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Positioning of a Nucleosome on Mouse Satellite DNA Inserted into a Yeast Plasmid Is Determined by Its DNA Sequence and an Adjacent Nucleosome

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Abstract—It has earlier been shown that multiple positioning of nucleosomes on mouse satellite DNA is determined by its nucleotide sequence. To clarify whether other factors, such as boundary ones, can affect the positionings, we modified the environment of satellite DNA monomer by inserting it into a yeast plasmid between inducible GalCyc promoter and a structural region of the yeast *FLP* gene. We have revealed that the positions of nucleosomes on satellite DNA are identical to those detected upon reconstruction *in vitro*. The positioning signal (GAAAAA sequence) of satellite DNA governs nucleosome location at the adjacent nucleotide sequence as well. Upon promoter induction the nucleosome, translationally positioned on the GalCyc promoter, transfers to the satellite DNA and its location follows the positioning signal of the latter. Thus, the alternatives of positioning of a nucleosome on satellite DNA are controlled by its nucleotide sequence, though the choice of one of them is determined by the adjacent nucleosome.

Key words: nucleosome, positioning, mouse satellite DNA, yeast cells

In the cell nucleus DNA is packed in nucleosomes including a histone octamer and a DNA fragment of 146 bp forming the 1.75 superhelical turns. In total, the nucleosome restricts the recognition of a DNA sequence by the factors of replication, transcription, recombination, etc. [1]. Obviously, random distribution of nucleosomes along a gene results in concealment of some of its regions, i.e., enhancers, promoters, other sites of interaction with regulatory proteins. However, as early as in pioneering studies, the location of nucleosomes with respect to individual genes was found to be predetermined, i.e., positioned [2].

Nucleosome positioning on the regulatory gene regions may be essential for regulation of transcription, this phenomenon being not necessary for repressive character. For example, a nucleosome located precisely on the promoter region stimulates transcription through drawing together recognition sites for a regulatory protein on the nucleosome surface or nucleosome entry and egress DNA sites [3, 4].

One of the most debatable points is on the positioning signal. In principle, any DNA sequence is capable of forming a nucleosome *in vitro*, though the affinity of various DNAs and synthetic oligonucleotides for histone octamer differs by dozens of times [5-7]. On the basis of these data, a determining part of the DNA sequence for positioning was postulated. Besides, numerous studies aimed at searching for the positioning motifs have been stimulated.

By now a group of such motifs exhibiting enhanced or reduced affinity for histone octamer, as compared to total nucleosome DNA, has been revealed [8-10].

The character of DNA positioning signal may stem from its geometrical features. Probably the ability to form a superhelical turn on the surface of a core particle is determined by local changes in the DNA structure (e.g., kinks), curvature of DNA sequence, flexibility of some DNA regions, etc. In total, the DNA signals are sufficient to ensure nucleosome positioning *in vitro*. However, nucleosome positioning in a cell is distinct from that observed in a reconstituted system [11].

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Less studied are other positioning signals which are unrelated to the DNA sequence. Possible candidates capable of playing this part are boundary factors, i.e., the structure of adjacent DNA, the presence of another nucleosome in the nearest vicinity, competition with some protein exhibiting high affinity for the DNA sequence, etc.

In this work, we have undertaken an effort to reveal possible changes in the nucleosome positioning on mouse satellite DNA inserted into a yeast plasmid. This DNA is a fragment of 234 bp that may be repeated in the genome about a million times. It has earlier been shown [12] that upon reconstruction *in vitro*, satellite DNA is characterized by pronounced multiple positioning of nucleosomes, namely nine positions per monomer with a 10-bp step. Under the *in vivo* conditions, nine extra positions are revealed which follows from tandem repetition of satellite DNA monomers [13]. In the construction used in this work (Fig. 1) such a monomer was inserted downstream of the GalCyc promoter of a yeast plasmid. Owing to this, we were able to estimate the contribution of the environment of this sequence to the positioning of nucleosomes under two metabolic states of the promoter, i.e., glucose-repressed and galactose-activated.

We have found that positions of nucleosomes on the satellite DNA monomer under artificial conditions are identical to those in a reconstituted system. However, the positioning signal of satellite DNA extends to the neighboring structural part of the *FLP* gene, but not to the GalCyc promotor. Upon galactose induction, nucleosome initially positioned on the promoter transfers to the adjacent region of satellite DNA, being positioned in conformity with the signal of this DNA fragment. The positioning signal of satellite DNA cannot be considered in affinity terms; on the other hand, the GAAAA motif restricts the initial and/or final site of nucleosome formation.

MATERIALS AND METHODS

Bacterial and yeast strains: construction of yeast plasmid YepMMS containing a mouse satellite DNA monomer. We used *Escherichia coli* strain JM109 (Promega, USA) and *Saccharomyces cerevisiae* strain 2805 (MATa, pep4::HIS3, prbL-δ, can-1, gal2, his3, ura 3-52) generously provided by C. K. Pu (Sang-Ki Rhii, GERI, Korea).

Cloning of the yeast YepMMS plasmid with monomeric satellite DNA insertion was performed as follows. Total mouse DNA was treated with *Mva*I. DNA fragments were separated in 1.5% agarose gel. A fragment of 234 bp was eluted. The 3′-sticky ends of the satellite DNA monomer were treated with Klenow fragment and ligated with the products of hydrolysis of pUC18 with *Sma*I. Orientation of the monomer in resulting plasmids was analyzed using *Sau*96I. In all the plasmids, the retained *Sau*96I site proved to be proximal to the *Hind*III site of pUC18 polylinker (the pMMS plasmid).

The 260-bp *BamH*I-*Sac*I fragment of pMMS was cloned into the major *Sac*I-*BamH*I fragment of the Yepsec1 plasmid. The resulting plasmid was designated YepMMS.

Yeast cells were transformed using LiCl, transferred into liquid YPD or YPGal (YPD with 2% galactose) medium, and cultivated (100-200 ml) for 16-18 h to A_{600} = 0.8-1.2.

Preparation of spheroplasts and partial hydrolysis of chromatin DNA with micrococcal nuclease. Yeast cells ($1 \cdot 10^9$) were harvested by centrifugation, washed with sterile water and buffer (10 mM Tris-HCl, pH 7.4, 1 M sorbitol, 0.5 mM β-mercaptoethanol), transferred into 2 ml tubes, and suspended in 1 ml of the same buffer. To prepare spheroplasts, enzyme Lyticase (Sigma, USA) was administrated into the yeast suspension at 20 mg/ml and incubated for 5-10 min at 30°C with gentle shaking. All other manipulations were carried out as described in [14].

Spheroplasts were suspended in 1 ml of buffer for digestion of chromatin by micrococcal nuclease. The buffer contained 1 M sorbitol, 50 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol, 0.5 mM spermidine, and 0.1% Nonidet NP-40.

Spheroplast suspension (portions of 200 μl) was transferred into centrifuge microtubes, and micrococcal nuclease was added in the amount of 150-350 units/ml. Incubation with nuclease continued for 5 min at 37°C. The reaction was arrested with 0.5 M EDTA (final concentration 25 mM) and 10% SDS (final concentration 0.5%).

Purification of DNA preparations. Southern blot hybridization. DNA samples were treated with ribonuclease A (100 μg/ml) and pronase K (100 μg/ml) for 1 h at 37°C. DNA was then deproteinized twice with phenol–chloroform (1 : 1) mixture and chloroform, and finally precipitated with ethanol. Purified DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0), and its concentration was determined. DNA sample prepared from spheroplasts, which were not preliminarily treated with micrococcal nuclease, was then subjected to the action of nuclease at 0.01-0.05 unit per μg DNA. Incubation continued for 1-3 min at 37°C, the reaction was arrested with 0.5 M EDTA (final concentration 25 mM), and DNA was precipitated with 96% ethanol. All the DNA samples were then treated with *Sac*I and *BamH*I (20 units/μg).

Following electrophoretic separation of DNA fragments in 1.5% agarose gel, their transfer onto nylon membrane filter (Nylon 66 plus, HSI, USA), prehybridization, and hybridization with ^{32}P-probe were carried out by routine procedures [15].

The radioactive probe was prepared using *Mva*I monomer of mouse satellite DNA (234 bp) and the Multiprime DNA labeling system (Amersham, USA). Hydrolyzates of pGEM-3 plasmid DNA (*Hpa*II) and phage λ DNA (*Hind*III) were used as markers upon electrophoresis of DNA and Southern blot hybridization. Southern blot radioautographs were analyzed with an Ultrascan XL (LKB, Sweden).

Preparation of nucleosomal DNA. DNA preparation obtained through deep hydrolysis of yeast spheroplast chromatin was loaded on 2% low-melting agarose gel, and DNA fragments were separated in Tris-acetate buffer containing 0.04 M Tris-acetate and 0.002 M EDTA. The Gene Ruler 100 bp DNA Ladder Plus (MBI Fermentas, Lithuania) system was used as size markers of DNA fragments. A 100-200 bp zone, which corresponds to mononuleosomal DNA, was excised from the agarose. Then the agarose block was melted for 5 min at 65°C, supplemented with two volumes of TE buffer, pH 7.5, heated for 5 min at 65°C, crushed, frozen two times in liquid nitrogen, and thawed with vortex homogenization (PPE-3; EKROS, Russia), and centrifuged for 5 min at 14,000 rpm. The procedure was completed by precipitation of DNA with 96% ethanol from resulting supernatant, drying the pellet, and dissolving it in a suitable volume of water.

Oligonucleotide end-labeling. The following oligonucleotides were used for upper strand:

5′-GAG-CAG-ATC-CGC-CAG-GCG-3′ ((−266)-(−248));

5′-AAT-GAG-AAA-TAC-ACA-CTT-TA-3′ (34-53);

and for lower strand:

3′-ACA-GGT-GAC-ATC-CTG-CAC-CT-5′ (102-121).

The primers were labeled at the 5′-ends using [γ-^{32}P]ATP (Obninsk, Russia) and T4-polynucleotide kinase. The incubation medium contained in 50 μl: 25 pmol DNA primer, 5 μl of 10× kinase buffer, 3.5 μCi [γ-^{32}P]ATP, and 20 units of T4-polynucleotide kinase. Labeled nucleotides were precipitated with ethanol after 1-h incubation at 37°C and dissolved in a suitable volume of water. The primers were used for the primer extension procedure with DNA isolated from nucleosomal fraction and for sequencing double-helical plasmid DNA using Taq polymerase (MBI Fermentas).

Primer extension procedure. A sample for amplification (25 μl) contained 0.1-0.2 pmol (300-600 ng) of mononucleosomal DNA isolated as described above, 2.5 μl 10× Taq buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 0.1% gelatin, and 5% NP-40), 3-5 pmol of labeled primer, 2.5 μl of 1.5 mM dNTP, and 5 units of Taq-pol. After preliminary denaturation for 2 min at 95°C, the samples were consecutively incubated for 1 min at 95°C, 1 min at 55°C (or 37°C), and 2 min at 72°C (30 cycles), which was followed by DNA precipitation with 96% ethanol overnight at −20°C. The DNA precipitate was dissolved in 5 μl of arresting solution (95% formamide, 0.1% bromophenol blue, and 0.1% xylene cyanole), boiled for 2 min, and loaded on 6% sequencing gel.

The cut points at the promoter and structural gene regions were estimated by comparing the primer extension DNA samples with the sequence ladder of DNA synthesized using the same primer.

Sequencing of double-strand plasmid DNA with Taq polymerase. Four tubes for PCR were loaded each with 2 μl of terminating mixtures including 15 μM of each dNTP and the optimum amount of corresponding ddNTP (G, A, C, and T mixtures contained 40 μM ddGTP, 1 mM ddATP, 1 mM ddCTP, and 1.5 mM ddTTP, respectively) and 4 μl of mixture composed of 2.5 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 0.1% gelatin, 5% NP-40), 3 pM (300 pg) DNA template, 5 units of Taq-pol, and water to final volume of 28 μl. The resulting mixture was incubated for 2 min at 95°C, which was followed by 30 cycles of consecutive incubation for 30 sec at 95 and 70°C. Then 3 μl of the arresting solution was added, and (after 2-min in boiling water bath) 4 μl aliquots were loaded on sequencing gel. The products of Taq-pol reaction were analyzed in 6% sequencing polyacrylamide gel with urea in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH 8.3). The gel was exposed to X-ray film overnight. Radioautographs were scanned with an LKB Ultrascan XL (Sweden).

RESULTS AND DISCUSSION

Multiple *in vivo* positioning of nucleosomes on satellite DNA monomer incorporated into a yeast plasmid. Figure 1 shows a scheme of nucleotide sequence of a region of the YepMMS yeast plasmid. The satellite DNA monomer is inserted between the inducible GalCyc promoter and partially deleted *FLP* yeast gene. Spacing between the 0-point (the entry of satellite DNA) and the nearest TATA box is 135 bp. We have undertaken an effort

Fig. 1. Scheme of analyzed sequence. 0-234 are entry and egress of satellite DNA monomer. Shaded squares are TATA boxes in GalCyc promoter. *1*, *2*, and *3* are primers used for amplification. Arrows show direction of synthesis.

to reveal whether the adjacent environment of satellite DNA affects the character of nucleosome positioning. Mapping of nucleosomes on the analyzed sequence was initially carried out by the indirect end-labeling method [16]. The precision of this method is not high enough and is limited by the accuracy of estimation of the size of the DNA fragment.

Chromatin in spheroplasts was cleaved with micrococcal nuclease and isolated DNA fragments were treated with *BamH*I and *Sac*I (the insertion sites are indicated in Fig. 1). To determine positions of nucleosomes not only on the octamer but also on the adjacent DNA regions, we varied the depth of DNA hydrolysis (Fig. 2). The length of resulting DNA fragments corresponded from one to six nucleosomes. The fragments were fractionated by agarose gel electrophoresis and blotted onto a nylon membrane. The blots were hybridized to a ^{32}P-labeled monomer of satellite DNA and visualized by radioautography (Fig. 3a). The length of DNA fragments and their relative number were estimated from laser scans of radioautographs (Fig. 3b). Since the GalCyc promoter is activated by galactose, we were able to compare the data on chromatin state for the repressed and induced promoter.

Figure 4 summarizes the results of analysis of nucleosome positioning by the indirect end-labeling method. It is seen that multiple positioning is characteristic for satellite DNA, and four to five positions span its monomer. Some of nucleosomes cover the structural region of the *FLP* gene, with some of them being only partially located on the satellite DNA region. Most of the positions are revealed upon indirect end-labeling with the use of *Sac*I. Perhaps the fact of existence of limited versions of positioning that are revealed with *BamH*I indicates the absence of nucleosomes at the boundary between the TATA box and the entry of satellite DNA monomer. The

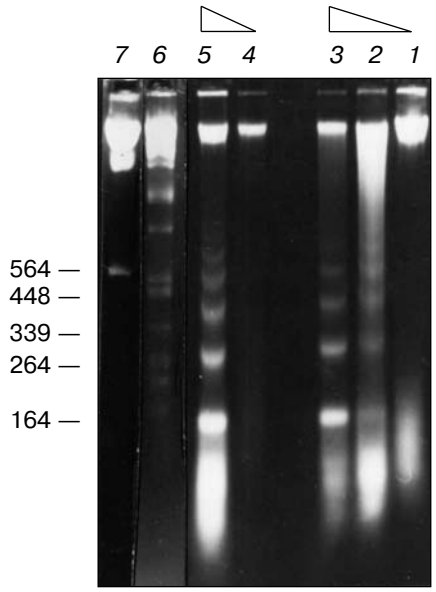

Fig. 2. Electrophoregram of DNA fragments after hydrolysis of yeast chromatin with micrococcal nuclease. ◺ shows the decrease in hydrolysis degree by diminishing nuclease/DNA ratio. Lanes: *1*) DNA from spheroplasts of yeast grown on glucose, without treatment with nuclease; *2*, *3*) the same as in *1* after enhancing hydrolysis with nuclease; *4*) DNA from spheroplasts of yeast grown on galactose, without treatment with nuclease; *5*) the same as in *4* after deep hydrolysis with nuclease; *6*) λ phage DNA restricted with *Pst*I; *7*) λ phage DNA restricted with *Hind*III. On the left, fragment length in bp.

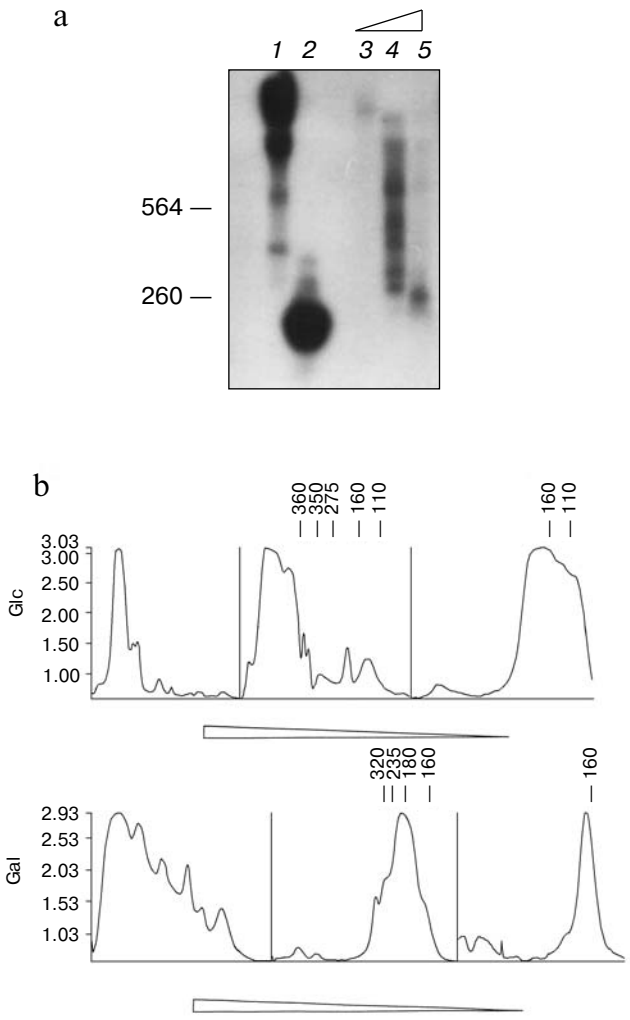

Fig. 3. Mapping of nucleosomes by the method of indirect end-labeling. a) Autograph of gel after electrophoresis of DNA fragments obtained by hydrolysis with micrococcal nuclease followed by treatment with *Sac*I and hybridization with ^{32}P-labeled satellite DNA monomer (234 bp). *1*) λ phage DNA treated with *Hind*III; *2*) satellite DNA fragment; *3-5*) enhancing hydrolysis. Numbers show the size of the fragments from the *Sac*I site. b) The results of scanning of autographs of gels after electrophoresis of DNA fragments obtained by hydrolysis with micrococcal nuclease followed by the treatment with *BamH*I. Glc and Gal are for yeast grown on glucose and galactose, respectively. Numbers show the size of fragments from the *BamH*I site. ◺, decrease of hydrolysis degree.

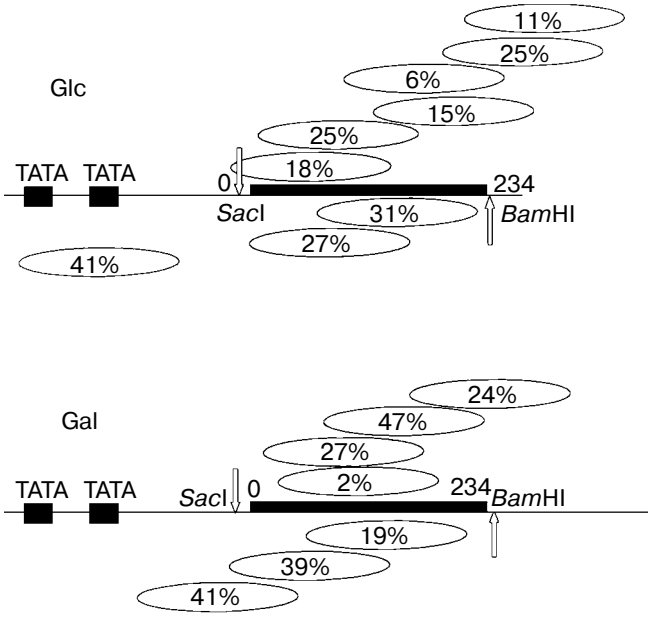

Fig. 4. Total scheme of nucleosome location on satellite DNA and its nearest environment. Shaded squares are for TATA box; 0, the entry of satellite DNA insertion. Arrows show restriction sites for indirect labeling. Ovals are for nucleosomes. Glc and Gal are for yeast grown on glucose and galactose, respectively.

data on relative intensity of different nucleosome positions on satellite DNA are also presented in Fig. 4. The multiple nucleosome positioning suggests the existence of multiple affinity signals on satellite DNA. If this were the case, the total affinity and hence the relative intensity of a nucleosome position would be higher the higher its overlap with satellite DNA sequence. As can be easily seen in Fig. 4, this is evidently not the case, since the relative intensity of nucleosomes having small overlap with satellite DNA and those having complete overlap are practically equal. As will be shown below a single histone border adjacent sequence motif is sufficient for nucleosome positioning on satellite DNA. The multiplicity of nucleosome positioning on satellite DNA suggests that nucleosomes are located on different parts of this fragment in different plasmids of a cell (which include 100-200 copies per cell). Besides, one should take into account that the yeast population is heterogeneous. The nucleosome position spanning both TATA boxes is detected at the promoter region adjacent to the satellite DNA fragment (Fig. 4). In the presence of galactose (induction), the nucleosome is absent at this place. However, a new place of nucleosome location is revealed between the promoter and satellite DNA with the nucleosome partially covering the monomer. The change in nucleosome location on the monomer is of interest. However, the question of whether the mode of positioning is variable remains unanswered, since the above method ensures estimation of nucleosome location only with the accuracy of 10-20 bp.

Specification of nucleosome positioning on satellite DNA and adjacent environment by the primer extension method. This method can be used to determine the location of the borders of nucleosome with accuracy of up to 1 bp. The experiment was carried out according to the following protocol. DNA in chromatin of yeast spheroplasts was digested with micrococcal nuclease. Purified DNA was fractionated by electrophoresis in low-melting agarose, which was followed by elution of the fragments corresponding in size to one or two nucleosomes. Purified

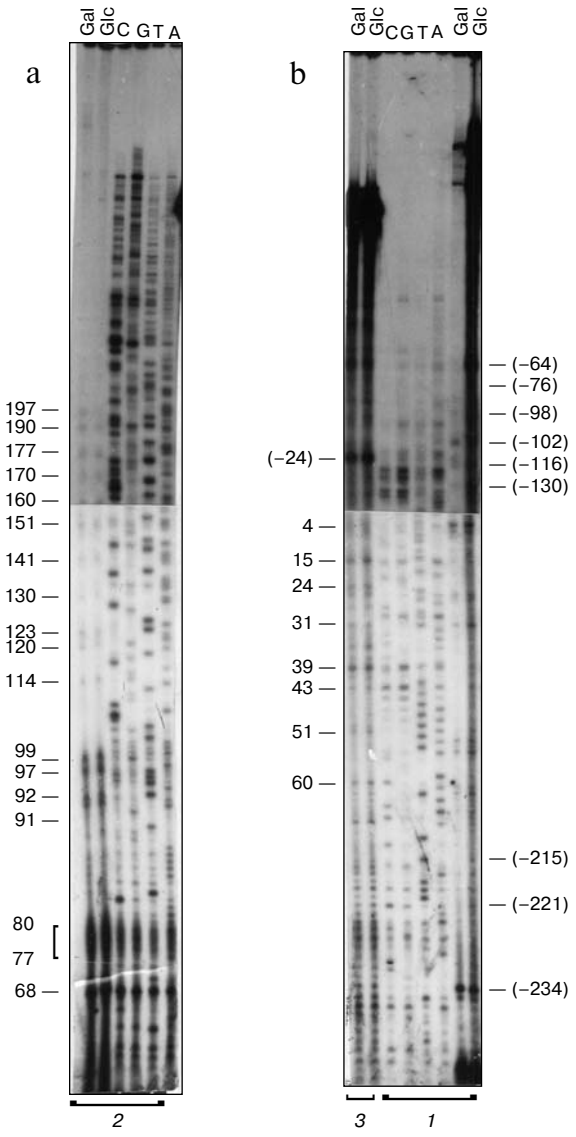

Fig. 5. Radioautograph of amplified DNA fragments after electrophoresis in 6% sequencing polyacrylamide gel. Numbers on the side show location of most dominant fragments. Glc and Gal, DNA from yeast grown on glucose and galactose, respectively. *1*, *2*, *3*) Separation of the fragments amplified with primers 1, 2, and 3, respectively. A, T, G, and C, sequence of polymeric DNA with the use of the same primers.

DNA served as a template for DNA amplification using Taq polymerase and ^{32}P-labeled primers. Precise positions of the nucleosome borders were established by sequencing polymeric DNA with the same primers (Fig. 5). According to the scheme in Fig. 1, such primer set provides estimation of positions of (–1) nucleosome on the promoter region and (+1) nucleosome on the satellite DNA insertion.

The pattern of separation of amplified DNA in sequencing gel is represented in Fig. 5. Most significant fragments are marked by arrows, and the results are summarized in a scheme (Fig. 6). The numbers in the scheme show nucleosome positions relative to the 0-point (see above). Obviously, there is multiple positioning of (+1) nucleosome on satellite DNA, with the data being different in presentation of individual positions for yeast cells grown in glucose- or galactose-containing medium. All these nucleosome positions coincide with those detectable upon the *in vitro* reconstruction experiments [12]. In other words, the character of nucleosome positioning on the monomeric satellite DNA inserted into the plasmid does not depend on its environment and is initiated by the motifs of the sequence itself; the 10-nucleotide periodicity in positioning is maintained. The distinctions in the presentation of various positions for the two metabolic states of yeast cells (glucose-repressed and galactose-induced) are difficult to interpret. One should take into account obvious heterogeneity of the cell population and, as a consequence, incomplete inducibility.

Three nucleosome positions on promotor (–1) nucleosome are encountered most often. They are situated at positions –98, –76, and –64 with respect to the satellite DNA monomer. All these positions completely cover two sequentially located TATA boxes. The minimum distance between (–1) and (+1) nucleosomes is 40 bp, while the maximum is 74 bp. A powerful positioning signal for (–1) nucleosome positioning is likely to be the DNA sequence occupied by the nucleosome. The same mode of positioning on promoters was earlier described in terms of translational positioning [12]. We have found that positioning of the (–1) nucleosome did not change upon substituting various sequences for that adjacent to the GalCyc promoter [17].

Upon galactose induction, both TATA boxes prove to be exposed, i.e., not protected by nucleosome, which in this case is situated at a different place. All the three most commonly occurring positions span the satellite DNA entry and are in accord with the positions that are usually revealed on satellite DNA *in vivo*.

Analysis of DNA sequences flanking (+1) and (–1) nucleosomes. Figure 7 shows DNA sequences flanking (+1) and (–1) nucleosomes or partially overlapping with them. The entry and the egress of the sequence included into nucleosomes are marked by parentheses. Evidently, the well-defined GAAAAA motif is located in this position (Fig. 7c). In some cases, this motif is degenerate,

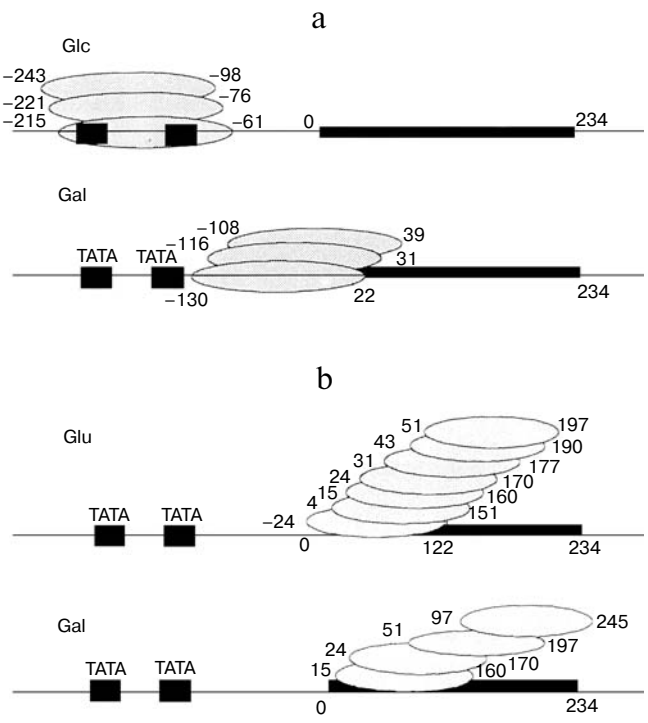

Fig. 6. Total scheme of mapping of nucleosome positioning on YepMMS by the method of primer extension. Bold line (0-234) shows satellite DNA monomer. Shaded squares are for TATA boxes, ovals are for nucleosomes. Numbers show location of nucleosomes DNA entry and egress respective to the 0-point (in nucleotide pairs). Glc and Gal are for yeast grown on glucose and galactose, respectively. a) Nucleosome (–1); b) nucleosome (+1).

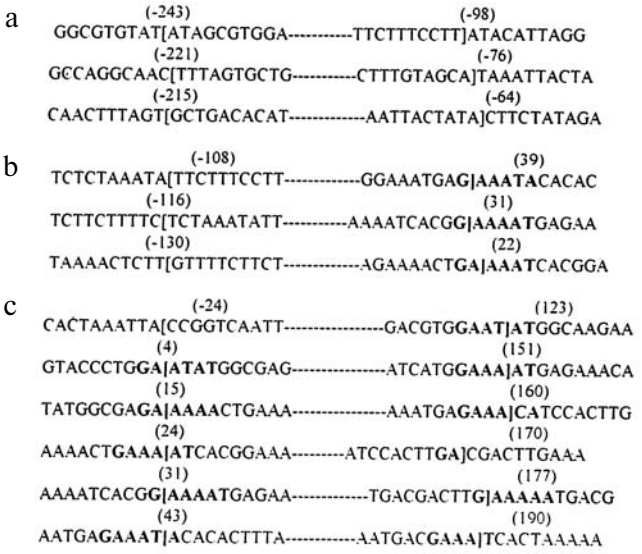

Fig. 7. a) Three versions of (–1) nucleosome positioning in glucose-grown cells. b) Three versions of (–1) nucleosome positioning in galactose-grown cells. c) The versions of (+1) nucleosome positioning in glucose-grown cells.

which manifests itself in substitution of T for the third or fifth A. This has earlier been shown with a model of nucleosome positioning upon their reconstruction on the satellite DNA monomer *in vivo* [13]. Here we have additionally revealed that this flanking signal is sufficient for positioning of nucleosome on the *FLP* gene region adjacent to the satellite DNA monomer: nucleosome occupying 75% of this region is positioned in the way to match the satellite DNA signal.

The (–1) nucleosome, located on the promoter region, is devoid of similar flanking signal in all the three most presentable versions of positioning (Fig. 7a). However, induction of the promoter results in dislocation of the (–1) nucleosome to an adjacent DNA region occupied by the satellite DNA monomer (Fig. 7b). In this case, nucleosomes in all the detected positions are flanked with the GAAAAT motif (substitution of T for A). Thus, satellite DNA is lacking in the motifs that could be affinity signal. Perhaps nucleosome can freely slide along this sequence to the flanking oligonucleotide, where it stops because of energetic difficulties to bend it at superhelix ends. The (–1) nucleosome is displaced from the TATA boxes by an inducer, which is probably accounted for by the interaction of this region with a new transcription factor exhibiting high affinity for it. This process might also be considered as sliding of the (–1) nucleosome along DNA and its arrest at one of the stop-signals of the satellite DNA. In all the three positions, nucleosomes leave the TATA block and occupy a part of the satellite monomer. As mentioned above, a stable and precisely located position of a nucleosome is determined by the positioning signal of satellite DNA, which constitutes a minimum of 15% of the region covered with the nucleosome. Presumably, blocking the DNA sites corresponding to the nucleosome borders occurs owing to geometrical restrictions for the start or the end of DNA winding around the octamer.

Of special interest is some hierarchy in the realization of the positioning signal. In fact, upon induction of the GalCyc promoter, (–1) nucleosome leaves it probably under the action of a transcription regulation factor characterized by higher affinity for this sequence. The (–1) nucleosome displaces the (+1) nucleosome located at the satellite DNA entry, being positioned by the DNA stop-signal therewith. On the other hand, the positioning signal is powerful enough to define nucleosome location on the adjacent sequence of the *FLP* gene.

By and large, the signal of nucleosome positioning on satellite DNA is determined by its structural features irrespective of nucleosome environment. However, positioning of a nucleosome on satellite DNA is affected by location of another nucleosome on adjacent DNA sequence.

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