MOLECULAR AND CELLULAR BIOLOGY, Jan. 1995, p. 456–465 Vol. 15, No. 1
0270-7306/95/$04.00+0

Nucleolin Is a Matrix Attachment Region DNA-Binding Protein That Specifically Recognizes a Region with High Base-Unpairing Potential

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Received 8 April 1994/Returned for modification 24 May 1994/Accepted 26 September 1994

A DNA affinity column containing a synthetic double-stranded nuclear matrix attachment region (MAR) was used to purify a 100-kDa protein from human erythroleukemia K562 cells. This protein was identified as nucleolin, the key nucleolar protein of dividing cells, which is thought to control rRNA gene transcription and ribosome assembly. Nucleolin is known to bind RNA and single-stranded DNA. We report here that nucleolin is also a MAR-binding protein. It binds double-stranded MARs from different species with high affinity. Nucleolin effectively distinguishes between a double-stranded wild-type synthetic MAR sequence with a high base-unpairing potential and its mutated version that has lost the unpairing capability but is still A+T rich. Thus, nucleolin is not merely an A+T-rich sequence-binding protein but specifically binds the base-unpairing region of MARs. This binding specificity is similar to that of the previously cloned tissue-specific MAR-binding protein SATB1. Unlike SATB1, which binds only double-stranded MARs, nucleolin binds the single-stranded T-rich strand of the synthetic MAR probe approximately 45-fold more efficiently than its complementary A-rich strand, which has an affinity comparable to that of the double-stranded form of the MAR. In contrast to the high selectivity of binding to double-stranded MARs, nucleolin shows only a small but distinct sequence preference for the T-rich strand of the wild-type synthetic MAR over the T-rich strand of its mutated version. The affinity to the T-rich synthetic MAR is severalfold higher than to its corresponding RNA and human telomere DNA. Quantitative cellular fractionation and extraction experiments indicate that nucleolin is present both as a soluble protein and tightly bound to the matrix, similar to other known MAR-binding proteins.

Considerable evidence suggests that eukaryotic chromatin forms discrete and topologically constrained loops which are attached at their base to the nuclear matrix (22, 29, 57). Active genes and replicating DNA have been shown to be physically associated with the matrix (38, 39, 66, 79). Recently it has been demonstrated that newly synthesized transcripts of viral and mammalian genes are localized within defined transcript domains, suggesting an association with the nuclear substructure (82), and visual evidence for the attachment of replication "factories" to a nucleoskeleton in HeLa cells was provided (35).

DNA sequences that have a high affinity for the nuclear matrix have been identified in different species. These sequences, called matrix or scaffold attachment regions (MARs or SARs), may act as boundary sequences by anchoring chromatin onto the nuclear matrix (63; reviewed in reference 29). MARs are often found close to regulatory sequences, including enhancers (15, 16, 27, 40). MARs are also common at the boundaries of transcription units, where they may delimit the ends of an active chromatin domain (7, 21, 27, 28, 50, 55, 64). MARs have been shown to buffer effects of flanking chromatin in stably transfected cell lines and transgenic animals (53, 65, 75) and in some cases to augment transcriptional activity (6, 45, 54, 65, 75).

It has been demonstrated that MARs readily relieve negative superhelical strain by becoming stably base unpaired under superhelical strain (6). A nucleation site for unwinding was identified in the MAR located downstream of the immunoglobulin heavy-chain (IgH) gene enhancer (46). Similar core-unwinding elements were also delineated in the MAR associated with the human beta interferon gene (6). Point mutations within these core sequences completely abolished unwinding in both cases. A concatemerized oligonucleotide containing the core-unwinding element of the MAR 3′ of the IgH enhancer exhibited a strong affinity for the nuclear matrix and augmented simian virus 40 promoter activity in stable transformants. Mutated concatemerized oligonucleotides that resisted unwinding showed weak affinity for the nuclear matrix and did not enhance promoter activity (6). These results suggest that the unwinding capability is important for MAR function. We recently cloned and characterized a protein, SATB1, which specifically binds to the synthetic MAR and to naturally occurring MAR fragments that share a propensity for unwinding under negative superhelical strain; however, this protein does not bind the mutated, A+T-rich DNA that resists unwinding (19). SATB1 selectively recognizes a special A+T-rich sequence context, which we named ATC sequences, in which one strand consists exclusively of mixed A's, T's, and C's but not G's and binds in the minor groove, making little contact with the bases. Clustering of ATC sequences confers a strong unwinding ability, and this feature is shared among various MARs. SATB1 is expressed predominantly in the thymus and is virtually undetected in other tissues, thus representing the first known example of a tissue-specific MAR-binding protein. Only a few MAR-binding proteins besides SATB1 are known to date. This short list includes attachment region-binding protein (81), SAF-A (67), SP120 (78), lamin B_1 (52), histone H1 (37), HMGI/Y (84), and topoisomerase II, which is a major structural component of mitotic chromosomes and interphase nuclear scaffolds (1, 4, 23, 30, 73). SAF-A, P120, and SP120

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were recently identified as heterogeneous nuclear ribonucleoprotein (hnRNP) U (24, 77, 80).

We describe here the purification of a 100-kDa MAR-binding protein from K562 cell extracts by use of a MAR affinity column. This protein was cloned and identified as nucleolin, the major nucleolar protein of actively dividing cells. The binding specificity of nucleolin was analyzed by determining the dissociation constants (K_ds) for a variety of naturally occurring MAR sequences, and in particular we compared the binding affinities of nucleolin for the double-stranded DNA, the single-stranded DNA, and the RNA derived from the same synthetic MAR sequence, as well as DNA and RNA derived from the human telomere repeat $(TTAGGG)_n$. We found that purified nucleolin binds various double-stranded MAR fragments with high affinity and exhibits a strict preference for a double-stranded MAR probe that has a high unwinding propensity over its mutated version that has lost this structural property. Nucleolin also shows a strong preference (approximately 50-fold) for the T-rich strand of the single-stranded synthetic MAR probe over the complementary A-rich strand and the double-stranded form of the MAR probe.

MATERIALS AND METHODS

Cell culture and cell extract preparation. The human erythroleukemia cell line K562 was grown at 37°C in an atmosphere of 5% CO_2 in a Cell Factory (Nunc) containing RPMI 1640 medium (Irvine Scientific) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 mg of streptomycin per ml. For harvest, cells were resuspended by repeated pipetting in their growth medium, centrifuged for 5 min at 2,500 rpm in a Beckman TJ-6 centrifuge and washed once in cold phosphate-buffered saline (PBS). The cell pellet obtained from a 500-ml cell suspension was resuspended in 10 ml of extraction buffer (0.4 M KCl, 10 mM sodium phosphate buffer [pH 7.4], 10% glycerol, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride [PMSF]; 10 μg of leupeptin per ml, 10 μg of aprotinin per ml). Cells were lysed in a Dounce homogenizer (50 strokes with pestle A). The extract was centrifuged for 1 h in a T865.1 rotor in a Sorvall centrifuge at 38,000 rpm, and the supernatant was either assayed directly for binding activity or used for affinity purification of MAR-binding proteins. The same procedure was followed for mouse thymus extract preparation, except that frozen thymus glands from 3- to 6-week-old mice were used.

Cell fractionation. Nuclei were isolated from 2×10^7 cells by a modification of the method of Dignam et al. (20), involving lysis in hypotonic buffer (10 mM Tris · HCl [pH 7.6], 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM PMSF) and homogenization in a Dounce homogenizer (40 strokes with pestle B) until approximately 90% of the cells were lysed as determined by trypan blue exclusion. The nuclei were then separated into two tubes and pelleted by centrifugation ($800 \times g$ for 5 min). One of the pellets (containing 10^7 nuclei) was resuspended in 1 ml of extraction buffer containing 0.4 M KCl (see preceding paragraph), homogenized in a Dounce homogenizer, and centrifuged by the same procedure used for the preparation of cell extract. The resulting supernatant represents nuclear extract.

The nuclear matrix was prepared from the second nuclear pellet (containing 10^7 nuclei) by the method of Smith and Berezney (71) with slight modifications. Briefly, 10^7 nuclei were resuspended in 1 ml of isotonic buffer (25 mM Tris · HCl [pH 7.6], 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM PMSF), digested with 200 μg of DNase I per ml for 16 h at 4°C, and centrifuged ($1,000 \times g$ for 10 min). The supernatant was the DNase I fraction. The pellet was extracted three times with 1 ml of low-salt buffer (10 mM Tris · HCl [pH 7.4], 0.2 mM $MgCl_2$, 1 mM PMSF) and centrifuged ($1,000 \times g$ for 10 min) to yield the low-salt supernatant. The remaining pellet was extracted with 1 ml of high-salt buffer (1.6 M NaCl, 10 mM Tris · HCl [pH 7.4], 0.2 mM $MgCl_2$, 1 mM PMSF) and centrifuged ($6,000 \times g$ for 10 min) to yield the high-salt supernatant. In a final step, the insoluble matrix pellet was extracted by the method of Kaufmann et al. (43) by incubation on ice for 30 min in the presence of 1% β-mercaptoethanol in 1 ml of high-salt buffer followed by centrifugation ($6,000 \times g$ for 15 min). The resulting supernatant represents the nuclear matrix. The insoluble pellet was resuspended in 1 ml of sodium dodecyl sulfate (SDS) sample buffer and boiled.

Nucleoli were purified by sonication and centrifugation through a sucrose step gradient as described by Bolla et al. (8) with some modifications. Nuclei (10^7) were resuspended in 1 ml of buffer B (10 mM Tris · HCl [pH 7.4], 10 mM $MgCl_2$, 150 mM NaCl) and sonicated four times for 30 s each at 50 W. The extract was treated with 20 μg of DNase I per ml for 1 h at 4°C and centrifuged at $800 \times g$ for 15 min through a 10-ml step gradient containing 5 ml each of 0.25 M and 0.88 M sucrose in buffer B. The pellet was resuspended in 1 ml of 1 mM Tris · HCl [pH 8.0]–1 mM EDTA, incubated on ice for 15 min, and centrifuged at $100 \times g$ for 5 min. The resulting pellet consisted of purified nucleoli.

Nucleolar matrix was prepared from purified nucleoli, corresponding to 10^7 nuclei as the starting material, exactly as described above for the matrix preparation from nuclei. The final pellet resulting from β-mercaptoethanol and high-salt treatment was resuspended directly in 1 ml of SDS sample buffer and heat denatured by boiling. Equivalent volumes for each fractionation step were loaded on an SDS–7.5% polyacrylamide gel.

Protein concentrations of whole-cell extracts were determined with a protein assay kit (Bio-Rad). Concentrations of purified proteins were determined with the Quantigold assay reagent (Diversified Biotech). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (7.5% polyacrylamide) (49) and visualized by silver staining.

Antibody production. The production of SATB1-specific polyclonal antibodies was described previously (19). Polyclonal antibodies against nucleolin were raised in BALB/c mice by four successive intraperitoneal injections of SDS-PAGE gel slices, each containing approximately 20 to 30 μg of affinity-purified nucleolin, with RIBI adjuvant (from RIBI Immunochemical Research, Inc.).

Western immunoblots. Proteins separated by SDS-PAGE were electrophoretically transferred to Immobilon P membranes (Millipore) in 20 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8). Biotinylated high-molecular-weight protein markers (Bio-Rad) were routinely included for internal size standards. The filters were blocked in 5% bovine serum albumin (BSA) in TST (20 mM Tris · HCl [pH 7.4], 0.5 M NaCl, 0.05% Tween 20), washed in TST buffer minus BSA, and incubated with 1:1,000-diluted rabbit anti-SATB1 serum for the detection of SATB1. After being washed in TST, the filters were incubated simultaneously with a 1:15,000 dilution of goat anti-rabbit antibody-conjugated horseradish peroxidase and avidin-labeled horseradish peroxidase (both from Bio-Rad). For detection of nucleolin, the blots were incubated with 1:1,000-diluted mouse antinucleolin serum and then with 1:3,000-diluted goat anti-mouse antibody-conjugated horseradish peroxidase. Following extensive washing in TST, the blots were incubated with ECL (enhanced chemiluminescence) reagent solutions (Amersham) and exposed to X-ray film for visualization of protein bands.

DNA affinity purification of proteins. The method described by Kadonaga and Tjian (42) for DNA affinity purification was used with some modifications. Briefly, the complementary oligonucleotides 5′-TCTTTAATTTCTAATATAT TTAGAAttc-3′ (T-rich strand) and 5′-TTCTAAATATATTAGAAATTAAA GAgaa-3′ (A-rich strand) (lowercase type indicates single-strand overhangs) were annealed, phosphorylated at their 5′ ends, and ligated to form concatemers. We routinely coupled 200 μg of the double-stranded oligomers to 1 ml of cyanogen bromide-activated Sepharose 6MB (Pharmacia). This high concentration of oligomers was necessary to obtain a high yield of purified nucleolin. Before being loaded onto the column, the cell extract was incubated with 500 μg of double-stranded poly(dI-dC) · poly(dI-dC) and 100 μg of denatured salmon sperm DNA for 10 min on ice, centrifuged at 10,000 rpm for 10 min in a Sorvall SS34 rotor, and diluted fourfold with buffer Z (42). This diluted extract was loaded directly onto the affinity column, which was then washed with buffer Z containing 0.1 M KCl. Bound protein was eluted with eight 0.5-ml fractions of 1 M KCl in buffer Z. The fractions containing binding activity were concentrated by centrifugation in Centricon-50 microconcentrators (Amicon). The samples were then desalted by dilution with PBS and centrifugation in Centricon-50 units. This last step was repeated three times.

Renaturation of gel-purified protein. A 100-μl portion of the affinity-purified protein fraction containing the highest binding activity was mixed with 100 μl of 2× SDS sample buffer and separated by SDS-PAGE (7.5% polyacrylamide). Protein bands were visualized by Coomassie blue fast stain (Zoion Research). Gel slices (2 to 4 mm wide) spanning a 30- to 200-kDa size range were cut out and eluted overnight at room temperature by the method of Hager and Burgess (32). The eluates were precipitated with acetone, and the pellets were dissolved in dilution buffer containing 6 M guanidine HCl. After a 20-min incubation at room temperature, the samples were diluted 50-fold with dilution buffer and allowed to renature for 2 h at room temperature. Protein was concentrated four- to fivefold in Centricon-30 concentrators (Amicon).

DNA mobility shift assay. The DNA probes used for protein-binding studies, as well as the sources of the various MAR fragments, are described in detail elsewhere (19). The double-stranded DNA fragments were end labeled at both ends with the Klenow fragment of DNA polymerase. For determining strand specificity, Bluescript plasmid containing wild-type $(25)_5$ or mutated $(24)_8$ inserted in the *Eco*RI or *Eco*RV site was cut and end labeled at the *Bam*HI or the *Hin*dIII site and then subjected to a second digestion with *Hin*dIII or *Bam*HI, respectively. The singly end-labeled fragments were gel purified and heat denatured by boiling before the binding reaction was performed. Labeled RNA was synthesized with T7 or T3 RNA polymerase and [^{32}P]UTP from linearized Bluescript plasmid containing the wild-type $(25)_5$ insert, using a transcription kit (Stratagene). The telomere DNA and RNA sequences were synthesized as oligomers and end labeled with T4 kinase.

Binding reactions were done in a total volume of 20 μl with the indicated amounts of cell extract or affinity-purified protein, exactly as described previously (19). Either double-stranded or heat-denatured poly(dI-dC) was added as a nonspecific competitor with double-stranded or single-stranded probes, respectively. Bound and free DNA was quantitated by laser densitometer scanning of short-exposure autoradiographs of the dried band shift gels. K_ds were calculated

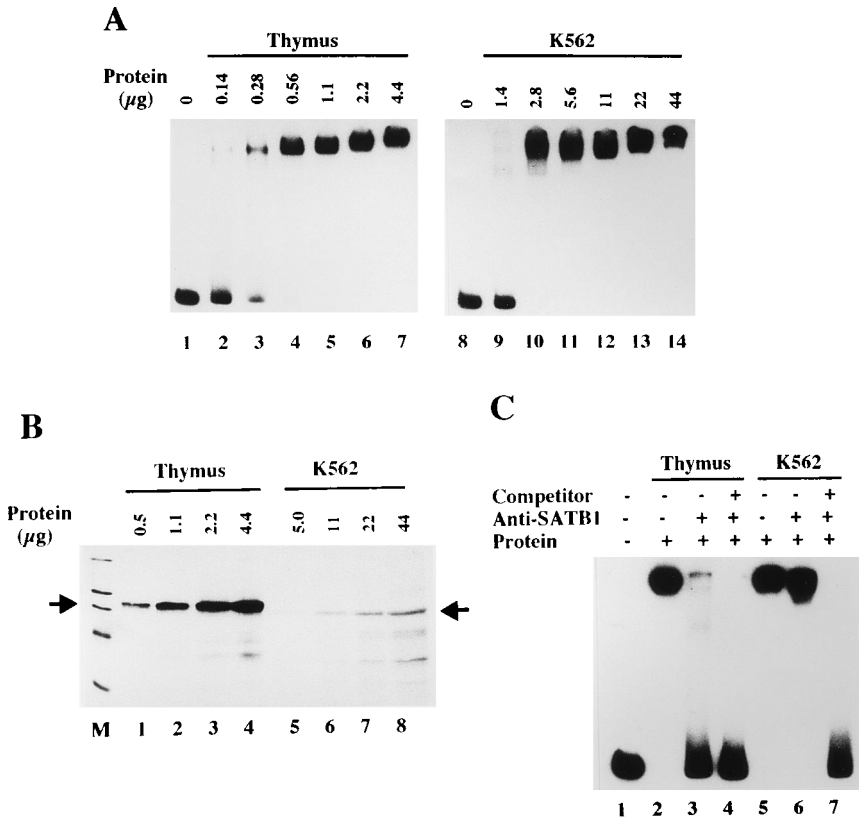


FIG. 1. Strong MAR-binding activity detected in K562 extract which is not due to SATB1. (A) Band shift analysis with cell extract from mouse thymus (lanes 2 to 7) and human K562 cells (lanes 9 to 14) with end-labeled wild-type $(25)_5$ as a MAR probe. A 10-fold excess of protein from K562 was used over protein from thymus. The protein concentrations (in micrograms per 20 μl) are given at the top of the gel. (B) Western blot of thymus extract (lanes 1 to 4) and K562 extract (lanes 5 to 8) with anti-SATB1 antibody. A 10-fold excess of protein from K562 extract was applied. The protein bands corresponding to the 103-kDa SATB1 from thymus and the 96-kDa SATB1 from K562 are indicated by arrows. The size markers (lane M) correspond to 200, 116, 97.4, 66, and 45 kDa, from top to bottom. (C) Antibody inhibition assay of binding activity to wild-type $(25)_5$. A band shift gel with 1 μg of total protein per lane from thymus extract (lanes 2 to 4) and 2 μg of total protein per lane from K562 cell extract (lanes 5 to 8) after preincubation with anti-SATB1 antibody (lanes 3, 4, 6, and 7) is shown. The minus and plus signs above the lanes indicate the absence or presence, respectively, of protein, antibody, or unlabeled wild-type $(25)_5$ competitor.

from mobility shift experiments with increasing protein concentration and a fixed probe concentration of 40 pM under a protein excess. The K_d value was estimated from the protein concentration required for a 50% shift of the probe (26, 48).

For antibody inhibition experiments, 3 μl of rabbit anti-SATB1 serum was added to the binding-reaction mixture containing cell extract (as indicated), the mixture was incubated for 30 min on ice, labeled probe was added, and the mixture was incubated for 15 min at room temperature.

For the distamycin inhibition experiment, an aqueous solution of distamycin was added to the binding reaction as indicated, prior to adding the DNA probe.

RESULTS

MAR-binding activity in extracts from K562 cells. In an effort to identify MAR-binding proteins in K562 cell extracts, we used a synthetic MAR probe derived from a 25-bp sequence within the MAR located 3′ of the IgH enhancer containing a nucleation site of unwinding (46). A concatemer containing five or more units of this sequence shows a high affinity for the nuclear matrix (6) and was previously used to isolate the human MAR-binding protein SATB1 (19). A concatemer of five repeats of the same sequence (described in Materials and Methods), named wild-type $(25)_5$, was used as a probe in band shift assays with cell extracts from different cell lines. We found significant binding activity to wild-type $(25)_5$ in whole-cell extracts from the human erythroleukemia cell line K562 (Fig. 1A, lanes 9 to 14). The binding activities of cell extracts from thymus (which is the major source of SATB1 protein) and from K562 cells were compared. With a constant probe concentration and increasing protein concentrations, a complete shift was observed with 0.56 μg of thymus extract (lane 4) and with 2.8 μg of K562 extract (lane 10), suggesting that the total MAR-binding activity in K562 whole-cell extract is almost 20% of the activity in thymus whole-cell extract.

To examine whether this relatively high MAR-binding activity in K562 extract was due to SATB1 or another protein(s), we determined the relative amount of SATB1 in extracts from thymus and K562 cells by Western blotting with rabbit anti-SATB1 serum (19). The rabbit anti-SATB1 antibody recognizes both human and mouse SATB1 with equal affinity (data not shown), and human and mouse SATB1 share 98% homology at the amino acid level (56). As expected, SATB1 was easily detectable in thymus extract even when only 0.5 to 4.4 μg of total protein was loaded (Fig. 1B, lanes 1 to 4). However, only faint bands were detected in K562 extract, even when 10 times as much total protein (5 to 44 μg) was loaded on the gel (lanes 5 to 8). Densitometer scanning of the autoradiograph revealed that the level of SATB1 in K562 cells is at least 100-fold lower than in thymus, strongly suggesting that the main portion of the MAR-binding activity in K562 extracts is caused by a protein different from SATB1. The protein detected by anti-SATB1 antibody in K562 extract has an apparent molecular mass of approximately 96 kDa (lanes 5 to 8), whereas SATB1 from thymus and in vitro synthesized SATB1

from the cDNA migrate at 103 kDa (lanes 1 to 4) (56). During this study, another group independently detected SATB1 in K562 cells and confirmed its identity by peptide sequencing (17). The 96-kDa SATB1 may represent a different isoform of thymus-specific SATB1.

We confirmed by an antibody inhibition assay that the MAR-binding activity in K562 cell extract was caused by a protein different from SATB1. Cell extract from thymus and from K562 was incubated with anti-SATB1 antibody prior to binding to the wild-type $(25)_5$ probe in a typical band shift assay. The results demonstrate that MAR-binding activity of K562 extract is virtually unaffected by anti-SATB1 serum (Fig. 1C, lane 6), whereas binding of thymus extract is almost completely inhibited (lane 3). Binding to wild-type $(25)_5$, however, is specific in both extracts, since the addition of a 200-fold molar excess of unlabeled wild-type $(25)_5$ effectively competes with binding (lanes 4 and 7). The competition experiment was done in the presence of anti-SATB1 antibody to make sure that any remaining MAR-binding activity, which was unaffected by anti-SATB1 antibody, was still specific. These results together strongly suggest that although K562 cells contain a low level of SATB1, the major portion of the MAR-binding activity can be attributed to a protein distinct from SATB1.

Affinity purification of a 100-kDa MAR-binding protein from K562 cells. We prepared a MAR DNA affinity column by coupling multimers of the wild-type $(25)_5$ sequence to activated Sepharose as described in Materials and Methods. This affinity resin was highly selective for the purification of MAR-binding proteins, since SATB1 was purified to near homogeneity from crude mouse thymus extracts. A major band of 103 kDa was detected in the eluted fractions by silver staining (Fig. 2A, lane 1); it was identified as SATB1 by immunostaining with anti-SATB1 antibody (Fig. 2B, lane 1). The same procedure was used for the purification of the MAR-binding protein from K562 cell extract. The fraction containing K562 protein eluted from the affinity column showed a major band migrating at 100 kDa (Fig. 2A, lane 2) which did not react with anti-SATB1 serum in a Western blot experiment (Fig. 2B, lane 2). A small amount of the 96-kDa SATB1 was detected in Western blots only when excess protein was loaded onto the gel (data not shown).

When individual fractions of K562 protein eluted from the column were simultaneously analyzed by mobility shift assays and SDS-PAGE, the peak of binding activity correlated with the peak of intensity of the 100-kDa polypeptide band (data not shown), suggesting that this protein was responsible for MAR binding. This was confirmed by a renaturation experiment with SDS-PAGE-purified protein. Gel slices containing affinity-purified protein from K562 extract ranging in size from 30 to 200 kDa were cut from an SDS-polyacrylamide gel, eluted, and renatured by the procedure described by Hager and Burgess (32), and the binding activity of each was tested in a mobility shift assay with wild-type $(25)_5$ as a probe. Binding activity was recovered mainly with the renatured 100-kDa protein (Fig. 2C, lane 5), similar to renatured SATB1 (lane 1). The 100-kDa value for the MAR-binding protein from K562 extract was further confirmed by UV cross-linking (data not shown). In summary, a 100-kDa protein was isolated from K562 cell extract as a result of its affinity to the double-stranded synthetic MAR wild-type $(25)_5$. This protein appears to be relatively abundant in K562 cells and is the major MAR-binding protein purified from the MAR affinity column, besides a very small amount of SATB1. Therefore, the relatively high MAR-binding activity of the K562 extract must be attributed, at least for the most part, to the 100-kDa protein under the conditions used for cell extract preparation. From 500 ml of K562 cell culture (7.5×10^8 cells), we routinely obtained 30 to 60 μg of purified 100 kDa protein, which corresponded to approximately 0.05 to 0.1% of the total protein of whole-cell extract. The yield of affinity-purified SATB1 from thymus extract was approximately 0.3% of the total protein.

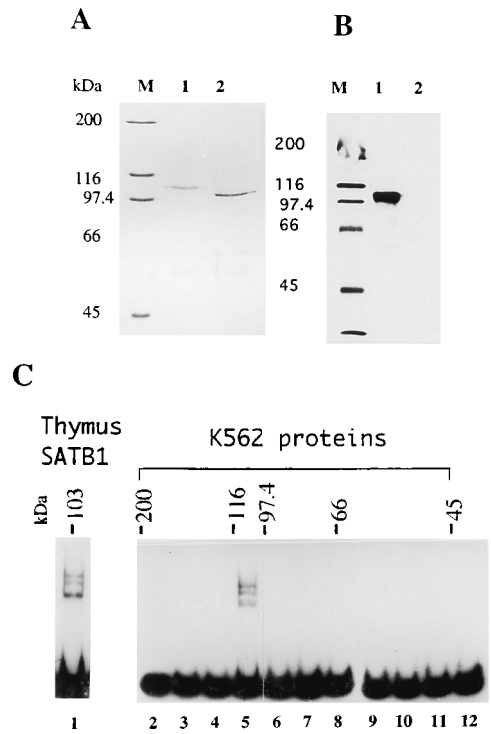


FIG. 2. Affinity purification of a 100-kDa MAR-binding protein from K562 cell extract. (A) Silver staining of an SDS–7.5% polyacrylamide gel with affinity-purified SATB1 from thymus extract (lane 1) and affinity-purified protein from K562 extract (lane 2). Size markers (lane M) are indicated in kilodaltons on the left. (B) Western blot of a duplicate gel to the one shown in panel A with anti-SATB1 antibody. (C) Mobility shift analysis with wild-type $(25)_5$ probe and affinity-purified protein, after elution and renaturation from an SDS-polyacrylamide gel. Lane 1, SATB1 from thymus; lanes 2 to 12, K562 protein eluted from gel slices ranging from 200 kDa to approximately 30 kDa. The size markers (in kilodaltons) above the lanes indicate the relative positions of the gel slices that were cut from the gel.

The MAR-binding protein is identified as nucleolin. Affinity-purified 100-kDa protein was isolated by SDS-PAGE and injected into mice to raise polyclonal antibodies, which were then used to screen a cDNA expression library from uninduced K562 cells. A total of 12 overlapping cDNA clones were obtained, ranging in size between 0.7 and 1.7 kb. Sequencing of approximately 200 bp of both 5′ and 3′ ends of each cDNA insert revealed their identity with the published sequence of human nucleolin cDNA (74). This result was confirmed by sequencing of three peptide fragments obtained by tryptic digestion of the gel-purified 100-kDa band. We therefore conclude that the MAR-binding protein from uninduced K562 cells is nucleolin.

Nucleolin selectively binds double-stranded MARs with high affinity. The sequence selectivity of nucleolin was tested by mobility shift analysis with the synthetic MAR wild-type $(25)_5$ as a probe and a non-MAR, mutated $(24)_8$, as a competitor. The mutated $(24)_8$ is a repeat of eight double-stranded units of the oligomer 5′-TCTTTAATTTCTACTG-CTTTAG AAttc-3′ and its complementary sequence 5′-TTCTAAAG -CAGTAGAAATTAAAGAgaa-3′, in which the core-unwinding element 5′-ATATAT-3′ (underlined) of the wild-type

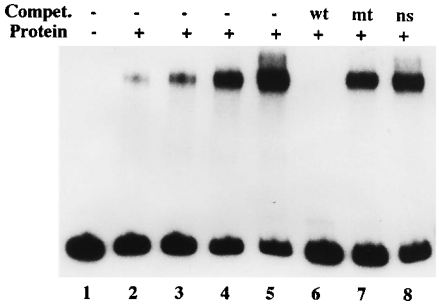


FIG. 3. Nucleolin selectively binds the wild-type MAR but not the mutated MAR. Gel retardation with wild-type $(25)_5$ probe. Nucleolin concentrations were 0, 1.5, 3, 6, and 12 ng/20 μl in lanes 1 to 5, respectively, and 12 ng/20 μl in lanes 6 to 8. Unlabeled wild-type $(25)_5$ (wt) (lane 6), mutated $(24)_8$ (mt) (lane 7), and a nonspecific 445-bp *Pvu*II fragment from Bluescript (ns) (lane 8) were added at a 200-fold molar excess as competitors (Compet.).

sequence (see the first paragraph of Results) was mutated to 5′-CTG–CT-3′ (the dash represents a base that was deleted). This mutated sequence is still A+T rich, but it lacks any of the properties of the wild type; i.e., it resisted unwinding under negative superhelical strain, it failed to bind the nuclear matrix or augment transcription (6) and it was not bound by thymus-specific SATB1 (19). When affinity-purified nucleolin was incubated with labeled wild-type $(25)_5$ and unlabeled competitor DNA, binding was completely inhibited by a 200-fold molar excess of unlabeled wild-type $(25)_5$ (Fig. 3, lane 6) but not by mutated $(24)_8$ (lane 7) or by a nonspecific sequence from the plasmid Bluescript (lane 8). Furthermore, double-stranded mutated $(24)_8$ probe (Fig. 4B, lanes 9 to 12) was not bound within the same range of nucleolin concentrations that efficiently shifted double-stranded wild-type $(25)_5$ (Fig. 4A, lanes 9 to 12). At least 10-fold-higher nucleolin concentrations were required to obtain a 10% shift of double-stranded mutated $(24)_8$ (data not shown) than were required for wild-type $(25)_5$. Because of this low binding affinity, an accurate K_d value could not be determined, but it was roughly estimated that the K_d was higher than 100 nM for the mutated probe. This strongly suggests that nucleolin is not just an A+T-rich sequence binding protein but can effectively distinguish a MAR sequence that can unwind from a non-MAR sequence that cannot unwind. These properties are very similar to those of SATB1, which specifically binds A+T-rich sequences containing exclusively A, T, and C in one strand (ATC sequences) (19).

MAR-binding proteins bind a variety of MARs from different species. This is to be expected, since MAR sequences share multiple segments with an ATC sequence context, even though there is no primary sequence consensus. We tested binding of nucleolin to various MAR fragments of human, mouse, plant, and yeast origin, using the mobility shift assay. These DNA fragments have been shown previously by different groups to specifically attach to the nuclear matrix (see reference 19 and references therein). All the double-stranded MAR fragments tested were efficiently bound by nucleolin; the K_ds ranged between 5×10^{-9} M for the yeast centromere and 2.4×10^{-8} M for the 5′ MAR of the IgH 5′ enhancer (IgH 5′-En MAR) (Table 1). SATB1 exhibited an overall higher binding affinity to the double-stranded MAR fragments tested, with K_d values ranging from 1×10^{-10} M for the IgH 5′-enhancer MAR to 1×10^{-9} M for the 3′ MAR of the IgH 3′ enhancer (IgH 3′-En MAR) and the plant MAR, ST-LS1. It is interesting that the two proteins have distinct preferences among the various MARs tested. The double-stranded fragments used in this study were made blunt ended by filling in overhangs with the

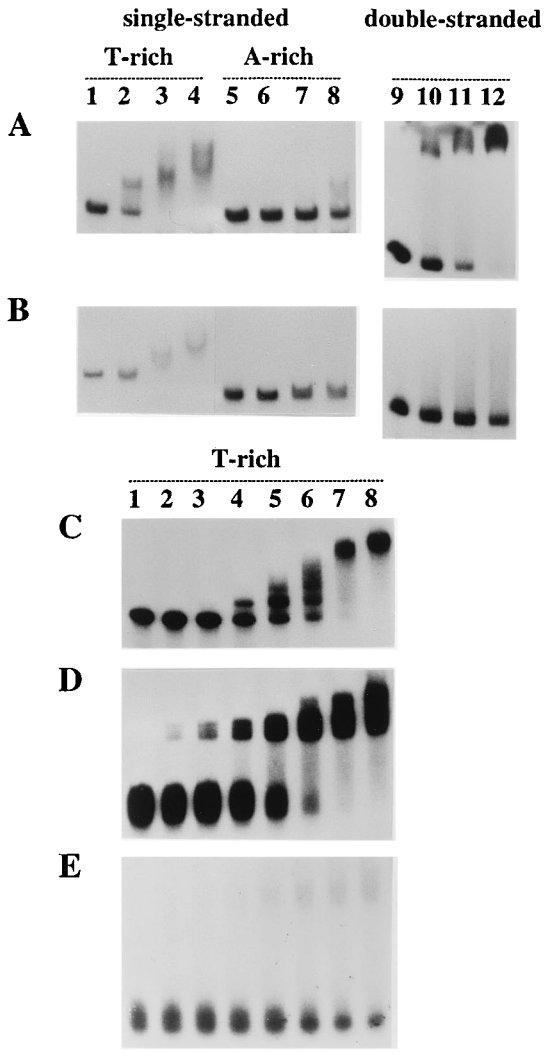


FIG. 4. Binding affinities of nucleolin to single-stranded DNA, double-stranded DNA, and RNA. (A) Wild-type $(25)_5$. (B) Mutated $(24)_8$. In panels A and B, T-rich (lanes 1 to 4) or A-rich (lanes 5 to 8) strands were incubated with 0, 0.15, 0.6, and 2.5 ng of nucleolin, and double-stranded probes were incubated with 0, 5, 10, and 20 ng of nucleolin (lanes 9 to 12). (C) U-rich strand of wild-type $(25)_5$ RNA. (D) Telomere single-stranded DNA $d(TTAGGG)_4$. (E) Telomere RNA $r(UUAGGG)_5$. Lanes 1 to 8 in panels C to E contain 0, 0.07, 0.15, 0.3, 0.6, 1.2, 2.5, and 5 ng of nucleolin, respectively.

Klenow fragment of DNA polymerase. Thus, binding to the double-stranded MARs was not due to single-stranded overhangs.

Nucleolin shows a strong preference for the T-rich single strand of the synthetic MAR probe. Since nucleolin has been shown to bind single-stranded DNA and RNA (2, 31, 34, 36, 68), we tested whether single-stranded MAR probes would be a better target than the double-stranded ones. Each strand of wild-type $(25)_5$ and mutated $(24)_8$ was end labeled separately, and the DNA was heat denatured immediately before the binding reaction. For both probes, nucleolin showed a strong preference for one strand over the other. For wild-type $(25)_5$, the strand that was bound with high affinity is distinguished by a T content of 54% versus 32% for the opposite strand (sequences are shown in Materials and Methods). For convenience, we refer to the specific strand that is preferred by nucleolin as the T-rich strand and to the other strand as the

TABLE 1. Binding affinities of purified human nucleolin and mouse thymus SATB1 to double-stranded MAR probes[a]

MAR probe	K_d (10^{-9} M) of[b]:		Nucleolin/SATB1 K_d ratio
	Nucleolin	SATB1	
CEN III	5	0.3	17
Wild-type $(25)_5$	9	0.3	30
ST-LS1 MAR	9	1	9
β-Globin MAR	10	0.3	33
H4 ARS	11	0.4	27.5
IgH 3′-En MAR	20	1	20
IgH 5′-En MAR	24	0.1	240

[a] The MAR fragments were described previously (19). Binding was analyzed by band shift experiments, and the K_d values were estimated as described in Materials and Methods.
[b] The values obtained represent means of three independent experiments.

A-rich strand. The T-rich strand of wild-type $(25)_5$ (Fig. 4A, lanes 1 to 4) was bound 45-fold more efficiently than the corresponding double-stranded DNA (Fig. 4A, lanes 9 to 12), with estimated K_d values of 2×10^{-10} and 9×10^{-9} M, respectively (Table 2). The A-rich strand was bound with lower affinity (Fig. 4A, lanes 5 to 8), approaching that for the double-stranded wild-type $(25)_5$ (compare Fig. 3, lane 3, and Fig. 4A, lane 8), and binding to the A-rich strand of mutated $(24)_8$ was not detected at the nucleolin concentrations used (Fig. 4B, lanes 5 to 8).

Nucleolin exhibited marginal sequence preference between the T-rich strand of wild-type $(25)_5$ and mutated $(24)_8$, since the T-rich strand of mutated $(24)_8$ was not shifted with the same nucleolin concentration that resulted in a 50% shift of the T-rich strand of wild-type $(25)_5$ (Fig. 4A and B, lanes 2). Moreover, at higher nucleolin concentrations a small shift was observed only for the A-rich strand of the wild-type probe but not for the A-rich strand of the mutated probe (Fig. 4A and B, lanes 8).

We also found that purified nucleolin binds a repeat of the single-stranded human telomere DNA sequence $d(TTAGGG)_4$ threefold less efficiently than it binds the T-rich strand of the wild-type MAR (Fig. 4D; Table 2). It was previously suggested that nucleolin binds human telomere DNA; however, there was no direct evidence for this when purified, undegraded nucleolin was used (36).

TABLE 2. Binding affinities of nucleolin to single-stranded DNA, double-stranded DNA, and RNA[a]

Probe	Type and strand of probe[b]	K_d (10^{-9} M)	Relative K_d[c]
Wild-type $(25)_5$	T-rich ss	0.2	1
	A-rich ss	10	50
	U-rich RNA	0.8	4
	ds	9	45
Mutated $(24)_8$	T-rich ss	0.4–1[d]	2–5[d]
	ds	>100	>500
$d(TTAGGG)_4$	ss	0.6	3
$r(UUAGGG)_5$	RNA	5	25

[a] The estimation of the K_d values and the preparation of the various forms of the probes are described in Materials and Methods.
[b] ss, single stranded; ds, double stranded. All probes are DNA unless specified otherwise.
[c] Relative to the value for T-rich single-stranded DNA for wild-type $(25)_5$.
[d] The affinity for single-stranded mutated $(24)_8$ may be overestimated, since the appearance of the shifted band was not linear, in contrast to the other probes.

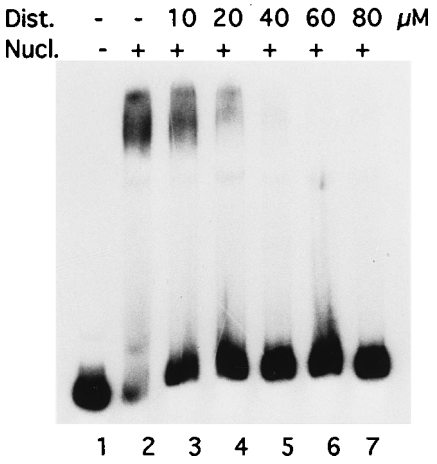


FIG. 5. Distamycin A inhibition of nucleolin binding. A mobility shift assay with double-stranded wild-type $(25)_5$ probe and 0 ng (lane 1) or 15 ng (lanes 2 to 7) of nucleolin per 20 μl was performed. The distamycin concentrations (micromolar) are indicated above the lanes. The minus sign indicates the absence of protein or distamycin; the plus sign indicates the presence of protein.

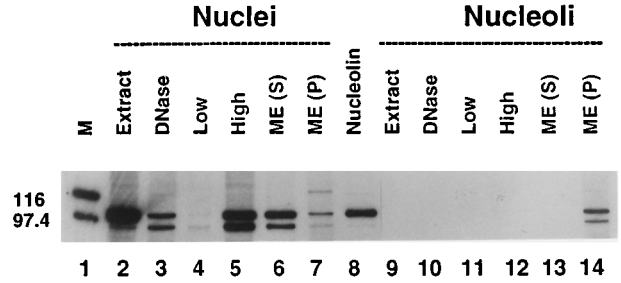
To test whether nucleolin prefers RNA over DNA, we synthesized RNA corresponding to the wild-type MAR in vitro. A similar preference of nucleolin for the U-rich strand over the A-rich strand was found with RNA (data not shown). The RNA corresponding to the U-rich strand of wild-type $(25)_5$ was bound by nucleolin with a fourfold-lower affinity than was the corresponding T-rich strand of single-stranded DNA (K_d, 0.8×10^{-9} M) (Fig. 4C; Table 2), and binding to a repeat of the RNA $r(UUAGGG)_5$, corresponding to the human telomere sequence was 25-fold less efficient than was binding to the T-rich strand of wild-type $(25)_5$ (Fig. 4E; Table 2).

In summary, these results show that nucleolin exhibits a distinct sequence preference for the double-stranded wild-type MAR over its mutated version. For single-stranded DNA, nucleolin shows a striking strand preference; however, only a marginal difference in binding between the wild-type MAR and the mutated DNA was seen for the preferred strand.

Nucleolin binds the minor groove of the double-stranded MAR. The MAR-binding protein SATB1 binds in the minor groove of DNA without extensive base contacts (19). It was therefore of interest to know whether recognition of double-stranded MARs by nucleolin also involves the minor groove. The minor groove-binding drug distamycin A was used as a competitor in band shift experiments with purified nucleolin and wild-type $(25)_5$. Distamycin A specifically binds the minor groove of A+T-rich DNA, without significantly changing the conformation of the DNA (47). Nucleolin binding to wild-type $(25)_5$ was greatly reduced even when a distamycin A concentration as low as 20 μM was used (Fig. 5, lane 4) and was completely inhibited by distamycin A concentrations of 40 μM and higher (lanes 6 and 7). This result strongly indicates that nucleolin binds the double-stranded MARs via the minor groove, similar to SATB1.

Subcellular localization of nucleolin and extractability with salt. Nucleolin was identified in the insoluble nucleolar matrix fraction by several independent groups (51, 61), but on the other hand it was also found to be released by nuclease treatment or high-salt extraction (60, 70). These results are apparently contradictory. Therefore, we determined the extractability of nucleolin from isolated nuclear matrix and nucleolar matrix in parallel and quantitated the relative amounts of total nucleolin in each extraction step. Nuclei and purified nucleoli

A



B

Fraction	% Nucleolin	
	Nuclei	Nucleoli
Extract	40	0
DNase	9	0
Low	1	0
High	24	0
ME (S)	17	0
ME (P)	9	10

FIG. 6. Nucleolin is both soluble and tightly associated with the nucleolar matrix. (A) Western blot with antinucleolin antibody and equivalent aliquots of each fraction. The starting material for the fractionation was isolated nuclei (lanes 2 to 7) or purified nucleoli (lanes 9 to 14) Lanes: 1, molecular size marker (M) (the sizes [in kilodaltons] are indicated on the left); 2 and 9, 0.4 M KCl extract (Extract); 3 and 10, supernatant after DNase I treatment (DNase), 4 and 11, supernatant after low-salt treatment (Low); 5 and 12, supernatant after high-salt treatment (High); 6 and 13, supernatant after treatment with 1% β-mercaptoethanol–1.6 M NaCl [ME (S)]; 7 and 14, pellet remaining after β-mercaptoethanol–NaCl treatment [ME (P)]; 8, affinity-purified nucleolin (50 ng per lane). (B) Relative amounts of the 100-kDa nucleolin band in each fraction.

of K562 cells were isolated and fractionated by digestion with DNase I and extraction with 1.6 M NaCl by the method of Smith and Berezney (71). The presence of nucleolin in each fraction was examined by Western blotting with mouse anti-human nucleolin polyclonal antibodies and quantitated by scanning densitometry. When nuclei were used as the starting material, approximately 40% of the total nucleolin was extracted in 0.4 M KCl extraction buffer (Fig. 6A [lane 2] and B), a minor amount (9%) was released by DNase I digestion (Fig. 6A [lane 3] and B), and it seemed almost completely resistant to low-salt extraction (Fig. 6A [lane 4] and B). About 24% was solubilized by 1.6 M NaCl treatment (Fig. 6A [lane 5] and B), but a significant level (17%) was released from the nuclear matrix when 1% β-mercaptoethanol was present in the high-salt wash (Fig. 6A [lane 6] and B). Approximately 9 to 10% remained in the insoluble pellet after β-mercaptoethanol treatment.

The same procedure was repeated with purified nucleoli as the starting material. Nucleoli were prepared from isolated nuclei by sonication and centrifugation through a sucrose step gradient as described previously (8). We included a DNase I treatment step (in 150 mM NaCl), which was shown to remove any contaminating nuclear DNA and associated proteins (8). The purified nucleoli were then further fractionated exactly as described for nuclei. It was found that nucleolin was resistant to extraction with 0.4 M KCl extraction buffer, DNase I, low-salt buffer, and high-salt buffer (Fig. 6A [lanes 9 to 12] and B). Addition of β-mercaptoethanol to the high-salt wash did not release nucleolin from the nucleolar matrix in the supernatant (Fig. 6A [lane 13] and B), in contrast to the results obtained with the nuclear matrix. Approximately 10% of the total nucleolin remained tightly associated with the insoluble pellet (Fig. 6A [lane 14] and B).

Figure 6A also shows a 95-kDa protein component which reacts with nucleolin-specific antibody in the high-salt extractions, in addition to the 100-kDa band. This protein could represent a different form of nucleolin. Two forms of nucleolin differing by 5 kDa in their apparent molecular masses have been reported previously (12), and they may reflect different states of phosphorylation.

DISCUSSION

DNA affinity purification of MAR-binding proteins. This report describes the use of a MAR affinity column for the identification and purification of MAR-binding proteins from whole-cell extracts. The column contained a concatemerized, 25-bp sequence derived from the core-unwinding element of the 3′ MAR of the IgH enhancer. The applicability of the MAR affinity column was demonstrated by purifying the known MAR-binding protein SATB1 to near homogeneity from crude mouse thymus extracts. A second MAR-binding protein of 100 kDa was purified from K562 cell extract because of its strong binding affinity to the MAR column. This protein was subsequently cloned, and it was identified as nucleolin on the basis of its cDNA sequence as well as peptide sequences of tryptic fragments. The MAR affinity column provides an efficient means for identifying MAR-binding proteins which are not detected by the commonly used DNA-binding protein blot assay or Southwestern (DNA-protein) analysis (52, 67, 78, 81). This assay system requires efficient renaturation of the binding activity of the protein after SDS-PAGE and after blotting to the membrane. Nucleolin was not detectable in a Southwestern blot assay with the double-stranded MAR probe, even after the filter was subjected to a denaturation-renaturation step with 6 M guanidine hydrochloride (data not shown). It is significant that with the same MAR affinity column, two distinct MAR-binding proteins, SATB1 and nucleolin, were identified from thymocytes and K562 cells, respectively. This demonstrates the general applicability of the MAR affinity column for the identification of different MAR-binding proteins from various sources.

Nucleolin was the major MAR-binding protein detected in 0.4 M KCl extracts from K562 cells. Although we showed that nucleolin is for the most part soluble at these salt concentrations, the presence of other, insoluble MAR-binding proteins in K562 cells cannot be excluded. It has been shown that several high-molecular-weight DNA-binding proteins were enriched in rat nuclear matrix and were not extracted by high-salt buffer (33). It therefore seems likely that several classes of MAR-binding proteins that differ according to extractability exist.

Nucleolin is an abundant nucleolar protein found in most rapidly proliferating cells, and it would also be expected to be a major MAR-binding protein in thymus extracts, together with SATB1. However, nucleolin was not found in the affinity-purified SATB1 preparation. Furthermore, only a minor amount of nucleolin was detected by Western blot analysis in thymus whole-cell extracts, obtained from 3- to 6-week-old mice, that contain a large amount of SATB1 (data not shown). We used antibody that was raised in rabbits against rat nucleo-

lin and was known to cross-react with mouse nucleolin (34, 58). This result is consistent with a previous report (62) showing that nucleolin was most abundant in tumor cell lines, solid tumors, and embryonic tissues. The level of nucleolin was substantially lower in the thymus than in tumor cell lines. This apparent low level of nucleolin in the thymus from 3- to 6-week-old mice, together with the lower MAR-binding affinity compared with SATB1, probably explains why nucleolin was not purified together with SATB1 from thymus extract.

MAR-binding activity of nucleolin. Nucleolin is thought to be a multifunctional protein (for a review, see reference 41). It presumably plays a role in the regulation of rRNA gene transcription and the assembly of ribosomes (10), and it may be important for cell growth control (3, 11, 13). Furthermore, nucleolin has been demonstrated to shuttle between the nucleus and cytoplasm, and it has been suggested that it transports ribosomal components across the nuclear envelope (9).

Consistent with a presumptive multifunctional role, nucleolin exhibits multiple nucleic acid-binding activities. Nucleolin binds specifically to RNA precursors (31, 34) and single-stranded DNA (2, 36, 68). Several other proteins were shown to have multiple binding activities; among these, TFIIIA is one of the best characterized (76). The present report demonstrates that nucleolin is also a MAR-binding protein that specifically recognizes the region with high base-unpairing potential. Only one report previously demonstrated that nucleolin can bind double-stranded DNA, with a preference for the nontranscribed spacer regions that separate the rRNA gene repeats from each other (59), but the basis for the interaction remained unknown. It is likely that the MAR-binding activity of nucleolin accounts for its interaction with spacer regions, since spacer regions are known to be tightly associated with the nucleolar matrix (8, 39, 72). The nontranscribed spacer region of rat ribosomal DNA (83) contains A+T-rich subregions represented by a cluster of ATC sequences (our unpublished observation). Clustering of ATC sequences is commonly found in MARs and was previously demonstrated to be important for SATB1 recognition (19). It has been proposed that ribosomal gene repeats are organized in loops, attached to the matrix by MARs located in the nontranscribed spacer regions (72). This could be similar to the loop organization of the histone gene cluster, where the attachment regions are also found in the nontranscribed spacer regions (55). The binding specificity of nucleolin for double-stranded MARs is very similar to that of the previously cloned MAR-binding protein SATB1 (19). Both proteins are also inhibited by distamycin A, indicating a minor groove recognition. SATB1 exhibits an overall higher affinity than nucleolin, whereas nucleolin shows a more distinct binding preference among the MARs tested than SATB1. SATB1 also binds certain MARs preferentially over others, but the distinction is less apparent in gel shift experiments, probably because of its very high affinity to MARs. These differences in affinity suggest that the natural target sites in vivo might be distinct for the two proteins and that they might differ in their functional roles.

Nucleolin shows strand specificity for the single-stranded synthetic MAR. We determined K_d values for nucleolin binding to the single-stranded forms of the wild-type and mutated MARs and for the corresponding RNA, as well as for human telomere DNA and RNA. The single-stranded telomere repeat was previously shown to associate with the nuclear matrix and therefore represents a MAR (18). Single-stranded DNA and RNA are bound with substantially higher affinity than are the double-stranded forms, and single-stranded DNA is preferred over RNA. This is similar to the major nuclear matrix proteins lamins B and C and matrins D, E, F, G, and 4, which reportedly showed a preference for binding single-stranded DNA over RNA and double-stranded DNA (33). Our results also show that purified, mostly undegraded nucleolin binds telomeric single-stranded DNA preferentially over telomeric RNA. A previous report showed that a proteolytic fragment of nucleolin was present within a group of purified proteins that bound a pre-mRNA splice site (UUAG/G) and the human telomeric sequence $(TTAGGG)_n$ with a preference for RNA over DNA (36). However, binding was not tested with purified, undegraded nucleolin.

It is demonstrated here that nucleolin has a strong preference for the T-rich strand of the synthetic MAR over the A-rich strand. For convenience, we designated each strand as T and A rich, respectively, although it is not yet known whether it is the T content per se that confers the high binding affinity to nucleolin. The precise mechanism underlying the strand specificity of nucleolin awaits further characterization. Nevertheless, this strand specificity may be very similar to that of the yeast proteins PUB1, PAB1 (14), and ssARS-T-binding protein (69), which specifically bind the T-rich strand of the autonomous replication sequence consensus, and to replication protein A from *Saccharomyces cerevisiae* and humans, which preferentially binds to the pyrimidine-rich strand of a homologous origin of replication (44). These similarities in binding specificities might suggest that nucleolin has functions in common with these proteins, although its precise role in vivo remains to be established. Replication protein A has been demonstrated to be essential for replication (44), but the biological significance of PUB1 is not clear (see reference 14 and references therein).

Nucleolin may be a new type of MAR-binding protein that is also an RNA-binding protein. The MAR-binding proteins SAF-A (24), p120 (80), and SP120 (77) were recently identified as hnRNP U, which was shown to bind to MARs. However, nucleolin appears to share more similarity with the yeast autonomous replication sequence consensus binding proteins, as described above, than with hnRNP-U, which binds double-stranded DNA with higher affinity than it binds single-stranded DNA and preferentially binds to poly(A) and poly(G) ribopolymers but not to poly(U).

Nucleolin is present both as a nucleolar matrix component and as a soluble protein. The cellular fractionation and salt extraction experiments were undertaken because MAR-binding proteins are commonly expected to be integral components of the nuclear matrix and therefore should be insoluble in high-salt buffers or detergent. Consistent with this, the MAR-binding proteins ARBP (81), SAF-A (67), and SATB1 (our unpublished results) were identified in the nuclear matrix fraction. However, these proteins were also detected in 0.4 to 0.5 M salt extracts, indicating that MAR-binding proteins can be soluble as well. In the present report we show that some nucleolin is tightly associated with the insoluble nucleolar matrix and cannot be extracted with β-mercaptoethanol but that a major portion of total nucleolin is salt soluble. Moreover, a small portion is solubilized by β-mercaptoethanol and high-salt treatment of nuclear matrix, strongly indicating that it is associated with the nuclear matrix. Previous reports showed that the nuclear matrix can be solubilized with β-mercaptoethanol and high salt (43), while the nucleolar matrix is resistant to extraction with sulfhydryl reagents (25). Thus, nucleolin is similar to other known MAR-binding proteins in that it is both salt soluble and matrix associated. This result, together with previous observations, demonstrates that MAR-binding proteins do not have to be restricted exclusively to the insoluble nuclear matrix but can be partitioned between various components of the nucleus. This is consistent with the notion that attachment

to the nuclear matrix may be dynamic; for example, it was shown that promoter elements of the bone-specific osteocalcin gene are attached to nuclear matrix proteins only during the time the gene is turned on in immature cells (5). These results suggest that nucleolin not only may function in the control of rRNA transcription and ribosome assembly in the nucleolus but may also be involved in processes taking place outside the nucleolus.

ACKNOWLEDGMENTS

This work was supported by grant ROI CA 39681 from NIH (to T.K.-S.) and an American Cancer Society Faculty Award (to T.K.-S.).

We thank J. M. Cunningham and A. W. Nienhuis for sharing unpublished data and Mark Olson for kindly providing rabbit anti-rat nucleolin antibody.

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