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The C-Terminal Domain of Nucleolin Accelerates Nucleic Acid Annealing†

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ABSTRACT: We report that the abundant nucleolar protein nucleolin accelerates nucleic acid annealing. Nucleolin accelerates annealing of complementary oligonucleotides and of oligonucleotides that contain a limited number of mismatches. The annealing activity of nucleolin can be localized to a C-terminal region consisting of two RNA binding domains (RBD3 and RBD4) and the RGG_9 domain (RBD3-RBD4-RGG_9). This same region mediates self-association of nucleolin. The RGG_9 domain of nucleolin, believed to mediate interactions between nucleolin and several ribosomal proteins, is neither sufficient for self-association, as determined by small-angle X-ray scattering, nor can it independently accelerate annealing. Acceleration of nucleic acid annealing by nucleolin is likely to depend on self-association of nucleolin molecules bound to nucleic acid.

In proliferating cells, the ribosomal DNA (rDNA)[1] and the proteins that associate with it comprise a specialized nuclear structure, the nucleolus [reviewed in refs (*1*−*6*)]. Both rDNA transcription and rRNA biogenesis occur within the nucleolus, and this nuclear compartment is also involved in other aspects of RNA transport and cell cycle regulation.

The most abundant protein in the nucleoli of vertebrate cells is nucleolin. Nucleolin is a highly conserved polypeptide composed of an unusual grouping of sequence and structural motifs (*7*). In the N-terminus there are several long and uninterrupted runs of acidic residues, as well as multiple sites for phosphorylation by both cdc2 kinase and nucleolar casein kinase CKII (*8*, *9*). The C-terminus contains four RNA binding domains (RBDs), and nine repeats of the tripeptide motif arginine-glycine-glycine (RGG_9). Both RBD domains and RGG repeats are found in a number of proteins that interact with single-stranded nucleic acids.

Consistent with the complex organization of the nucleolin polypeptide, biochemical and cell biological analyses support the view that this protein carries out multiple functions within the nucleolus [reviewed by (*5*, *6*, *10*)]. High-affinity interactions of nucleolin with G-G-paired DNAs can be mapped to a domain comprised of RBD3-RBD4-RGG_9 (K_D = 0.5 nM) and to the isolated RGG_9 domain (K_D = 3 nM) (*11*), suggesting that nucleolin may function as an architectural factor during transcription of the G-rich rDNA nontemplate strand. Nucleolin also binds to a site in pre-rRNA that is targeted for the first cleavage during rRNA processing, in an interaction that involves RBD1 and RBD2 of nucleolin (*12*−*15*). In addition, nucleolin interacts with a subset of ribosomal proteins (*14*). The RGG_9 domain appears to be involved in some of these interactions, and is sufficient for interactions with ribosomal protein L3. The RGG_9 domain also mediates nonspecific interactions of nucleolin with nucleic acids (*16*).

The rDNA of most eukaryotes is organized into tandem arrays, each of which contains multiple copies of the rDNA repeat. Each repeat unit includes the regions that template mature 18S, 5.8S, and 28S rRNAs, as well as transcriptional regulatory elements and external and internal transcribed spacer regions. In the course of normal cell proliferation, mutations could in principle accumulate within the rDNA that alter elements essential for rDNA transcription, pre-rRNA processing, or rRNA structure. Recombination of the rDNA is thought to prevent accumulation of mutations by ensuring that repeat sequences remain homogeneous. In *S. cerevisiae*, rDNA recombination is dependent upon the conserved *RAD52* gene (*17*−*21*). Our laboratory has recently found that, in mammalian cells, Rad52 is predominantly localized to the nucleolus during S phase (*22*), suggesting that mammals, like *S. cerevisiae*, use a Rad52-dependent pathway for rDNA recombination. In addition, we have identified nucleolin as one component of the heterodimeric factor LR1 (*23*, *24*). LR1 is a transcriptional regulator specific to activated B cells (*25*−*27*) that also appears likely to function in immunoglobulin heavy chain class switch recombination (*27*−*30*). These results have prompted us to analyze in greater detail properties of nucleolin that might contribute to function in recombination.

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[1] Abbreviations: RBD, RNA binding domain; RGG, arginine-glycine-glycine; rDNA, ribosomal DNA; MBP, maltose binding protein; GST, glutathione *S*-transferase; ssDNA, single-stranded DNA; SAXS, small-angle X-ray scattering; BSA, bovine serum albumin.

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Here we report that nucleolin accelerates the annealing rate of single-stranded DNA (ssDNA), and is capable of self-association. By deletion mapping, we localize the annealing activity of nucleolin to a C-terminal region consisting of RBD3-RBD4-RGG_9. Self-association is mediated by this same region and requires the RGG_9 domain. Although RGG-rich regions have in some cases been shown to mediate protein/protein interaction (*14*, *31*), we find, using small-angle X-ray scattering, that the RGG_9 domain of nucleolin cannot self-associate. These results taken together suggest that self-association of nucleolin molecules bound to nucleic acids provides a plausible mechanistic basis for acceleration of nucleic acid annealing by nucleolin.

MATERIALS AND METHODS

Plasmid Construction. The plasmid pNuc-1,2,3,4-RGG_9 [construction of which was described in ref (*11*) where it was referred to as pMalNuc] carries human nucleolin residues 284−709, including all 4 RBDs and the RGG_9, fused at the N-terminus to *E. coli* maltose binding protein (MBP). This plasmid was the backbone for construction of a series of deletion mutants expressed as MBP fusion proteins, as described in detail elsewhere (*11*). pGST-1,2,3,4-RGG_9, a fusion construct which expresses nucleolin (residues 284−709) fused at the N-terminus to glutathione *S*-transferase (GST), was constructed by transfer of the *Eco*RI nucleolin insert from pNuc-1,2,3,4-RGG_9 into the pGST vector (Clontech) digested with *Eco*RI. As we have previously reported (*23*), the N-terminus of nucleolin could not be expressed in *E. coli*, and it was therefore not included in the recombinant nucleolin constructs.

Protein Purification. Full-length (106 kDa) murine nucleolin was purified starting with nuclear extract prepared from PD31 pre-B cells and chromatographed on heparin agarose resin as described (*24*). Fractions containing nucleolin were identified at this and subsequent steps by blotting with anti-nucleolin antibodies (*23*) and purified as previously described (*11*). All recombinant proteins were produced by overexpression and purified as previously described (*11*, *23*). All purified fusion proteins were visualized as single species on SDS−PAGE. Protein concentrations were determined by Bradford microassay (BioRad).

DNA Annealing Analysis. DNA annealing assays were carried out in 15 μL reactions containing 10 mM HEPES, pH 7.5, 150 mM KCl, 1 mM EDTA, 1 mM spermidine, 5% glycerol, 0.1% NP40, 0.17 mg/mL BSA, 1 nM purified or recombinant protein, 1 fmol of ^{32}P-labeled DNA, and 4 fmol of unlabeled complementary DNA strand. Reactions were incubated at 37 °C for the times indicated, and DNA was resolved by electrophoresis on 6% polyacrylamide (29:1 acrylamide:bis-acrylamide), 1 × TBE (100 mM Tris−borate, 1 mM EDTA, pH 8.2) gels at 5 V/cm for 3 h at 4 °C. DNA renaturation was quantitated by phosphoimager analysis of the dried gels. Percent reannealing was calculated as the fraction of labeled DNA that had formed duplex during the reaction.

Oligonucleotides used for annealing analysis were the following:

M11, GATCCGAGCTGGGCTAGGCTGAGC-TGAGCTAAACTA;

M12, GATCTAGTTTAGCTCAGCTCAGCC-TAGCCCAGCTCG;

TOP-0, GATCCGGTACACCTGTCAAACGG-TAACGCAGGTGTC;

TOP-2, GATCCGGTACACCTTTCAAACGG-TACCGCAGGTGTC;

TOP-8, GATCCGTTACCCCTTTCACACGTT-AAAGCATGTGGC;

BOT-0, GATCGACACCTGCGTTACCGTTT-GACAGGTGTACCG;

BOT-4, GATCGACACCTTCGTTACCTTTT-GACATGTGTACAG

The M11 and M12 oligonucleotides were derived from the immunoglobulin Sα switch region. TOP and BOT oligonucleotides were derived from the murine rDNA sequence and were designed to result in 0, 4, 6, 8 or 12, mismatches when TOP series oligonucleotides are annealed to BOT series oligonucleotides (mismatch positions are underlined in the sequences above).

Nucleolin Homooligomerization Analysis. Beads carrying either GST or GST-nucleolin fusion protein (GST-1,2,3,4-RGG_9) were prepared by washing 30 μL of glutathione agarose beads (Sigma) 5 times with 1 mL of TBS (10 mM Tris, pH 7.9, 60 mM NaCl), followed by incubation with lysate from bacterial cells overexpressing GST-1,2,3,4-RGG_9 or GST containing micrococcal nuclease (90 units/mL) and 15 mM $CaCl_2$. After incubation for 30 min at 4 °C with gentle agitation, excess lysate was removed, and the beads were washed 3 times with 1 mL of TBS containing 0.1% Tween-20 (TBST). To assay nucleolin/nucleolin interactions, purified recombinant MBP-nucleolin fusion proteins were incubated with beads bound to GST or to GST-1,2,3,4-RGG_9 for 1 h at 4 °C. The supernatant was removed, the beads washed 3 times with 1 mL of TBST, and bound proteins were eluted by boiling in SDS−PAGE loading buffer. Proteins were resolved on a 4−15% gradient Phast Gel (Pharmacia), transferred to nylon membranes, and visualized by blotting using anti-MBP antibodies (NEB) and the ECL detection system (Amersham), as described in the ECL manual.

Small-Angle X-ray Scattering (SAXS) Measurements and Data Analysis. The SAXS instrument and experimental methods were as previously described (*32*). Briefly, the X-ray source was a Rigaku RU-300 rotating anode generator operating at 50 kV and 180 mA producing 1.5 Å characteristic Cu-K_α radiation selected by a pyrolitic graphite monochromator. The beam was pinhole collimated with an incident beam diameter of 1.2 mm. The detector was a two-dimensional multiwire detector with 256 × 144 pixels and a sensitive area of 290 × 288 m^2. The sample-to-detector distance was 2.3 m. This enabled a Q range from 0.01 to 0.35 $Å^{-1}$ to be covered, where $Q = 4\pi \sin(\theta/2)/\lambda$ is the magnitude of the scattering vector, θ is the scattering angle, and λ is the wavelength of the X-rays. Protein samples were in Buffer L with 150 mM NaCl or 800 mM NaCl as specified; concentrations are indicated, and all measurements were at 25 °C. The molecular weight and the radius of gyration, R_g, were obtained by Guinier approximation:

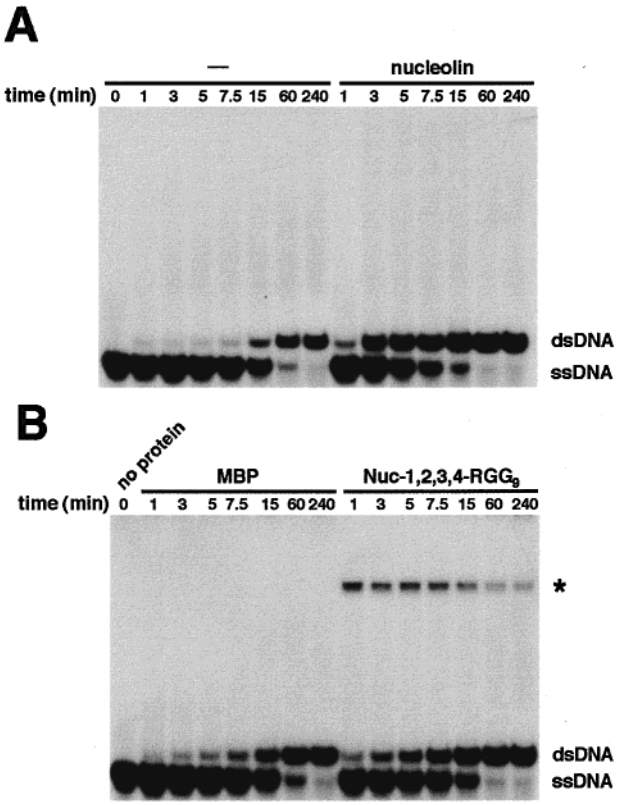

FIGURE 1: Nucleolin accelerates DNA annealing. (A) Kinetics of duplex DNA formation in the presence (right) or absence (left) of purified murine nucleolin (1 nM), analyzed by native gel electrophoresis. Duplex formation by oligonucleotides M11 (^{32}P-labeled) and M12 is evident. Mobilities of ssDNA and dsDNA are indicated. (B) Kinetics of duplex DNA formation in the presence of MBP (1 nM) or of the recombinant MBP-nucleolin fusion protein, Nuc-1,2,3,4-RGG$_9$ (1 nM). The asterisk (*) denotes a complex of Nuc-1,2,3,4-RGG$_9$ bound to ssDNA. Conditions and symbols as in panel A.

$I(Q) = I(0) \exp^{-1/3}Q^2R_g^2$, where $I(Q)$ is the scattered intensity at a certain Q value and $I(0)$ is the forward scattered intensity and is related to the protein molecular weight by $I(0) = Kc/Mw$, where Kc is related to the instrument configuration, transmission of the X-rays by the sample, the partial specific volume of the protein, and the electron density contrast of the protein. The factor Kc can be calibrated by a protein molecular weight standard, BSA, dissolved in the same buffer as Nuc-RGG$_9$.

RESULTS

Nucleolin Accelerates Annealing of Complementary Oligonucleotides. The ability of nucleolin to stimulate nucleic acid annealing was assayed using nucleolin purified from the murine pre-B cell line PD31. Annealing was assayed in standard conditions (see Materials and Methods) using ^{32}P-labeled oligonucleotide M11 (66 pM) and unlabeled oligonucleotide M12 (270 pM). M11 and M12 are synthetic 36-mers which anneal to form a 32 bp duplex with 5′ overhangs. In the absence of added nucleolin, formation of duplex was first evident at about 7.5 min; the reaction neared completion at about 60 min and was complete within 4 h (Figure 1A). Addition of murine nucleolin (1 nM) stimulated annealing, so that formation of duplex was first evident by 1 min, about half the labeled oligonucleotide was duplex by 5 min, and the reaction was complete before 60 min.

To determine whether the observed annealing activity was affected by posttranslational modification, annealing was assayed in reactions which contained either recombinant *E. coli* maltose binding protein (MBP), or Nuc-1,2,3,4-RGG$_9$ (1 nM), which is MBP fused to nucleolin residues 284−709, including all 4 RBDs and the RGG$_9$ domain. Kinetic analysis showed that MBP did not significantly enhance the annealing rate, but that Nuc-1,2,3,4-RGG$_9$ significantly accelerated annealing (Figure 1B).

Mobility shift analysis of the annealing reactions containing Nuc-1,2,3,4-RGG$_9$ revealed a highly retarded complex at early time points. This complex was due to interaction of the recombinant protein with the single-stranded oligonucleotide, and essentially represents a "gel shift" caused by the formation of a stable complex of labeled DNA and Nuc-1,2,3,4-RGG$_9$. The fraction of single-stranded oligonucleotide bound by recombinant Nuc-1,2,3,4-RGG$_9$ decreased with increasing reaction times, suggesting that duplex formation resulted in the release of DNA from Nuc-1,2,3,4-RGG$_9$. No protein/DNA complex was evident in assays of annealing by endogenous nucleolin. This may be a result of the rapid annealing kinetics observed with the endogenous nucleolin. Additionally, the endogenous nucleolin might dissociate from ssDNA more rapidly than recombinant protein, so that a protein/DNA complex cannot be readily visualized in a gel mobility shift. An alternative explanation, that binding of purified murine nucleolin to ssDNA is rate-limiting for annealing, is made unlikely by the observation that annealing is more rapid in reactions containing endogenous nucleolin than in reactions containing recombinant Nuc-1,2,3,4-RGG$_9$.

These results show that nucleolin stimulates the rate of annealing of complementary sequences approximately 4-fold. Stimulation is comparable by purified endogenous nucleolin, and a fusion protein expressing the four RBDs and the RGG$_9$ domain (RBD1-RBD2-RBD3-RBD4-RGG$_9$).

Nucleolin Accelerates Annealing of Mismatched Sequences. To determine whether nucleolin can assist in the annealing of sequences which carry mismatched bases, a series of 36-mer oligonucleotides were synthesized carrying 0, 2, 4, 6, 8, and 12 evenly spaced mismatches within a 32 bp region of complementarity. Annealing of these oligonucleotides was then analyzed in the presence of nucleolin purified from murine pre-B cells, in reactions which contained 70 pM labeled oligonucleotide and 4-fold excess of the complementary strand. Figure 2A shows that, under these conditions, about 80% of the labeled oligonucleotide annealed in a 4 h reaction carried out in the presence of BSA as carrier, but no added nucleolin. As expected, at higher levels of unlabeled complementary strand the reaction kinetics were more rapid and annealing reaches 100% (data not shown). Addition of purified murine nucleolin accelerated the rate of annealing of synthetic oligonucleotides which were 100% complementary, consistent with results presented in Figure 1. Strikingly, nucleolin greatly accelerated the rate of formation of duplexes in which there were either 4 or 6 mismatches (Figure 2A). In the presence of nucleolin, annealing of these mismatched oligonucleotides was rapid, and the annealing reaction approached a maximum essentially as rapidly as did annealing of a perfect duplex. In contrast, formation of duplexes by the mismatched oligonucleotides occurred very slowly in the absence of nucleolin (Figure 2A).

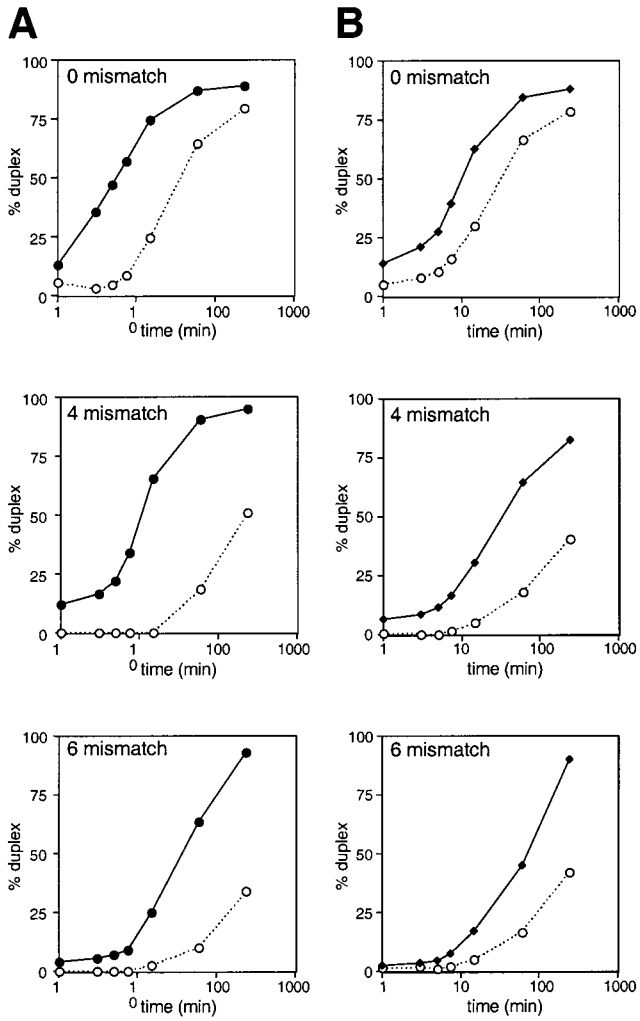

FIGURE 2: Nucleolin can stimulate annealing of duplexes containing mismatches. (A) Kinetics of annealing of mismatched oligonucleotides in reactions containing nucleolin purified from PD31 murine pre-B cells. Synthetic oligonucleotides were designed to form duplexes which contained 0, 4, or 6 mismatches. Duplex formation was analyzed by native gel electrophoresis, and the fraction of DNA that had become duplex was determined by phosphorimaging. Filled circles, purified endogenous nucleolin (1 nM); open circles, no protein. (B) Kinetics of annealing of mismatched oligonuncleotides in reactions containing the recombinant MBP-nucleolin fusion protein, Nuc-1,2,3,4-RGG$_9$. Filled diamonds, Nuc-1,2,3,4-RGG$_9$ (1 nM); open circles, MBP (1 nM). Conditions as in panel A.

Annealing experiments with oligonucleotides carrying 8 and 12 mismatches resulted in no detectable duplex DNA formation in the presence or absence of protein (data not shown).

The ability of recombinant Nuc-1,2,3,4-RGG$_9$ to accelerate annealing was assayed in reactions with the same series of oligonucleotides (Figure 2B). Because Nuc-1,2,3,4-RGG$_9$ was expressed as an MBP fusion protein, its ability to accelerate annealing was compared to that of MBP. Nuc-1,2,3,4-RGG$_9$ accelerated annealing of mismatched oligonucleotides, although not as effectively as endogenous nucleolin. It is possible that annealing was slowed by the stable interactions between Nuc-1,2,3,4-RGG$_9$ and the DNA substrate evident in the gel mobility shift in Figure 1.

Annealing activities of endogenous and recombinant nucleolin are compared in Table 1. As is evident from these data, both endogenous and recombinant protein enhanced annealing of not only perfect but also mismatched DNA duplexes. Even though both purified murine nucleolin and Nuc-1,2,3,4-RGG$_9$ accelerated the rate of duplex formation by mismatched oligonucleotides, the destabilizing effects of the incorporated mismatches were still observed as an overall decrease in duplex DNA formation.

Table 1: Purified and Recombinant Nucleolin Stimulate Annealing of Duplexes Containing Mismatches[a]

mismatches	−	MBP	nucleolin	Nuc-1,2,3,4-RGG$_9$
0	24.4	29.8	74.3	62.3
4	0	4.63	65.1	30.1
6	2.2	4.67	25.0	16.7

[a] Synthetic oligonucleotides were designed to form duplexes which contained 0, 4, or 6 mismatches. Duplex formation was analyzed by native gel electrophoresis, and the fraction of DNA that had become duplex at 15 min was determined by phosphorimaging. This time point was in all cases within the linear range of the reaction for 1 nM nucleolin.

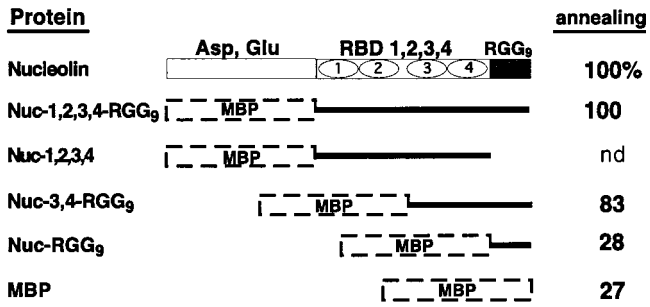

FIGURE 3: DNA annealing by deletion mutants of nucleolin. The top line shows a schematic diagram of nucleolin showing the acidic N-terminal domain, the central domain containing the 4 RBDs, and the C-terminal RGG$_9$ domain. Subsequent lines diagram deletion mutants, showing the regions of the nucleolin polypeptide that are included in each expression construct. All recombinant proteins were expressed as fusions to the *E. coli* maltose binding protein (MBP), represented as a box within dashed lines. Annealing reactions were carried out for 20 min at 37 °C using 1 nM protein. Values at right indicate the acceleration of annealing relative to endogenous nucleolin. Annealing by Nuc-1,2,3,4 could not be measured because this protein formed a stable complex with DNA which inhibited duplex formation, indicated by nd (not determined).

Stimulation of Annealing by Nucleolin Requires the C-Terminal Region Comprised of the RBD3-RBD4-RGG$_9$ Domains. To determine which domains of nucleolin are required for annealing of complementary sequences, deletion mutants were expressed as recombinant MBP fusion proteins and assayed for the ability to stimulate annealing (Figure 3). The annealing activity of each protein was expressed relative to a comparable reaction containing nucleolin purified from murine pre-B cells.

As seen in Figures 1B and 3, stimulation of annealing of perfect duplexes by purified murine nucleolin and Nuc-1,2,3,4-RGG$_9$ was comparable. The highly charged N-terminal domain of nucleolin therefore does not contribute substantially to the ability of nucleolin to stimulate annealing. Deletion of the RGG$_9$ domain to yield Nuc-1,2,3,4 resulted in such a strong increase in ssDNA binding at all protein concentrations assayed that it was not possible to measure stimulation of annealing. The annealing activity of Nuc-3,4-RGG$_9$ was comparable to that of Nuc-1,2,3,4-RGG$_9$, indicating that RBDs 1 and 2 are not essential for the observed annealing activity. The RGG$_9$ domain alone (Nuc-RGG$_9$) did

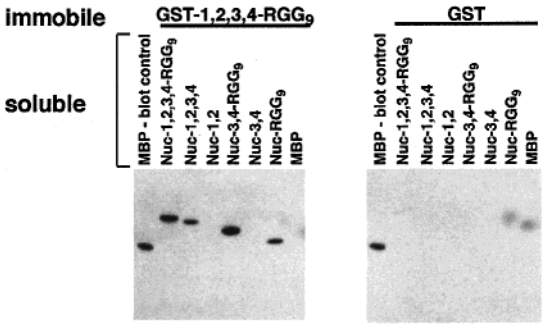

FIGURE 4: The 4-RBD and RGG_9 domains of nucleolin can mediate nucleolin self-association. Western blot to identify deletion mutants of nucleolin bound to GST-nucleolin resin. Nucleolin deletion mutants were allowed to interact with protein immobilized on the affinity resin, and bound protein was resolved on SDS−PAGE and identified by western blotting with anti-MBP antibodies.

not stimulate DNA annealing. Nuc-3,4-RGG_9 therefore represents the minimal nucleolin sequence capable of stimulating the annealing of complementary sequences.

The data summarized in Figure 3 show that the RBDs of nucleolin play an important role in the observed accelerated rate of duplex formation. These data further show that although the RGG_9 domain of nucleolin alone does not affect the rate of duplex formation by complementary sequences, this domain does play a role in the observed stimulation of ssDNA annealing, because this region is essential for dissociation of nucleolin from DNA.

Two Domains of Nucleolin Contribute to Nucleolin−Nucleolin Self-Association. Nucleolin interacts nonspecifically with single-stranded nucleic acid, and this interaction is mediated by the RGG_9 domain (*16*, *33*, *34*). Thus, one possible mechanism for the observed acceleration of DNA annealing postulates that duplex formation is driven by protein/protein interactions between nucleolin molecules bound to DNA. We set out to test this model by asking if nucleolin can self-associate in a "pull-down" assay. GST-tagged nucleolin (GST-1,2,3,4-RGG_9, which carries RBDs 1, 2, 3, and 4 and the RGG_9 domain) was bound to glutathione resin and assayed for interaction with purified MBP-nucleolin chimerae. The MBP-nucleolin fusion proteins were incubated with GST-1,2,3,4-RGG_9-beads or GST-beads, followed by elution of the bound protein and detection by immunoblotting using anti-MBP antibodies to detect the presence of the MBP tag.

As shown in Figure 4, GST-1,2,3,4-RGG_9 interacted well with both Nuc-1,2,3,4-RGG_9 and Nuc-3,4-RGG_9, and to a lesser extent with Nuc-1,2,3,4 and Nuc-RGG_9. Neither Nuc-1,2, Nuc-3,4, nor MBP interacted with GST-1,2,3,4-RGG_9. In control reactions, resin bearing GST alone was shown not to retain the MBP-chimerae (Figure 4, right). To rule out the possibility of nucleic acid mediated tethering of proteins, the lysates used in Figure 4 were treated with micrococcal nuclease. Additionally, pull-down experiments carried out in the presence of ethidium bromide, which by virtue of its ability to alter nucleic structure upon interacalation, can eliminate nonspecific protein/nucleic acid interactions (*35*). Concentrations of ethidium bromide as high as 0.8 mM had no effect on interactions (data not shown), verifying that the results shown in Figure 4 were not the result of tethering.

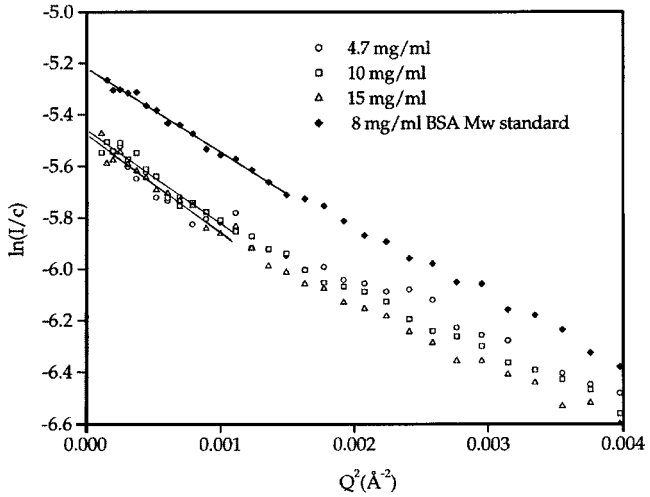

FIGURE 5: Guinier plot of SAXS analysis. Nuc-RGG_9 solutions were measured at 4.7, 10, and 15 mg/mL, respectively. At these concentrations, the intermolecular weight interaction effect was negligible. BSA was measured at 8 mg/mL. Previous SAXS measurements have shown that for concentrations of BSA between 1 and 10 mg/mL the intermolecular interaction efect is small and can be ignored. The BSA-calibrated molecular mass of Nuc-RGG_9 was 52 000 ± 7000 daltons, indicating that the protein was a monomer. The radius of gyration was 33.5 ± 0.5 Å.

We conclude that the domain required for efficient self-association is RBD3-RBD4-RGG_9. This is the same minimal domain active in annealing.

The RGG_9 Domain of Nucleolin Cannot Self-Associate. The isolated RGG_9 domain of nucleolin was capable of interacting with Nuc-1,2,3,4-RGG_9 (Figure 4). As it has previously been reported that the RGG_9 domain of nucleolin can mediate interactions between nucleolin and ribosomal protein L3 (*14*), it was important to determine whether nucleolin self-association could occur via RGG_9−RGG_9 interactions. We therefore performed small-angle X-ray scattering (SAXS) on recombinant Nuc-RGG_9 to examine self-association of the RGG_9 domain. Figure 5 shows the Guinier plot of data generated by SAXS analysis of BSA (known to be monomeric), and of Nuc-RGG_9 at concentrations ranging from 4.7 to 15 mg/mL, in 150 mM NaCl. The BSA-calibrated molecular mass of Nuc-RGG_9 was 52 000 ± 7000 daltons, indicating that the protein was a monomer. Similar results were obtained in low salt (150 mM NaCl, shown) and high salt (800 mM NaCl, data not shown). These data rule out the possibility that nucleolin self-association is mediated by RGG_9−RGG_9 interactions. As the RGG_9 domain is clearly critical for self-association of nucleolin (Figure 4) but does not interact with itself, it must interact with a region of nucleolin that is contained within the four RBDs.

DISCUSSION

Our results show that nucleolin accelerates DNA annealing. This property localizes to a C-terminal region consisting of RBD3-RBD4-RGG_9. The effects of nucleolin were particularly striking in assays that measured annealing of oligonucleotides that were not perfectly base-paired duplexes. In standard reaction conditions, nucleolin accelerated annealing of oligonucleotides differing by 4 or 6 mismatches by a factor of 10-fold or more.

The C-Terminal Domain of Nucleolin Accelerates Annealing. The minimal domain of nucleolin capable of accelerating annealing is the C-terminal region consisting of RBD3-RBD4-RGG_9. The RBDs alone do not accelerate annealing; instead, they become bound to single strands and prevent duplex formation. The RGG_9 domain has been shown to mediate nonspecific interactions of nucleolin with nucleic acids (*16*), but does not by itself accelerate annealing. These observations, along with the observation of nucleolin self-association (Figure 4), provide a basis for a working model for the mechanism by which nucleolin accelerates annealing. The first step in annealing would involve binding of single-stranded nucleic acid by the RGG_9 domain. In the next step, protein interactions between nucleolin molecules bound to nucleic acid would increase the frequency of encounters between strands, allowing annealing to occur.

Nucleolin as a Nucleic Acid Chaperone. The domain of nucleolin that accelerates annealing, RBD3-RBD4-RGG_9, is distinct from the RBD1-RBD2 domain that is involved in nucleic acid recognition at the first cleavage step of rRNA processing (*12, 13, 15, 36*). It is possible that annealing may contribute to nucleolin function in rRNA processing at the first cleavage step, for example, by stimulating interactions within the pre-rRNA, or between the pre-rRNA and the snoRNAs involved in rRNA biogenesis. In this reaction, the ability to promote annealing of mismatched sequences might enable nucleolin to function as a chaperone that transiently alters nucleic acid structure.

Our experiments point to an unanticipated property of nucleolin that is essential to its ability to accelerate annealing: its ability to *dissociate* from ssDNA. This appears to be mediated by the RGG_9 domain, as Nuc-1,2,3,4-RGG_9 accelerated DNA duplex formation; while the derivative without the RGG_9 domain (Nuc-1,2,3,4) formed a stable protein/DNA complex that prevented formation of a free DNA duplex (Figure 3). In the intact polypeptide, the high-affinity ssDNA binding activity of the four RBDs (Nuc-1,2,3,4) may be modulated by the acidic N-terminal region in addition to the RGG_9 domain, as a *myc*-tagged nucleolin derivative, containing the N-terminal acidic domains but lacking the C-terminal RGG_9 domain, failed to demonstrate nucleic acid binding in Southwestern blotting (*37*). Alternatively, the apparent deficiency in binding in those experiments may reflect poor renaturation of the recombinant protein following denaturation by SDS−PAGE and transfer to nitrocellulose.

Differences in DNA dissociation were also evident in comparisons of endogenous nucleolin with recombinant, bacterially produced Nuc-1,2,3,4-RGG_9. Nucleolin purified from murine pre-B cells did not form a detectable stable protein/DNA complex under the conditions of our assay (Figure 1A), while Nuc-1,2,3,4-RGG_9 expressed in *E. coli* did (Figure 1B). As shown in Figure 1, the kinetics of annealing were slower in the presence of the recombinant Nuc-1,2,3,4-RGG_9 than in the presence of the purified murine nucleolin. Taken together, these observations suggest that the protein/DNA complex observed with recombinant Nuc-1,2,3,4-RGG_9 may represent an intermediate in the annealing reaction that is observable due to the depressed kinetics of the reaction. In fact, it is possible that the stability of this protein/DNA complex is the underlying cause of the slow annealing kinetics observed with Nuc-1,2,3,4-RGG_9. The rapid annealing kinetics observed in the presence of purified endogenous nucleolin make it impossible to visualize this protein/DNA intermediate complex and emphasize the role of DNA dissociation in the reaction.

It has previously been reported that nucleolin exhibits DNA helicase activity (*38*). These assays were carried out at 20−100 nM protein, a concentration 20−100-fold higher than that used in our analysis (1 nM), and several orders of magnitude higher than typically used in assays of helicase activities in vitro, raising the possibility that the observed helicase activity was due not to nucleolin but to a contaminating polypeptide in the preparation. Alternatively, the helicase activity of nucleolin may be regulated by self-association or DNA binding, reflected by a requirement for high protein concentrations for activity in vitro. In that case, the renaturation activity we have documented might be opposed by the helicase activity under certain conditions or at certain times during the cell cycle.

In eukaryotic cells, nucleolin is normally highly phosphorylated (*8, 9*), and arginines within the RGG_9 domain are dimethylated (*39, 40*). The differences we observe in annealing kinetics in reactions containing purified murine nucleolin and recombinant Nuc-1,2,3,4-RGG_9 may reflect the absence of posttranslational modifications in protein expressed in vitro. Alternatively, the N-terminus may modulate the structure of the entire polypeptide to enhance certain activities. This acidic domain could, for example, enhance DNA dissociation by competing for a basic region of the polypeptide that interacts with DNA.

A Role for Nucleolin in Recombination and Maintenance of rDNA Sequence Homogeneity. In most vertebrates, the rDNA is a tandemly repeated gene family. The sequence homogeneity of the rDNA must be maintained, because mutations within the rDNA repeats could compromise transcriptional regulation of the rDNAs, rRNA biosynthesis, and ultimately cell viability. In yeast and very likely in mammals, rDNA recombination and maintenance depend in part upon the recombination protein Rad52 (*17*−*22*). Rad52 can accelerate the annealing of ssDNA (*41, 42*) and also synergize with Rad51 in homologous DNA pairing reactions (*43*−*46*). As both Rad52 and nucleolin localize to the nucleolus and accelerate annealing, their functions may be redundant. Alternatively, the ability to accelerate annealing of *mismatched* as well as perfect duplexes may distinguish nucleolin from proteins which function in homologous recombination, like Rad52. This property would, in particular, enhance the ability of nucleolin to function in recombination events that maintain homogeneity within a repeated gene family. In contrast to homologous recombination processes, which are inhibited by DNA mismatches, nucleolin may stimulate pairing between heteroduplexes that would be targets for subsequent repair.

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