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Human satellite 3 (HS3) binding protein from the nuclear matrix: isolation and binding properties

O. Podgornaya [a,*], R. Dey [b], I. Lobov [a], N. Enukashvili [a]

[a] *Institute of Cytology, Russian Academy of Sciences, St. Petersburg 194064, Russia*
[b] *SUNY at Buffalo, Buffalo, NY 14260, USA*

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Abstract

Satellite DNA (satDNA) is the main component of residual DNA in nuclear matrix (NM) preparations. Gel mobility shift assay (GMSA) revealed specific human satellite 3 (HS3) binding activity in NM extracts. An HS3 binding protein was purified using diethylaminoethyl (DEAE)-cellulose and preparative GMSA. The binding was specific, although other satDNA fragments compete to some extent for the binding. DNase I footprinting and methylation interference revealed multiple points of protection distributed throughout the HS3 fragment with periodicity of about 10 bp, mostly inside an AT island. Polyclonal antibodies (AB) were raised against HS3-protein complexes cut from the preparative GMSA gel. On immunoblots, AB recognise a protein, which is not lamin, with apparent molecular mass 70 kDa, the same as revealed by purification (p70). In in situ nuclear matrix preparations combined immunofluorescence (AB) and fluorescent in situ hybridisation (HS3) shows that HS3 and p70 areas correspond to each other. The localisation of this protein detected with AB in interphase nuclei coincides with the heterochromatic regions which surround nucleoli in correspondence with the known HS3 position in the nuclei.

Keywords: Nuclear matrix; Satellite DNA binding protein; Antibody; Immunocytochemistry (human)

1. Introduction

There are tandemly organised, highly repetitive sequences of satellite DNA (satDNA) in all eukaryotic genomes. SatDNA is a special type of highly condensed, transcriptionally silent, constitutive heterochromatin in interphase nuclei [1]. The primary sequences of satDNA differ substantially in different species, and the level of their evolutionary variability is relatively high [2], although they also have common structural features [3,4]. The conservative structure recognised by specific proteins may lead to chromatin organisation in the heterochromatic regions which is of a particular nature, common but to different species [5–7].

The chromosome centromeric (CEN) region DNA of primates belongs to the family of alphoid satellite

Abbreviations: HS3, human satellite 3; NM, nuclear matrix; GMSA, gel mobility shift assay; satDNA, satellite DNA; AB, antibodies; CEN, centromere; α-satDNA, alphoid satellite DNA; TM-2, 20 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$; DEAE, diethylaminoethyl; 0.2DE, active fraction from DEAE chromatography; MAR, matrix attachment regions; NOR, nucleolus organiser region; TBS, Tris-buffered saline; CBB, Coomassie brilliant Blue; DAPI, 4′,6-diamino-2-phenylindole

* Corresponding author. Fax: +7-812-247-03-41 or +7-812-520-97-03; E-mail: podg@ivm.stud.pu.ru

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DNA (α-satDNA) [8]. In most chromosomes, α-satDNA arrays are surrounded by arrays of so called big, or classical, satDNA, to which human satellite 3 (HS3) belongs. This pericentromeric region may have a specific, high-order chromatin structure and be responsible for chromatin spatial organisation.

The non-random distribution of interphase CENs recently has been established. CENs in T-lymphocytes are shown to be at the nuclear periphery during the G_1 phase. Transition from G_1 to G_2 phase is accompanied by extensive chromosome movement, with CENs assuming a more interior location [9]. Heterochromatic homogeneously staining regions also display positional changes during cell cycle [10]. It is not yet clear which of the satDNAs is involved in the heterochromatic region movement and rearrangement. It has been shown that the CEN part of the α-satDNA array is not responsible for the chromatids association [11] and separated by the other satDNA [12].

The arrays of HS3 and 2 are based on variants of the ATTCCA monomeric unit [13,14]. HS3 and 2 in highly variable amounts are present in pericentromeric regions of most (possibly all) human chromosomes, and their position appears to be highly conserved throughout evolution [15]. A suggestion was made that they might be functionally important components of the CENs, together with the protein specific in binding to them [16]. A number of chromosome-specific HS3 subfamilies has been reported [14,15]. An HS3 fragment specific of chromosome 1 has been used as the main probe in the current work [17].

The residual DNA of the nuclear matrix (NM) is shown to be enriched in satDNA [18–20]. That means that the NM of the interphase nucleus should contain proteins that can bind HS3 satDNA. The purpose of the present work was to find out whether the NM contains proteins that can specifically bind HS3 in vitro and to define its localisation in vivo.

HS3-binding protein of apparent molecular mass 70 kDa (p70) was purified from the NM extract and antibodies (AB) raised against it stain in the interphase nuclei of the HeLa cells some patches that surround nucleoli in correspondence with the known HS3 position in the nuclei [21,22].

2. Materials and methods

2.1. Plasmids and gel mobility shift assay (GMSA)

The initial plasmid with 1.77 kb *Eco*RI fragment of HS3 was the kind gift of Dr. H. Cooke (MRC, Edinburgh, UK). The fragment was digested with *Sau*3A and subfragments of the HS3 sequence were cloned into the *Bam*HI site of pUC 19. A 0.3 kb fragment from the plasmid called pRT336 was the main probe. The fragments were end-labelled by Klenow fragment DNA polymerase (Sibenzym, Novosibirsk, Russia) in the presence of [α-^{32}P]dATP and were isolated by agarose gel electrophoresis [23]. GMSA basically followed the procedure described [24]. Usually the incubation mixture of 20 μl contained about 1 ng of labelled probe and 1–10 μl of protein solution (∼1–20 μg of protein). Unless stated otherwise, *Escherichia. coli* DNA ultrasonicated, on average to 0.5 kb, was used as a competitor.

2.2. NM preparation and extraction

Nuclear and NM preparations were obtained from HeLa cultured cells as described [25], with some modifications. All the procedures were carried out at 4°C. All buffer solutions contained 0.5 mM PMSF. The nuclear preparations were checked for purity by phase contrast microscopy. For NM preparations the nuclei were resuspended in TM-2 (20 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$) buffer and treated with DNase I (20 units DNase I per 1 mg of nuclear DNA) for 30 min at room temperature, followed by a high salt extraction of soluble proteins with 0.2 M $(NH_4)_2SO_4$. NM was pelleted by centrifugation for 15 min at $2000 \times g$ through the cushion of a TM-2 buffer containing 300 mM sucrose and 0.2 M $(NH_4)_2SO_4$ and resuspended either in a buffer for extraction or in a TM-2 buffer containing 50% glycerol for storage at −70°C. The NM extracts were prepared as follows: 100 mg of the NM pellet were incubated overnight with stirring at 4°C in either (a) the low salt extract buffer (20 mM Tris–HCl, pH 8.5, 10 mM EDTA, 4 mM DTT) or (b) the high salt buffer, the same as (a) but with 0.8 M NaCl added.

After centrifugation, the extracts were used for subsequent steps.

2.3. Column chromatography

Ion exchange chromatography was performed using a diethylaminoethyl (DEAE)52 cellulose (Chemapol, Czech Republic) in a solution of 15 mM Tris–HCl, pH 8.0; 0.1 mM EDTA; 0.5 mM DTT; 0.1 mM PMSF, 5% glycerol, 50 mM NaCl. The protein bound to the column was eluted by a stepwise NaCl gradient, from 0.1 to 0.7 M, with steps of 50 mM. The activity tested by GMSA was detected at 0.2 M NaCl (active fraction from DEAE chromatography (0.2DE)).

The 0.2DE were loaded onto a gel filtration column with Sephacryl S300 (Pharmacia, Sweden) in 20 mM Tris–HCl, pH 7.5, 0.5 mM DTT, 1 mM PMSF, 150 mM NaCl. The column was calibrated using a Bio-Rad (USA) protein kit (N 151-1901) containing thyroglobulin 670 kDa, γ-globulin 158 kDa, ovalbumin 44 kDa, myoglobin 17 kDa, and vitamin B12 1.35 kDa.

2.4. Large scale GMSA

Mixture volumes for the GMSA were scaled up 10–20 times and loaded into wells of 10 mm thick acrylamide or agarose gels [5]. The whole gel was transferred to a nitrocellulose membrane (BA83, Schleicher and Schuell, Germany) with the help of vacuum blotting and the filter was exposed to X-ray film for 10–60 min. The spots corresponding to the complexes were cut out. Nitrocellulose filters with immobilised complexes were boiled in a minimal volume (about 200 μl) of loading buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [26]. Each piece was removed from the Eppendorf tube after 3 min of boiling. About 10 spots were used for each type of complex. Nitrocellulose pieces corresponding to the free DNA fragment were used as a control.

2.5. DNase I footprinting and G-methylation interference

Sequencing of the central 0.3 kb fragment of HS3 was carried out according to Maxam and Gilbert [23]. An asymmetrically labelled fragment was isolated using a 4% polyacrylamide gel. For DNase I footprinting, the binding reactions contained 100 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0,1% Triton X-100, 4% glycerol, 0.5–1.5 μg of protein of 0.2DE fraction, 20 000–60 000 cpm of end-labelled fragment and an excess of competitor DNA. After 20 min of incubation, $MgCl_2$ and $CaCl_2$ were added to 5 mM, and DNA was digested for 30 s with 0.01 units of DNase I. EDTA was added to a final concentration of 10 mM. DNase I digestion products were extracted with phenol:chloroform, precipitated, and analysed by autoradiography after electrophoresis on an 8% polyacrylamide/8 M urea sequencing gel.

For G-methylation interference [27], the labelled probe was modified for 5 min at 22°C with dimethyl sulphate in 50 mM sodium cacodylate, pH 8.0, containing 1 mM EDTA. The methylation reaction was stopped with 1.5 mM sodium acetate, pH 7.0, containing 1 mM β-mercaptoethanol, and DNA was precipitated. The methylated probe was incubated with column-purified (0.2DE) proteins as described above for DNase I footprinting, and the reactions were loaded onto a 4% GMSA gel. After electrophoresis, both bound and free bands were cut out and eluted in 0.5 M ammonium acetate containing 1 mM EDTA and 0.1% SDS. DNA recovered from gel was cleaved at methylated guanine residues by treatment with 1 M piperidine for 30 min at 90°C. After precipitation, cleavage products were analysed on a sequencing gel, as described above.

2.6. Antibodies (AB)

After determining optimal conditions of 0.2DE for GMSA, a preparative 4% gel (18×24 cm) was run. The gel was dried between cellophane and exposed on X-ray film (Kodak XAR-5, USA). DNA–protein complexes were cut out according to the autograph, homogenised in the Tris-buffered saline (TBS) with complete Freund's adjuvant in a 1:1 ratio, and injected into the guinea pig. Two male guinea pigs, each 3 months old and weighing approximately 300 g, were immunised. The complexes from one gel were used for each boost. The same injection was repeated after 14 days, and blood was drawn from the heart a week after the last injection. Serum

was obtained and stored at 4°C in the presence of sodium azide.

2.7. Immunoblotting

SDS-PAGE was carried out as described [26]. Proteins were electrotransferred to a nitrocellulose membrane (BA 83, Schleicher and Schuell, Germany) at 150 mA in an electrophoresis buffer containing 10% methanol. The membrane was blocked with 5% nonfat dry milk for 2 h in TBS/Tween (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 0.1% Tween 20). The primary AB at dilutions indicated in the figure legends in TBS/Tween was applied for 1 h at room temperature. After washing with TBS/Tween the secondary AB (biotinylated goat anti-guinea pig (Sigma, USA)) at 1:2000 dilution was applied for the same time. The streptavidin-conjugated alkaline phosphatase was used for detection of the secondary AB. Neither secondary AB nor streptavidin-alkaline phosphatase itself produced any staining. Monoclonal AB against lamin B and lamins A/C (Vector Labs, USA) were also used.

2.8. Indirect immunofluorescence

HeLa cells were grown overnight on coverslips. The cells were washed twice with TBS and were either permeabilised with 4% formaldehyde solution on TBS with 0.5% Tween 20 for 20 min at room temperature or used to obtain in situ NM. In the latter case the coverslips were treated for 10 min at room temperature with DNase I (50 units/ml of TBS containing 5 mM $MgCl_2$ and 0.5 mM $CaCl_2$), washed, and extracted for 2 min with 0.2 M $(NH_4)_2S0_4$ in the same buffer. The permeabilised cells and in situ NM were rinsed in TBS–Tween 20 and covered with AB (1:100) in TBS for 2 h at room temperature, washed three times with TBS–Tween, and covered with goat anti-guinea pig-biotin conjugated AB (1:100). Streptavidin–FITC or avidin–alkaline phosphatase conjugates followed by NBT-BCIP staining were used to reveal the primary AB. The coverslips were washed four times with TBS–Tween, sometimes counterstained with 4′,6-diamino-2-phenylindole (DAPI), and mounted in 10 mM sodium phosphate, pH 7.2, 150 mM NaCl, and 50% glycerol, and observed under a LUMAM R-8 (LOMO, Russia) fluorescence microscope. Slides processed for AB staining were fixed in 4% formaldehyde in TBS prior to fluorescent in situ hybridisation (FISH) procedure.

2.9. FISH

HS3 fragment was labeled with biotin-16-dUTP (Boehringer Mannheim, Germany) by random prime nick-translation according to conditions described by the manufacturer. For in situ hybridisation the chromosomal DNA was denatured in 70% formamide/2×SSC (0.03 M sodium citrate, 0.3 M NaCl, pH 7) at 70°C for 3 min followed by dehydration through ice-cold 70, 90 and 100% alcohol. Probe DNA was heat denatured for 5 min, ice-cooled for 3 min and hybridised overnight in 50% formamide/2×SSC at 37°C with sonicated salmon sperm DNA as a carrier. The slides were then washed in 2×SSC at 50°C. Texas red avidin D (Vector Labs, Burlingame, CA, USA) was used to detect the labeled probe on the chromosomal DNA. The DNA was finally stained with DAPI. A Zeiss Axioplan fluorescence microscope was used for viewing and taking pictures. Images were assembled into plates using Adobe Photoshop software.

3. Results

3.1. The specificity of binding

In our previous work it was shown using the nitrocellulose binding assay that HS3 specific binding activity was present in NM preparations and could be extracted [28]. The HS3 fragment ~1.8 kb in length forms DNA–protein complexes revealed by GMSA (Fig. 1A); however, such a long fragment is not convenient to work with, so the subcloned *Sau*3A fragments were made. All of them have the ability to form DNA–protein complexes in GMSA (Fig. 1B,C). The complexes are multiple, which may be due either to different proteins or to multimerisation of one protein. With the same amount of the NM extract, complexes with 0.3 and 0.4 kb HS3 fragments are prominent up to 10^3 fold weight excess of *E. coli* DNA (Fig. 1C, lanes 1, 2, 5, 6, and data not shown) but more than 2×10^3 excess is required

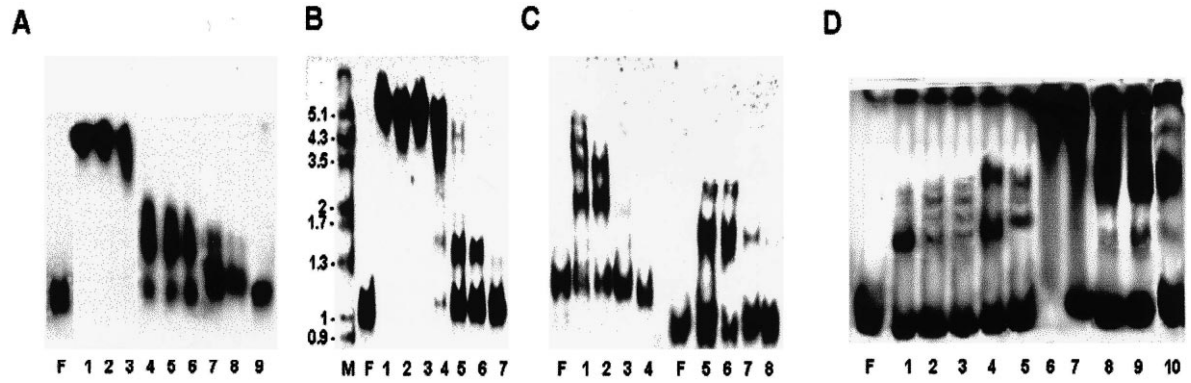

Fig. 1. GMSA of the NM extracts. The 1.8 kb (A), 1 kb (B), and 0.4 kb and 0.36 kb (C) HS3 fragments were used as a probe. 1 ng of end-labelled fragment and 5–10 μl (~5–10 μg of protein, constant volume in each experiment) were used in all the experiments. F: Free fragment here and everywhere. (A) 1, 0; 2 ×50; 3, ×100; 4, ×300; 5, ×500; 6, ×1000; 7, ×1500; 8, ×2000; 9, ×3000 fold excess of competitive DNA was added. (B) M: Size of marker fragments are given in kb; 1, 0; 2, ×50; 3, ×100; 4, ×200; 5, ×300; 6, ×500; 7, ×1000 fold excess of *E. coli* DNA was added. (C) 1, 2 and 5, 6×50 and ×100 fold excess of linearised pUC was added, respectively; 3, 4 and 7, 8×50 and ×100 fold excess of linearised plasmids with the inserts corresponding to the labelled ones was added, respectively. (D) 0.3 kb HS3 fragments and high and low salt NM extracts. 1–5: low salt NM extract (5 μl, ~5 μg of total protein), 6–10 high salt extract (5 μl, ~20 μg of total protein); 1, 6: 5×10^2; 2, 7: 10^3; 3, 8: 3×10^3; 4, 9: 5×10^3; 5, 10: 10^4 weight excess of poly (dIdC) added.

to destroy complexes of 1.8 kb HS3 fragment (Fig. 1B). The specific competitor, i.e. the unlabeled fragment itself, competes with the complexes at 20 fold weight excess (Fig. 1C, lanes 3, 4, 7, 8). So the binding activity is length dependent; since the shortest HS3 subfragment of 0.3 kb displays quite specific binding, it was used in the subsequent experiments. There is specific activity in high salt as well as in low salt NM extracts (Fig. 1D), and the sequential extractions exhaust the activity from NM [28]. Both extracts were used in DEAE column chromatography in different experiments. All the fractions obtained were tested by GMSA (Fig. 2) and the rest

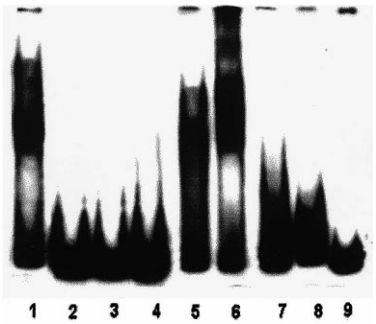

Fig. 2. Testing of ion exchange stepwise chromatography fractions by GMSA. All mixtures contain ×100 fold excess of *E. coli* DNA. 1: 5 μl of low salt NM extract; the rest contain 10 μl of 2: 50 mM; 3: 0.1 M; 4: 0.15 M; 5, 6: 0.2 M; 7: 0.3 M; 8: 0.4 M; 9: 0.5 M NaCl fractions.

of the experiments were done with 0.2DE. Among synthetic oligonucleotides, poly(dIdC) is a very weak competitor, while poly(dAdT) competes nearly as efficiently as HS3 itself; poly(dGdC) does not compete for binding (data not shown). The single stranded HS3 could not compete with complexes to the same extent as the double-stranded fragment, so the activity prefers double-stranded DNA (data not shown). In competition experiments, other satDNAs (murine major and minor satDNAs, human α-satDNA) compete for the binding to some extent: about 10^2 weight excess of these fragments is necessary to destroy the complexes (data not shown). But HS3 remains preferable in binding, for only HS3 fragments could compete with the complexes at less than 10^2 weight excess (Fig. 1C, lanes 3, 4, 7 and 8).

Methylation interference (Fig. 3A) and DNase I footprinting (Fig. 3B) were carried out to determine how protein interacts with DNA. In footprinting, free DNA has different intensities of residues along the fragment due to the sensitivity of DNase I to the DNA secondary structure. On the lanes where the samples with protein were loaded there were no long areas of protection, but the pattern of more and less intensive residues changes along the fragment. All the protected residues read in 0.3 kb HS3 are shown on the sequence (Fig. 3C). Most of the protected residues are 'A' and, therefore, in accor-

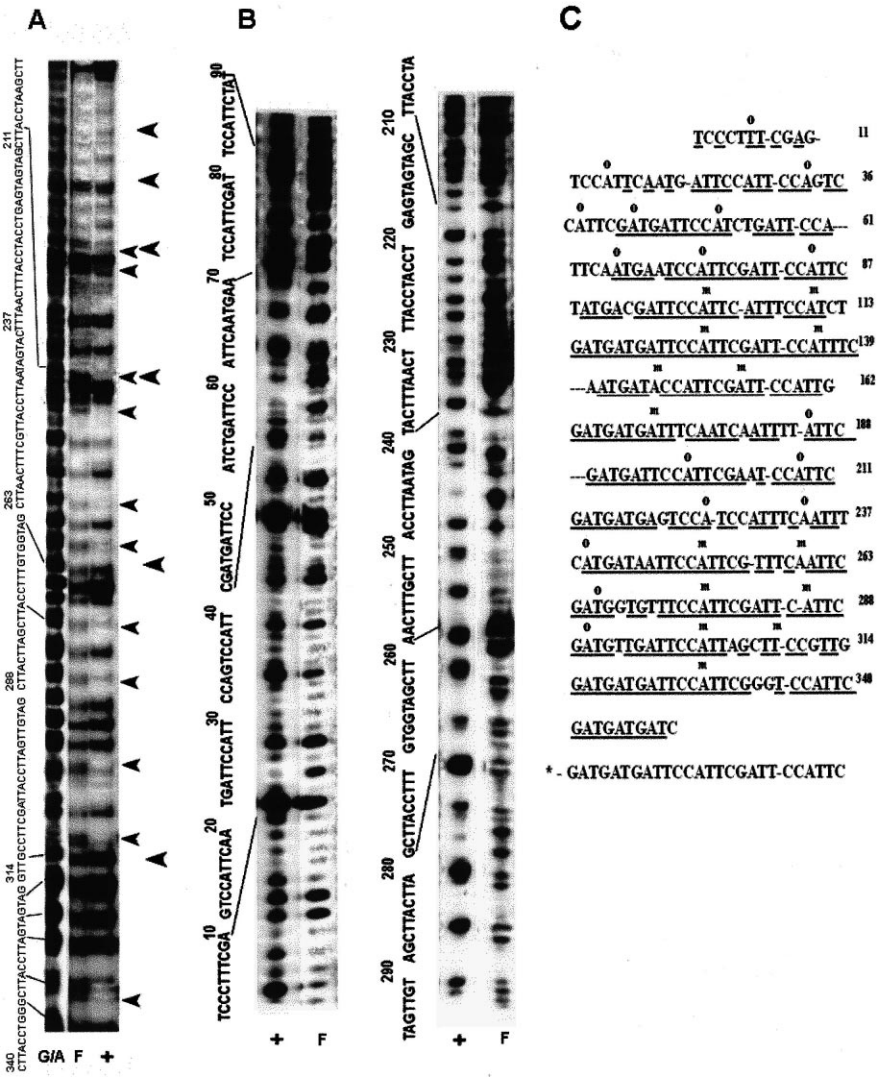

Fig. 3. Methylation interference (A) and footprinting (B) of p70 and 0.3 kb HS3. (A) G/A-G/A cleavage reaction of Maxam and Gilbert [26]; F: free fragment; +: lane from p70-HS3 complex. The first row of arrows marks protected residues, the second row of arrows marks internal repeats. (B) Two examples of DNase protection. F: Free fragment; +: after incubation with 0.2DE. (C) Protected residues are marked on the sequence aligned according to the internal repeat (*: consensus is given below and underlined). o: Footprinting, m: methylation.

dance with the competition of synthetic oligonucleotides (see above). There are protected residues in nearly every 10 bp, approximately at the same place in the internal repeat. Specific DNA binding, but without defined binding site in the DNA fragment, was also shown in the case of matrix attachment region (MAR)/SAR interaction with NM/scaffold proteins [29].

HS3 is one of a well studied family of highly repetitive sequences in the human genome. It has been shown that DNA sequences of this family consist mainly of simple and diverged repeat, ATTCCA [13]. However, during the sequencing of the 1.8 kb *Eco*RI fragment of HS3 the presence of another subsequence, GATGATGAT, was found [17]. The authors reported that this subsequence was located irregularly, although some regular pattern was observed in free fragment line on methylation interference (Fig. 3A). The sequence was aligned according to the subrepeat with its consensus shown in Fig. 3c. It is noteworthy that the subrepeat revealed differs from the previously published flanking ones and

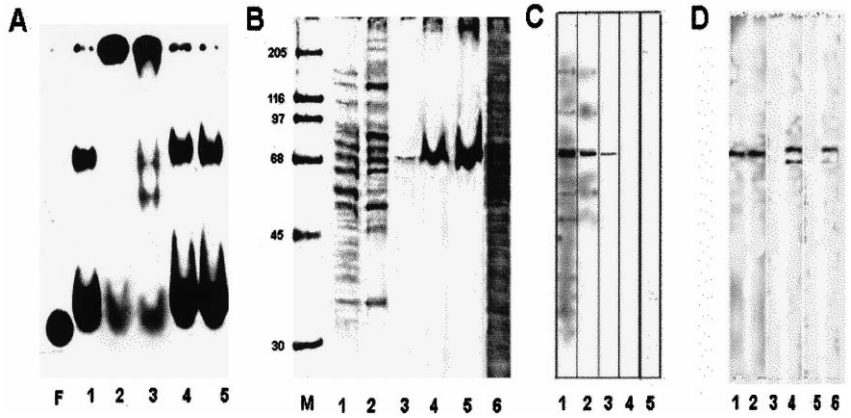

Fig. 4. HS3-binding p70 purification (B), testing of the AB raised by GMSA (A) and immunoblotting (C) and comparison with AB against lamins (D). (A) GMSA test of the AB raised. 1: Mixture contains 1 ng of 0.3 kb HS3 labelled, 5 μl of 0.2DE and ×100 fold excess of *E. coli* DNA; 2, 3: immune serum was added to the same mixture at dilutions 1:100 and 1:200, respectively; 4, 5: preimmune serum was added at the same dilutions. (B) SDS-PAGE Coomassie brilliant Blue (CBB) stained gel of 1: 0.2DE, 2: the first peak of HS3-binding activity from the second gel filtration column, 3: the second peak of HS3-binding activity from the gel filtration column, 4, 5: protein from the complexes of a low and high mobility, respectively, 6: raw NM extract. (C) Immunoblot of the NM extract with AB raised at dilutions 1:50, 1:500, 1:1000 (1–3, respectively) 4: preimmune serum, 5: alkaline phosphatase alone. (D) 0.2DE (1, 3, 5) and NM extract (2, 4, 6) were stained with AB raised (1, 2), AB against lamins A/C (3, 4) and lamin B (5, 6),

agrees well with the central one [17]. The difference may reflect some hierarchical order of repeats in the HS3 array.

Methylation interference shows that, at least in some internal repeats, the protein protects residues nearly at the same places with the periodicity of about 10 bp in the same way as is seen in DNase I footprinting. A very similar picture has been obtained during interaction of chicken W-satDNA with the W-protein which binds it [5].

3.2. P70 is responsible for the activity

0.2DE was used for the gel filtration in the attempt to perform the second step of column chromatography purification. Two peaks of activity were found by GMSA. The first was located in the area of molecular mass over 670 kDa and the second between 58 and 144 kDa. The latter peak shows one polypeptide chain with a molecular mass of around 70 kDa (p70) on SDS-PAGE (Fig. 4B, lane 4). The first active fractions (Fig. 4B, lane 3) also contain this protein as a major component, but there are some other proteins in this fraction and so p70 is included in large protein complexes. The ability to form complexes in vitro under conditions close to physiological ones is a property of W satDNA binding protein [5], the mouse major satDNA/MAR binding p120 [30], and well may be a universal property of the NM proteins.

The large scale variant of GMSA was performed to find out which proteins are involved in multiple complexes. The result does not depend on the gel used – agarose or acrylamide, or the type of complexes – with low or high mobility; the only protein found was the same: p70 (Fig. 4B, lanes 5, 6). Thus, the mobility of the complexes depends on the protein multimerisation under conditions close to physiological ones, and it is p70 which is responsible for the complex formation. The extent of the p70 purity in the complexes make them suitable for AB production.

3.3. Antibodies against HS3-p70 complexes

The antiserum added to the mixture for GMSA caused the hypershift of the complexes, while preimmune serum added in the same amount did not change its mobility (Fig. 4A). The immune sera added to the free fragment in the absence of proteins did not affect its mobility (data not shown). Thus, the AB raised does react with protein but not with DNA. In immunoblot with NM proteins loaded, AB recognise the only polypeptide chain of the same molecular mass, 70 kDa (Fig. 4C, lanes 1–3). No signal was obtained when preimmune serum or sec-

ondary ABs alone were used as a control (Fig. 4C, lanes 4, 5). Serum raised against complexes cut from GMSA did contain AB against the same p70 revealed during protein purification. The molecular mass of p70 was similar to that of lamins, whose location is shown to coincide with that of constitutive heterochromatin (i.e. satDNA) [31]. This was the reason for the following experiments. The immunoblotting of the active fraction and the NM loaded were done with AB against lamins B and A/C (Fig. 4D). The lamins were detected by immunoblotting in NM but not in 0.2DE. The p70 coincided with one of the lamin A/C zones but was not detected by anti-lamin A/C AB (Fig. 4D). Addition of anti-lamins A/C AB to the incubation mixture for GMSA did not produce a hypershift effect (data not shown).

AB were used for the immunofluorescent staining of the entire HeLa monolayer and in in situ NM preparation (Fig. 5). The AB stained individual granules in the circle in the middle of each nucleus and at the nuclear periphery. Sometimes granules were twined and of a different intensity (Fig. 5A). The alkaline phosphatase-staining makes it possible to see that the most prominent granules are located around nucleoli. The peripheral staining of the nucleus is also observed (Fig. 5B). The staining pattern looks very similar to that obtained when the HS3 probe was used for in situ hybridisation [32]. The AB staining shows that p70 remains in NM despite the loss of most DNA after in situ NM preparation (Fig. 5C). The staining pattern looks like numerous little granules scattered and attached to residual nucleoli in the nucleus. It looks like the protein covers and abut onto the residual DNA stained by DAPI (Fig. 5C, 3). One can see that in the nontreated interphase nuclei stained with DAPI the pattern of AB staining corresponds to the main heterochromatic regions, but sometimes the main signal looks like large patch at the rim of the bright DAPI spot (Fig. 5D). It is possible that DNase and high salt treatment employed in NM in situ isolation lead to the chromatin structure disruption; dense large patches went to pieces and the correspondence of the clouds of granules scattered to the residual heterochromatic areas became more obvious (Fig. 5C). To clarify the relationship between p70 and HS3 areas their localisation on in situ NM preparations were established. Examples of two nuclei with central

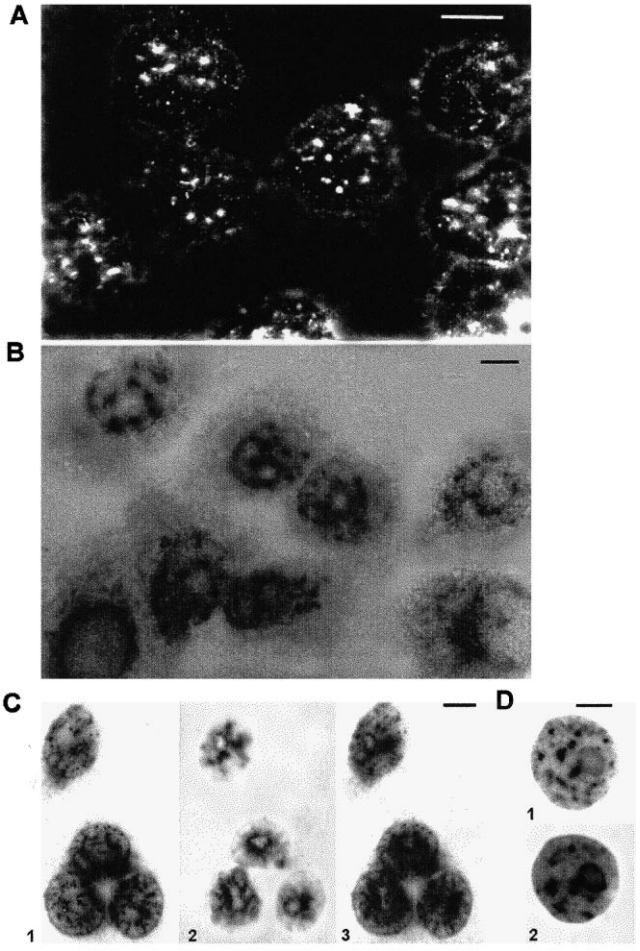

Fig. 5. HeLa cells monolayer stained with p70 AB (1:100). Avidin–FITC (A, C, D); or alkaline phosphatase (B) staining protocols were used for the detection. (C) In situ NM stained as on A, negative image, 1: AB revealed by FITC conjugate, 2: residual DNA revealed by DAPI, 3: merged image of 1 and 2. (D) Negative images of the nontreated interphase nuclei stained with 1: AB revealed by FITC conjugate, 2: DAPI. Bar: 5 μm.

or peripheral position of the residual DNA are shown (Fig. 6). Combined immunofluorescence (p70) and FISH (HS3) shows that HS3 and p70 areas correspond to each other (Fig. 6). The residual DNA of the NM is enriched with HS3 in the same way as it was shown for other satDNA [18–20]. Although the p70 patches became less evident, the colocalisation of p70 and HS3 is obvious (Fig. 6). NM does contain p70 that can specifically bind HS3 in vitro, so the biochemical procedure of p70 identification has been successful due to its presence in the NM together with HS3.

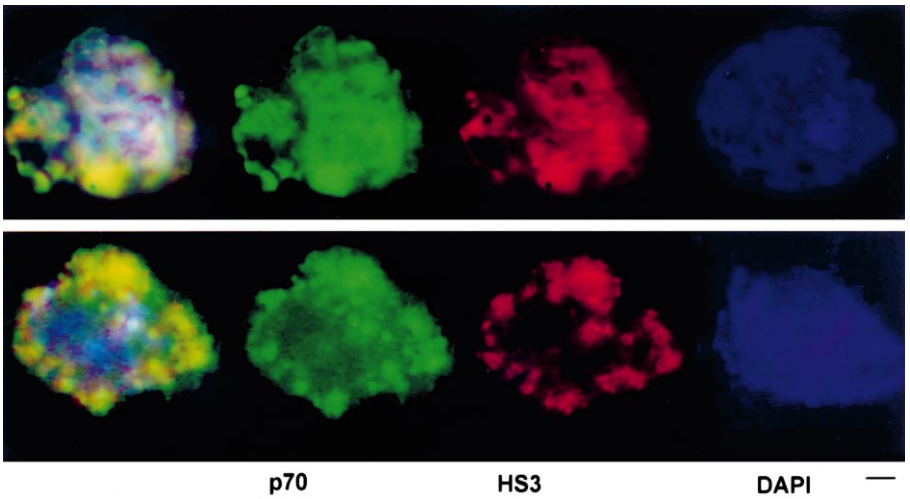

Fig. 6. In situ NM preparations stained with p70 AB (p70), applied to FISH with HS3 (HS3) and stained with DAPI (DAPI). 2 nuclei are shown and first image in each row is the merged one. Bar: 1 mm.

4. Discussion

The spatial organisation of chromatin is maintained by the NM [31,33–36]. The MARs are thought to be distributed along the entire length of chromosomal DNA and to cause its association with NM [34,36]. DNA is also attached to NM through the enzyme complexes – replicative and transcriptional machines associated with NM [35]. The mechanisms and functions of such interactions have been intensively investigated. Less well studied is the association of NM with pericentromeric and peritelomeric constitutive heterochromatin, formed on the base of satDNA and located not randomly inside the nucleus [28,31]. But such interactions between NM and satDNA should take place in the nuclei, according to the NM's role as a nucleus spatial organiser [31,37] and the location of satDNA sequences in the interior of the interphase nuclear chromosome domains [38].

Among satDNA-binding proteins the group of CEN proteins is the best investigated. CENPB (80 kDa) is so far the only protein known to bind α-satDNA in a site-specific manner. It binds a 17 bp sequence, CENP-B box, inside the 170 bp α-satDNA monomer [39]. The sequence specificity of CENP-B led to the suggestion that it is this protein which is responsible for CEN recognition in the α-satDNA array during kinetochore formation. CENP-B has been shown to be partially located in the NM [40]. The absence of the CENPB-box in the HS3 sequence as well as the molecular mass of p70 (70 kDa) rule out the possibility of p70 being identical to CENP-B.

Peripheral undermembrane localisation of constitutive heterochromatin formed by satDNA is a well-documented cytological fact [1,31]. It has been shown that lamins, the major nuclear lamina component, bind DNA, and their DNA-binding domains are well defined [31]. It is found that lamins bind MAR DNA [41]. The association of heterochromatic regions with the submembrane lamina suggested that the lamins could interact with satDNA. The HS3-binding p70 revealed has a molecular mass very similar to that of lamins. However, the negative result of 0.2DE immunoblotting and the absence of a hypershift effect in GMSA with anti-lamin AB prove that lamins B and A/C are not present in HS3-p70 complexes. The possibility of interaction between p70 and lamins that not only underlie the membrane but also are components of the inner NM [31] cannot be excluded and remains to be investigated.

Certain features of p70 are similar to those of W protein, with the specificity in binding to bird W-chromosome satDNAs [6]. Like p70, W protein forms large aggregates under conditions close to physiological ones. A number of satDNAs homologue by less than 60% are positive in binding and bent, i.e. they possess some secondary structure like the HS3 fragment. W protein binds only fragments longer than 300 bp; inhibition of the binding by Hoechst 33258 indicates this binding to occur

through A/T residues in the minor groove. The model based on footprinting shows that W-satDNA is bent around W protein aggregate by multiple contacts of about 10 bp periodicity [5], as in the case of p70 (Fig. 3). The molecular mass reported for W protein (72 kDa) is very similar to that of p70. We suggest that p70 on the basis of its binding mode and molecular mass similarity, might be an analogue of W-protein in mammals. W-protein was isolated from the nucleoplasm. NM is enriched with p70 about 100 times more than the nucleoplasm, according to immunoblotting and quantitative GMSA (data not shown), but p70 could be purified from nucleoplasm as well. The ability of p70 to form large aggregates under conditions close to physiological is confirmed by gel filtration chromatography. So far, neither an AB against W protein nor its sequence have been reported, so the opportunity to compare it with p70 is restricted to the experiments described.

Localisation of p70 in vivo according to the AB raised corresponds to the HS3 pattern obtained by in situ hybridisation. HS3 is present in the pericentromeric regions of most human chromosomes. It may be a functionally important component of CEN and is definitely located close to the CEN α-satDNA [15]. The interphase CEN is associated with nucleoli in human cells not only in the nucleolus organiser region (NOR)-bearing, but also in the non-NOR-bearing chromosomes [21,22]. In mammalian interphase cells the CENs tend to congregate near the nuclear periphery and around nucleoli. These locations are not static. Rather, within the cell type, the CEN distribution in relation to the two nuclear landmarks changes reproducibly during the cell cycle progression [42], in response to the functional [43] and transcriptional states of the cell [22,44]. The patterns of staining observed in HeLa cell line monolayer correspond to the expected CEN and HS3 localisation, i.e. clusters around nucleoli and close to the nuclear periphery. Immunostaining also confirmed the NM association of p70, although the exact staining pattern correspondence to the precise stage of the cell cycle remains to be investigated.

The data obtained over the last decade suggest that transcriptionally silent sequences, such as long tandem repeats, are actively used in interphase to organise specific groups of chromatid in mammalian nucleus and thus are involved in spatial organisation of the nucleus [45]. The data of the current work show that the NM contains p70 that is specific in binding to HS3 in vitro and in in situ NM preparations double staining shows the colocalisation of the p70 and HS3 areas. In HeLa cell monolayer, i.e. in vivo, p70 adjusted to the heterochromatic regions, some of which are composed of HS3. The aggregation of p70 into large complexes revealed in the current work could provide a functional advantage, especially in cementing particular patterns of expression in cells of different lineage [21,45] by taking part in heterochromatin organisation.

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