7, 8), Гърция (9) и България (1).

През последните години няколко вида от род *Sideritis* бяха широко изследвани с фитохимични и фармакологични методи. Основните вторични продукти, интересни за медицината, са етеричните масла, дитерпените и флавоноидите (10).

Етеричните масла на *Sideritis* са съсредоточени основно в жлезистите клетки на власинките, като тяхното количество и качество варира в зависимост от вида, анализираните органи или тъкани и мястото и сезона на събиране (11, 12, 13. 14, 15,16). Приложението на флавоноиди

МОЛЕКУЛЯРНО-ГЕНЕТИЧНИ ИЗСЛЕДВАНИЯ ПРИ ФАМИЛЕН РАК НА МЛЕЧНАТА ЖЛЕЗА

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Въведение

Ракът е генетично заболяване, дължащо се на мутации или неправилна експресия на специфични гени, участващи в контрола на клетъчния цикъл. Тяхното нарушено функциониране потенциално може да отключи канцерогенетичен процес. Ракът на млечната жлеза (РМЖ) е най-масовото хетерогенно злокачествено заболяване при жените [1]. В България годишно заболяват около 3500 жени и приблизително 1600 умират от РМЖ, като заболеваемостта показва непрекъсната тенденция към нарастване. Съществена роля в патогенезата на РМЖ имат някои туморно супресорни гени и прото-онкогени – *BRCA1*, *BRCA2*, *p53*, *ATM*, *p65*, *HER2* [2].

Туморно супресорните гени *p53* и *BRCA1* са локализирани в хромозома 17, а *ATM* (ataxia telangiectasia mutated) генът – в хромозома 11. *ATM* е сензор на двойноверижни повреди в ДНК, причинени от йонизираща радиация. При наличието на такива повреди, ATM-киназата директно фосфорнлира p53 и BRCA1 [3]. Активирани, тези протеини осъществяват адекватен отговор на възникналите промени, чрез контрол върху специфични фази на клетъчния цикъл [4-6]. Въз основа на това, се предполага наличие на директна функционална връзка между *ATM*, *p53* и *BRCA1* гените (схематично отразена на Фиг. 1).

Фиг. 1. *Схематично представяне на функционалните взаимодействия между туморно супресорните гени* ATM, p53 *и* BRCA1.

МОДЕЛ ЗА ГЕНЕТИЧНО КОНСУЛТИРАНЕ ПРИ ФАМИЛЕН РАК НА МЛЕЧНАТА ЖЛЕЗА

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Въведение

На клетъчно ниво ракът на млечната жлеза (РМЖ) е генетично заболяване, резултат от засилена и качествено променена експресия на ключово регулаторни гени (туморно супресорни гени и прото-онкогени). Установено е, че от първичните карцинноми около 70% са спорадични форми, между 15-20% – фамилни и останалите 5-10% – наследствени. В нашата страна заболеваемостта от РМЖ ежегодно нараства и поради късно първично диагностициране, смъртността сред засегнатите жеии е висока. Едновременно с това се повишава здравната образованост на българската жена и отговорността й към собственото здраве, което поражда интерес и търсене на услугите на медико-генетичната коисултация (МГК).

Фамилното предразположение към РМЖ се унаследява доминантно с 50% вероятност. Актнвното наблюдение на клинично здрави лица с висок теоретичен риск и болни, при които е идентифицирана мутация в специфични туморно супресорни (TS) гени, дава възможност за откриване на карцинома в ранен клиничен стадий (Т0 и Т1), когато леченнето е по-просто, икономически по-ефективно и с по-добра прогноза по отношение качеството на живот, съхраняване на психичния и соматичен статус на заболелите. Внедряването на генетично консултиране (ГК) и молекулярна диагностика в онкологичната практиката ще подпомогнат лекаря в диагностичния и терапевтичен подход и ще допринесат за достигане на европейските стандарти по отношение на основни демографскн и здравни показатели като заболеваемост, смъртност, нрежнвяемост и др.

В настоящото съобщение споделяме нашия опит в първата за страната регламентирана МГК за фамнлен рак на млечната жлеза (ФРМЖ) и нейното приложно значение.

Цел на дейността на МГК

Създаване и популяризиране па ГК в България за болни с ФРМЖ. Оценка на индивидуалния риск за заболяване. Сформиране на селектирана група от фамилпо обременени болни с РМЖ и техни клипично здрави родственици, за активно наблюдение, раниа диагностика и своевременно лечение.

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MOLECULAR GENETIC APPROACH IN STUDYING THE ROLE OF P53 MUTATIONS IN BULGARIAN PATIENTS WITH CANCER

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Key words: Tumor suppressors, p53, PCR-SSCP, carcinogenesis, mutation.

Abstract: The here-reported results represent screening in four groups of Bulgarian patients with a different type of cancer for the presence of p53 gene mutations. The p53 status of the patients was determined by the use of an optimized PCR-SSCP analysis. Tumor and/or blood samples of the patients were analyzed. The detected frequency of p53 mutations is comparable within the studied groups and varies between 16.67% and 33.33%. To date, no clear data on the p53 mutation frequency in Bulgarian patients with cancer have been reported. Our results are in support to the concept that the incidence of mutations in the p53 tumor suppressor gene leads to a loss of the normal p53 function and is one of the reasons for the initiation and progression of the carcinogenic process.

A current concept in studying the molecular mechanisms involved in carcinogenesis is based on determination of consistent genetic alterations in two groups of genes: proto-oncogenes and tumor suppressor genes. The activation of the first and the inactivation of the second are associated with the initiation and progression of the carcinogenic process. To date, at least 24 tumor suppressor genes and 100 proto-oncogenes are known but the number of the new genes identified as oncogenes and tumor suppressors is steadily increasing. The study of the tumorigenic process via molecular genetic approaches will not only reveal the genesis of transformation but will also give possibilities of prophylaxes, early detection of the malignant process and a more successful way of cancer therapy in the future.

The tumor-suppressor gene p53 is the most frequent target of genetic alterations in human cancers. Initial studies have classified p53 as a dominant-acting oncogene, while current data have definitely shown that wild type p53 inhibits neoplastic transformation and acts as a typical tumor suppressor gene [1, 2].

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Structural and Functional Organization of Maize Chromatin During Embryo Germination

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1 INTRODUCTION

The huge amount of eukaryotic DNA as well as the other nuclear components must be organized in a tiny nucleus in a highly ordered way to achieve a perfect synchronization and accuracy of the various structural/functional processes occurring in chromatin. The basic structural chromatin subunit is the nucleosome which consists of about 160 bp of DNA wrapped in approx. two turns around two molecules of each of the four core histones (H2A, H2B, H3 and H4). In both animals and plants, DNA is arranged in a 10 nm nucleosomal fiber which is further wound in a 30 nm solenoid[1]. At the next organizational level, the DNA is attached to the nuclear matrix[2]. Despite the tremendous progress that has been made in defining the steps of chromatin and nuclear assembly, our knowledge is still far from an exact understanding of how this complex chromatin structure can, under tight control, modulate the regulatory mechanisms through development of organisms. Nowadays one of the most challenging problems of molecular biology is the understanding of how genes are activated from a repressed into an actively transcribed state.

During the last few years we studied the structural and functional relationships in plant genome using germinating plant embryos as a model system. The germination of plant embryo is a differentiation process in which the cell nucleus undergoes changes from an extremely low metabolism in quiescent embryos to a state of fully restored activity after the start of seed imbibition. This process therefore represents a sequence of molecular events that transform the heterotrophic embryo into a complex autotrophic organism. Hence, germinating plant embryos are a good, natural system for unraveling the mechanisms maintaining and regulating structural and functional organization of plant genome.

The present report summarizes our investigations on the mechanisms involved in the regulation and organization of maize chromatin during the embryo germination process.

1.1 Cell Cycle Analysis

Studies of cellular and molecular events during plant embryo germination require a detailed analysis of nuclear processes and cell cycle parameters. In view of the limited and often controversial data we set out to study the cell cycle distribution of maize embryo cells at different time points of germination process by DNA flow cytometry[3,4] and some of