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Nucleolin forms a specific complex with a fragment of the viral (minus) strand of minute virus of mice DNA

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ABSTRACT

Nucleolin, a major nucleolar protein, forms a specific complex with the genome (a single-stranded DNA molecule of minus polarity) of parvovirus MVMp *in vitro*. By means of South-western blotting experiments, we mapped the binding site to a 222-nucleotide motif within the non-structural transcription unit, referred to as NUBE (nucleolin-binding element). The specificity of the interaction was confirmed by competitive gel retardation assays. DNaseI and nuclease S1 probing showed that NUBE folds into a secondary structure, in agreement with a computer-assisted conformational prediction. The whole NUBE may be necessary for the interaction with nucleolin, as suggested by the failure of NUBE subfragments to bind the protein and by the nuclease footprinting experiments. The present work extends the previously reported ability of nucleolin to form a specific complex with ribosomal RNA, to a defined DNA substrate. Considering the tropism of MVMp DNA replication for host cell nucleoli, these data raise the possibility that nucleolin may contribute to the regulation of the parvoviral life-cycle.

INTRODUCTION

Parvoviruses are small non-enveloped lytic viruses, that can infect insects, birds and a variety of mammals, including humans (1). Minute Virus of Mice (prototype strain, MVMp), a member of autonomous parvoviruses, has a linear single-stranded DNA genome that comprises about 5 kilobases and is bracketed by terminal palindromic regions (2). Viral DNA replication, which uses these terminal hairpins to prime the formation of double-stranded intermediates (1), takes place in infected cell nuclei, more particularly in association with nucleoli, as shown by *in situ* hybridization (3) and biochemical studies (4).

The low genetic complexity of parvoviruses makes the replication and expression of their genome subordinate to helper factors. Interestingly, intracellular factors controlling the metabolism of parvoviral DNA appear to depend on the host cell's proliferative and developmental programs for their production and/or functioning (5). In this respect, it is noteworthy that many transformed cells of human and murine origin are more susceptible to the killing effect of MVMp than corresponding normal cells. This enhanced sensitivity is often associated with a stimulation of virus replication (reviewed in 6). To date, only a few cellular protein effectors or modulators of the parvoviral life-cycle have been identified. With regard to gene expression, parvoviral promoters contain RNA polymeraseII transcription signals (2) and were recently shown to be regulated by cellular transcription factors, i.e. MLTF (7) and YYI (8) for Adeno-Associated Virus 2 (AAV2) and Sp1 for MVMp (9) and B19 (10) viruses. On the other hand, cellular DNA polymerases (11) and topoisomerase I (12) have proved to be involved in parvoviral DNA amplification. Yet, host components controlling parvoviral DNA replication, including functions available transiently during the S-phase of the cell-cycle (2), remain largely unknown. It should also be stated that nuclear proteins were found to be part of complexes with MVMp (13), bovine parvovirus (14) and AAV 2 (15, 16) terminal DNA sequences which play an essential role in the production of parvoviral DNA.

The coincidence of MVMp DNA amplification centers with nucleoli (4) suggests that some host factor(s) modulating virus replication may be located in this nuclear compartment. Consistently, conditions promoting MVMp replication, in particular cell transformation, were found to be accompanied by changes in the number and morphology of nucleoli (4). Moreover, one of the cellular proteins associated with MVMp DNA termini proved to be of nucleolar origin (13). In a previous report (17), we have identified a protein (p102) that can be preferentially extracted from normal (versus transformed) human fibroblasts and forms a high affinity complex with MVMp single-stranded DNA. The present report identifies p102 with nucleolin and demonstrates the specificity of the binding of this protein for an intragenic region of MVMp single-stranded DNA of minus polarity (corresponding to encapsidated genomes). Nucleolin is

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a major nucleolar phosphoprotein that was proposed to be involved in the synthesis and processing of ribosomal RNA (18) and in nucleolar chromatin organization (19, 20). Interestingly, the production and the posttranslational modifications of nucleolin are tightly coupled with the ongoing cell-cycle (21, 22, 23). Together with the fact that nucleolin and MVMp DNA replication share their subcellular localization and dependence on cell growth, the data presented lead one to speculate about a possible role of this protein in the control of the parvoviral life-cycle.

MATERIALS AND METHODS

Preparation of nuclear extracts

MRC-5, a finite-life strain of human lung fibroblasts, was grown at 37°C in Eagle's Minimal Essential Medium supplemented with 5% fetal calf serum and was used at passages 26–34. Nuclei were collected from subconfluent cultures and extracts were obtained essentially as described by Hennighausen and Lubon (24). The protein concentration was determined according to Lowry.

Fractionation of nuclear proteins and purification of nucleolin

Nuclear extracts were fractionated by CsCl density-gradient isopycnic centrifugation for 15 h, at 90 000 rpm in the TL-100 Beckman rotor, at 20°C. Fractions (0.5 ml) were collected and dialyzed for 3 h at 4°C against 20 mM Hepes pH 7.9, 75 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.5 mM phenyl-methyl-sulfonyl-fluoride. Nucleolin was purified as described by Belenguer et al. (21). Briefly, the crude nuclear extract was applied to a heparin-sepharose column and fractions containing nucleolin were further purified by anion-exchange chromatography.

Preparation of MVMp DNA fragments

pBR322 and pMM984A, a pBR322 recombinant plasmid containing the entire genome of MVMp (25), were used to prepare DNA probes. pBR322 was digested with BamHI and AvaI endonucleases, and the 1054-base pair (bp) restriction fragment was recovered. The pMM984A clone was digested with BamHI and EcoRI enzymes, and the 1084-bp 3′ terminal region of MVMp DNA (referred to as MVM3′) was isolated. Nine subfragments were obtained by digestion of MVM3′ with several restriction endonucleases (see Results). DNA fragments were separated by agarose gel electrophoresis and recovered by electro-elution. 3′ end-labelling was performed with the Klenow fragment of *E.coli* DNA polymerase I, in the presence of [α-^{32}P] dNTP, as previously described (17). Selective labelling of MVMp DNA on either viral or complementary strand was achieved by using nucleotide precursors that were specific for corresponding 3′ ends.

Subcloning of the nucleolin-binding element (NUBE)

The PstI-StyI fragment was isolated from MVM3′ and the StyI site was filled in with DNA polymerase I (Klenow fragment). Plasmid pULBS was obtained by ligation of this fragment into PstI- and SmaI-digested pUC18 vector.

South-western blotting analysis of nuclear proteins

South-western blotting experiments were performed essentially as described by Avalosse *et al.* (17), except that the incubation buffer contained 150 mM NaCl. Briefly, nuclear proteins were electrophoresed in a sodium dodecylsulfate-10% polyacrylamide gel (SDS-PAGE), blotted to a nitrocellulose membrane, incubated with the ^{32}P-labelled DNA probe and visualized by auto-radiography.

Screening of a human cDNA expression library with MVMp DNA

Proteins expressed from a human lung fibroblast (IMR90) cDNA library constructed in the λ bacteriophage vector gt11 (Clontech Laboratories, Inc., CA) were screened for their ability to bind heat-denatured MVM3′ DNA, as described by Singh et al. (26). The specificity of interaction was assessed by incubating immobilized proteins from plaques generated by recombinant bacteriophages with either MVM3′ or pBR322 count-matched probes and comparing bound radioactivities after washing the membranes in the presence of 150, 250 or 400 mM NaCl for 30 min at room temperature. The cDNA insert of a positive candidate was sequenced, using the dideoxy-mediated chain-termination method (27), and the SWISS-PROT database was searched for homology with the deduced amino-acid sequence, according to Pearson and Lipman (28).

Western blotting analysis of nuclear proteins

Nuclear proteins were analyzed by SDS-PAGE as described by Laemmli (29), and were electrophoretically transferred to a nitrocellulose membrane (30). Incubation of the blot with rabbit antiserum (1:100 dilution) directed against nucleolin (21), and detection of bound antibodies by autoradiography after reaction with ^{125}I-labelled protein A (Amersham) were carried out according to Bugler *et al.* (31).

Gel retardation assays

Crude nuclear extracts (5 μg), fractionated proteins (1 μg), or purified nucleolin (0.3 μg) were incubated with 10^4 cpm of 3′-end labelled native or heat-denatured probe, for 30 min at room temperature, in 20 μl of 20 mM Tris-HCl pH 7.4, 4 mM $MgCl_2$, 200 mM KCl, 20% Glycerol, 1 mM dithiothreitol, 4 μg/ml bovine serum albumin and 1 μg of denatured poly (dI-dC) added as non-specific competitor. DNA-protein complexes were analyzed by electrophoresis on 5% polyacrylamide gels and revealed by autoradiography.

Computer-assisted conformational analysis

Potential DNA secondary structures were calculated by the algorithm of Zucker and Stiegler (32), using the Fold program available from the University of Wisconsin Genetics Computer Group (33) and run on a Digital Equipment Corporation 5000/200 work station.

DNaseI and nuclease S1 probing

End-labelled probes (10^4 cpm) were heat-denatured and incubated, or not, in the presence of 2.5 μg of purified nucleolin in a total volume of 50 μl, under the conditions described for gel retardation assays. Following incubation, the samples were supplemented with 50 μl of 10 mM $MgCl_2$, 5 mM $CaCl_2$. Probe digestion was performed with various quantities of DNaseI ranging from 0.2 to 1 ng per μl, for 30 sec at room temperature. For nuclease S1 digestion, 50 μl of 30 mM Na acetate, 100 mM NaCl, 1 mM $ZnCl_2$ were added to the binding mixture and the probe was digested with 4–20 enzyme Units per μl, for 15 min at room temperature. The reactions were stopped by the addition of 100 μl of 50 mM EDTA, 0.5 μg/μl proteinase K, followed by incubation for 30 min at 37°C and 5 min at 90°C. DNA was

phenol-chloroform extracted, ethanol precipitated and analyzed on a 8% polyacrylamide sequencing gel.

RESULTS

Specific interaction of p102 with the viral (minus) DNA strand *in vitro*

Most encapsidated MVMp genomes consist of a single DNA strand (referred to as viral) of minus polarity. The reproduction of this viral DNA in infected cells involves the formation of duplex intermediates through the synthesis of a complementary (plus) strand. We have previously shown, by South-western blotting experiments, that the 3′-terminal portion of cloned MVMp duplex DNA (MVM3′) binds to a specific nuclear protein, p102, provided that DNA is first denatured (17). In order to determine the strand specificity of p102, the MVM3′ probe was selectively labelled on either viral or complementary strand, heat-denatured and incubated with blots of electrophoretically fractionated nuclear extracts. As shown in Fig. 1, p102 was strongly revealed by the viral (V) strand but hardly by the complementary (C) strand or denatured pBR322, although count-matched probes with similar specific radioactivities were used. An equimolar mixture of C and V strand-labelled probes (V+C) gave less p102-bound radioactivity than the latter probe alone, most probably as a result of a competition between the unlabelled and labelled V strands generated upon denaturation of the former and latter probes, respectively. These data clearly indicate that the viral (minus) strand of MVMp DNA is involved in a specific interaction with p102.

Mapping of the viral DNA strand element recognized by p102

To map the site of interaction of p102 with the viral DNA strand, nine segments of MVM3′ (Fig. 2A, fragments b−j) were individually purified, V strand-labelled and used in South-western blotting experiments after denaturation. Some fragments retained the ability to bind to p102 (Fig. 2B, lanes c, d, f and g), while others were hardly recognized by the protein, as did pBR322 (Fig. 2B, lanes b, e, h, i, j and pBR). The fragments that formed a specific complex with p102 shared a 222-nucleotide region (Fig. 2A, fragment g) whose affinity for p102 was similar to that of denatured MVM3′ (compare lanes a and g in Fig. 2B). This region (nt 412−633 in the MVMp genome) is located within the non-structural transcription unit (2) and will be referred to as NUBE for reasons explicited below. Cleavage in the middle of NUBE generated two subfragments that lacked specific recognition by p102 (Fig. 2B, lanes i and j), suggesting that the core or even the whole of NUBE may be required for binding.

Identification of p102 as nucleolin

As a first step towards the identification of the cellular protein that binds to the V-strand of MVMp DNA, a human fibroblast cDNA library constructed in the bacteriophage λgt11 expression vector was screened by plaque South-western blotting, using heat-denatured MVM3′ as a probe. From a library consisting of 2×10^5 clones, a few candidates were revealed by the DNA probe, picked, replated and retested for their ability to react with MVM3′. A single clone, referred to as λ IMR-8ZN, scored as

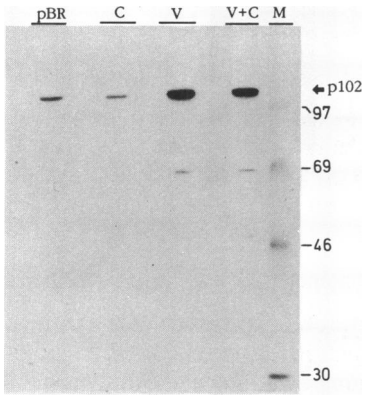

Figure 1. South-western blotting analysis of the interaction of p102 with MVMp DNA strands. Protein samples (40 μg) from a MRC-5 nuclear extract were fractionated by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane and incubated with ^{32}P-labelled DNA. Probes were 3′ end-labelled to the same specific activity and heat-denatured prior to use. pBR, 100 ng of plasmid pBR322 BamHI-AvaI 1054 bp fragment labelled on both strands; C, 100 ng of cloned MVMp DNA BamHI-EcoRI fragment labelled at the EcoRI site (C-strand); V, same as C, but labelled at the BamHI site (V-strand); V+C, mixture of 100 ng each of V and C probes. M, ^{14}C-labelled molecular weight (in kDa) marker proteins.

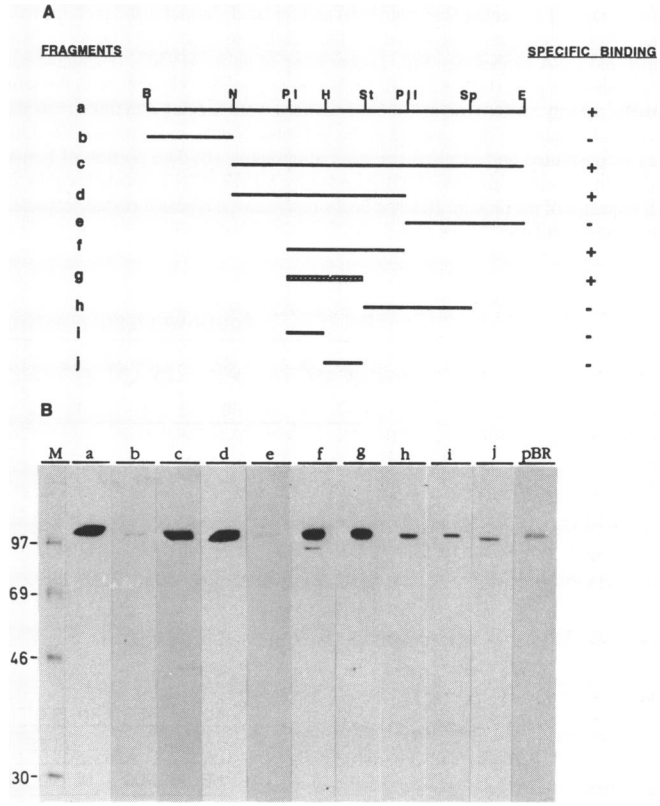

Figure 2. Identification of a p102-binding element in the MVMp viral DNA strand. (A) Restriction map of the 3′-terminal portion of MVMp DNA (nt 1−1084) and location of fragments (a−j) used for South-western blotting experiments. The '+' and '−' signs on the right-hand side indicate the respective ability and failure of individual fragments to form a specific complex with p102 (see B). B, BamHI; N, NcoI; PI, PstI; H, HinfI; St, StyI; PII, PvuII; Sp, SpeI; E, EcoRI. (B) South-western blots of nuclear extracts (40 μg) incubated with the restriction fragments indicated in panel A, after ^{32}P-labelling of the V-strand 3′ end and heat-denaturation. Probes of similar specific radioactivity were used in equimolar amounts. pBR is a plasmid DNA fragment serving as a control of unspecific binding (see Fig. 1). M, ^{14}C-labelled molecular weight (in kDa) marker proteins.

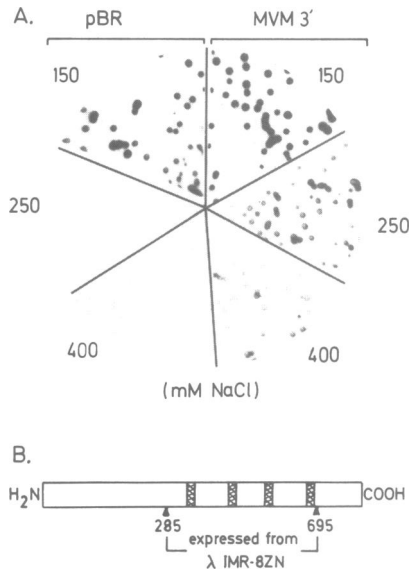

Figure 3. Characterization of a human cDNA clone encoding a MVM3'-binding fusion protein. (A) The λ IMR-8ZN recombinant bacteriophage was picked after a first round of screening (see main text) and replated. Immobilized proteins from bacteriophage plaques were tested for their ability to react with 100 ng of heat-denatured DNA probes (see Fig. 1) consisting of either MVM3' (righthand part of the filter) or pBR (lefthand part). Probes were 3'-end-, ^{32}P-labelled to the same specific activity. After reaction, the membranes were divided into three parts that were each washed in the presence of the indicated NaCl concentration. The filter was reconstituted and exposed for autoradiography. (B) The portion of human nucleolin (amino-acids 285–695) encoded by λ IMR-8ZN is located within the full sequence of the protein. Hatched boxes represent the repeated ribonucleoprotein consensus sequence.

positive in this second round of screening and was further characterized. As illustrated in Fig. 3A, the human polypeptide encoded by λ IMR-8ZN as part of a fusion protein with β-galactosidase, had an especially high affinity for MVMp DNA. Indeed, the binding of a size-matched heat-denatured pBR322 DNA fragment used as a nonspecific probe, became insignificant under washing conditions (i.e. in the presence of 250 or 400 mM NaCl) that left a major proportion of the MVMp DNA probe still attached to the immobilized proteins.

The 1.2 kbp cDNA insert from λ IMR-8ZN was sequenced. As shown schematically in Fig. 3B, the deduced amino-acid sequence proved to be identical to an internal portion of human nucleolin (34). Interestingly, the nucleolin fragment encoded by λ IMR-8ZN contained four copies of an 8-residue ribonucleoprotein consensus sequence that is thought to be involved in specific binding to RNA (18). These results, together with the fact that nucleolin has an apparent molecular mass of about 100 kDa (31), suggested that this protein was a possible candidate for p102.

In order to verify this possibility, nucleolin was purified from human cells and tested for its ability to react with DNA, in comparison with total nuclear extracts and nuclear fractions enriched in p102. These fractions were obtained by CsCl gradient equilibrium centrifugation of nuclear extracts and corresponded to the 1.35–1.375 range of densities at which most p102 banded (Fig. 4A), in contrast with the overall nuclear protein population that was distributed across the gradient, in particular at lower densities (Fig. 4B).

As illustrated in Fig. 4C, South-western blotting experiments indicated that nucleolin shared with p102 from crude or partially purified nuclear extracts, the capacity for specific interaction with

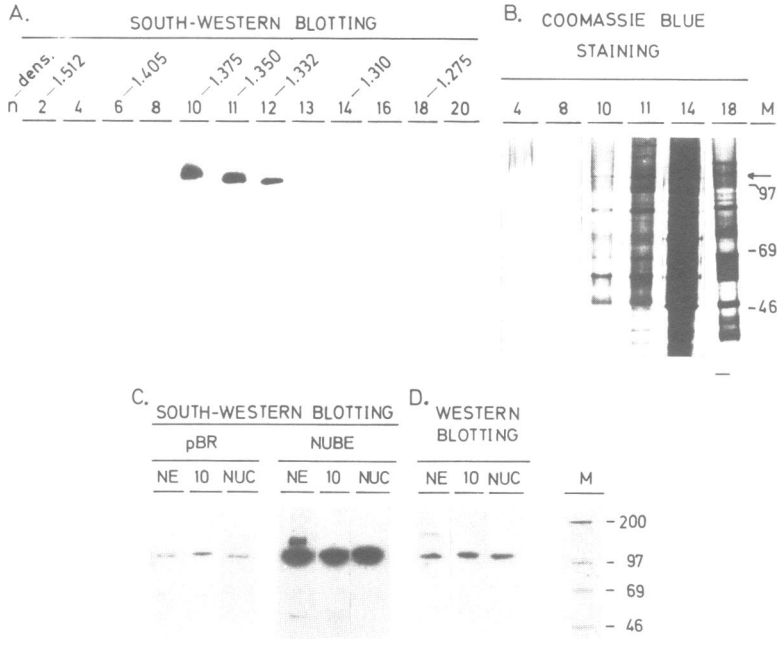

Figure 4. Comparison of purified nucleolin with crude and enriched nuclear extracts for their interaction with NUBE. (A, B) Nuclear protein extracts were fractionated by CsCl gradient equilibrium centrifugation. Fractions were collected from the bottom of the tube and are shown from left to right with their number (n) and density (dens. in g cm^{-3}) on top of lanes. Aliquots were taken and subjected to SDS-PAGE. Proteins were either blotted, incubated with radiolabelled NUBE (heat-denatured fragment g from Fig. 2) and revealed by autoradiography (panel A), or visualized by Coomassie blue staining (panel B). (C) South-western blots of a total nuclear protein extract (NE), CsCl gradient fraction 10 from panel A (10) and purified human nucleolin (NUC), using NUBE or pBR (see Fig. 1) probes that were 3'-end labelled to the same specific activity. The samples were matched for their NUBE-binding capacity. (D) Western blots of duplicate samples from panel C, after incubation with antinucleolin antiserum, reaction with ^{125}I-labelled protein A and autoradiography. M, ^{14}C-labelled molecular weight (in kDa) marker proteins.

the NUBE element of MVMp DNA. The signal generated by pBR322 DNA used as a control for nonspecific binding, was at least 10-fold lower with both p102 and nucleolin, as determined by densitometric scanning of the autoradiograms. It should also be stated that nucleolin could not be distinguished from p102 with respect to its electrophoretic mobility.

On the other hand, p102-enriched nuclear fractions from CsCl gradients contained nucleolin, as shown by Western blotting analysis using an antiserum which was directed against nucleolin and failed to cross-react with other nuclear proteins that were present in these fractions (Fig. 4D, lanes 10 and NUC). Importantly, samples of total nuclear proteins, enriched p102 and purified nucleolin that were matched for their NUBE-binding capacity (Fig. 4C), reacted to a similar extent with the anti-nucleolin serum (Fig. 4D).

Altogether, these data showed that nucleolin formed a specific complex with MVMp DNA and was sufficient to account for the observed trapping of NUBE at the p102 position (hence the reference to this motif as *nu*cleolin *b*inding *e*lement). If additional proteins were to participate in the p102 activity of nuclear extracts, they should be indistinguishable from nucleolin by their apparent molecular mass and relative ability to associate with MVMp DNA versus antinucleolin antibodies. In subsequent experiments, nucleolin from enriched CsCl fractions and purified preparations was used to further characterize the interaction of this protein with NUBE.

Gel retardation analysis

Band shift experiments were carried out with crude nuclear extracts, CsCl fractions enriched in nucleolin or purified nucleolin. A characteristic retarded band was observed when heat-denatured NUBE was incubated with either of these preparations (Fig. 5A, arrow). This retarded complex was not detected with the double-stranded NUBE probe (Fig. 5B), consistently with previously reported South-western blotting experiments (17). An additional retarded band was visible with both heat-denatured and native NUBE exposed to crude nuclear extracts (Fig. 5 A and B, lanes 2) but not to enriched or purified nucleolin samples, and was not further investigated.

In order to assess the specificity of nucleolin association with NUBE, competition experiments were performed with homologous and heterologous DNA. As illustrated in Fig. 6, unlabelled NUBE was an efficient competitor for the retardation of the probe, whereas a significant amount of complexes remained in the presence of a 500-fold molar excess of heterologous DNA. It therefore appears that nucleolin formed a specific complex with NUBE under the native conditions of gel retardation assays, i.e. in the absence of protein denaturation and renaturation that take place in South-western blotting experiments.

Computer-predicted secondary structure of NUBE

Fresco et al. (35) first showed that single-stranded RNA may fold back onto itself in structures stabilized by hydrogen bonds between complementary bases. By homology, the algorithm of Zucker and Stiegler (32) was used to determine whether NUBE (minus DNA strand) could also adopt a thermodynamically stable secondary structure. Indeed, the computer-predicted conformation of lowest free energy, shown in Fig. 7B, consisted of four stem-loop regions (S1−L1 to S4−L4) connected by an essentially double-stranded bridge (B). Although the ΔG of this NUBE structure was quite low (−29.7 kcal), it is noteworthy that S3 and S4 stems contained a number of mismatches which may destabilize them, as documented below.

Nuclease probing of NUBE structure

In order to experimentally confirm the occurrence of the computer-predicted structure, NUBE was tested for its sensitivity to DNaseI and nuclease S1 that preferentially cleave double- and single-stranded DNA, respectively. Heat-denatured and quick-cooled NUBE was partially digested with either nuclease and analyzed on a sequencing gel (Fig. 7A, lanes −). As indicated in Fig. 7B (lines), DNaseI sensitivity fairly correlated with computer-predicted base-pairing and mostly concerned the putative stems S1 and S2, bridge B and double-stranded segments of stem S3. Conversely, nuclease S1 attacked the putative loops L1 and L2 as well as the mismatched portion of the stems and bridge (Fig. 7B, dots). Stem S3 was made of alternating and often overlapping DNaseI and nuclease S1-sensitive segments, suggesting that it was unstable and coexisted in several folded

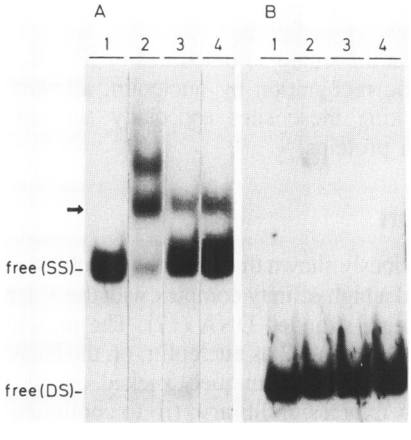

Figure 5. Gel retardation analysis of nucleolin association with MVMp DNA. NUBE DNA was ^{32}P-labelled on the V-strand and analysed by polyacrylamide gel electrophoresis without prior treatment (lanes 1) or after incubation with crude nuclear extracts (lanes 2), CsCl fractions enriched in nucleolin (lanes 3) or purified nucleolin (lanes 4), in the presence of excess poly (dI-dC). The probe and poly (dI-dC) were both heat-denatured (A), or not (B), before use. The NUBE-nucleolin complex is indicated by the arrow, while unbound single-stranded (SS) and double-stranded (DS) probes are marked 'free'.

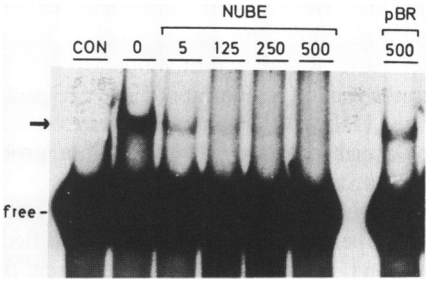

Figure 6. Competitive gel retardation analysis of the specificity of NUBE-nucleolin complex formation. NUBE (0.1 ng) was 3′ end-labelled on the V-strand, heat-denatured and incubated with 0.3 μg of nucleolin in the presence of 1 μg of poly (dI-dC) and various amounts of unlabelled denatured competitor DNA (NUBE or an unrelated 185 nt pBR322 fragment). Lane CON, without nucleolin; lane 0, with nucleolin and without competitor; lanes 5−500, with nucleolin and indicated molar excesses of competitor. The NUBE-nucleolin complex is indicated by the arrow.

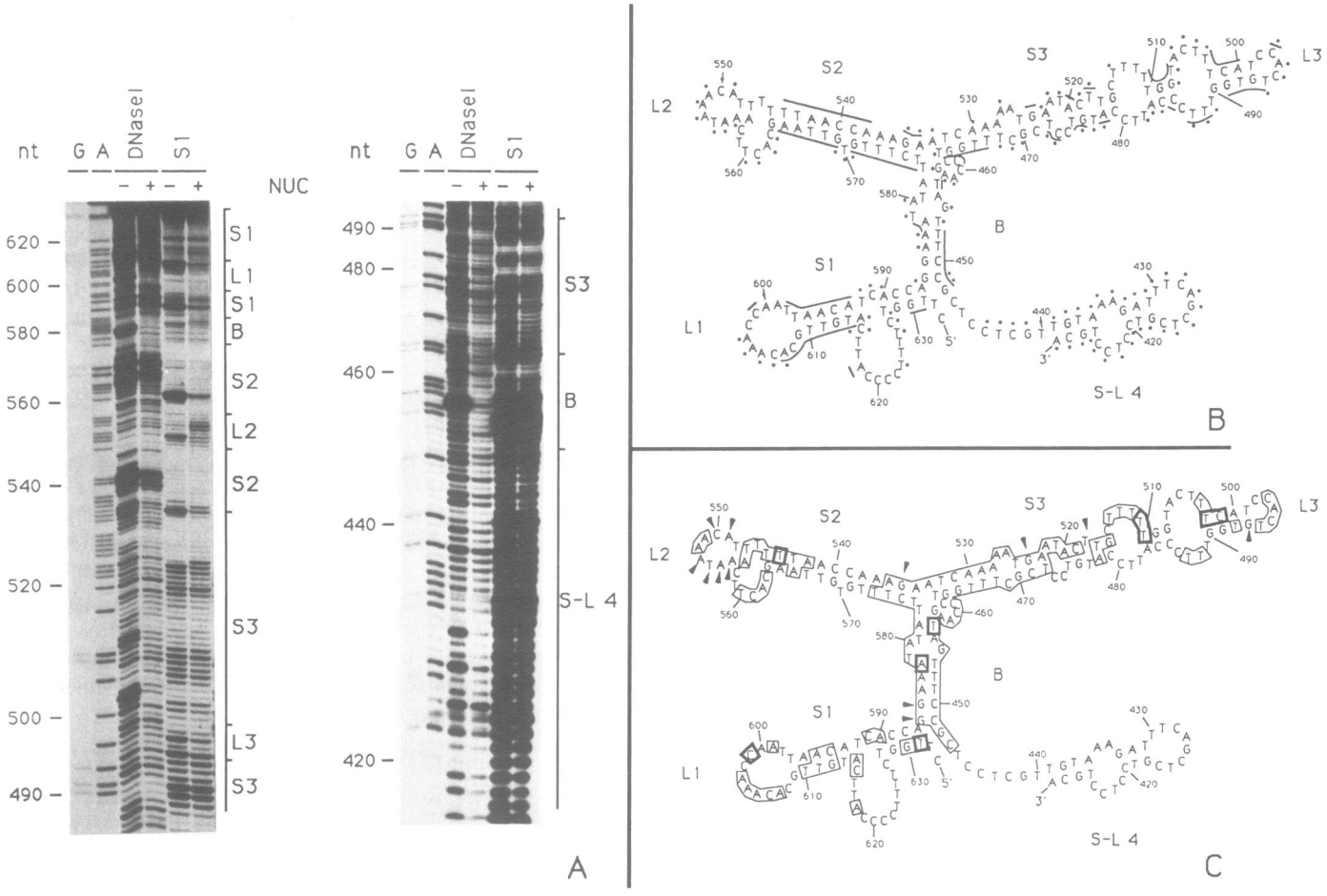

Figure 7. Nuclease probing and computer analysis of NUBE. (A) NUBE was labelled on the V-strand, heat-denatured, incubated (lanes +) or not (lanes −) in the presence of nucleolin (NUC), digested with DNaseI or nuclease S1 and analyzed by electrophoresis on a sequencing gel. Lanes G and A, Maxam and Gilbert G and G+A sequence ladders respectively. Bars on the right indicate the computer-predicted stem (S), loop (L) and bridge (B) regions (see B). MVMp nucleotides are numbered according to Cotmore and Tattersall (2). (B) The distribution of DNaseI (−) and nuclease S1 (●)-sensitive sites in the absence of nucleolin, determined according to panel A, is shown on the computer-predicted secondary structure of NUBE. (C) Protection (□) and sensitization (▼) towards digestion by DNaseI or nuclease S1, in the presence of nucleolin (see panel A), are indicated on the conformational model of NUBE. Heavy boxes mark sites that are hyperprotected against DNaseI attack.

forms in solution. The 3′ terminal region S-L 4 was fully sensitive to nuclease S1 only and therefore appeared to lack a secondary structure, at least under *in vitro* conditions. It should be stated that, although they were in fair agreement, experimental and theoretical data showed some discrepancies. Indeed, DNaseI-sensitive sites were found within loops L1 and L3, whereas putative double-stranded regions at nt 528−533 and nt 585−589 proved to be DNaseI-resistant. Such inconsistencies may tentatively be ascribed to local B-helix minor groove mimicry or distortion (inaccessibility), respectively.

When it was incubated with purified nucleolin prior to nuclease probing, NUBE exhibited a markedly modified pattern of nuclease digestion (Fig. 7A, lanes +). As apparent from Fig. 7C, protected (boxes) and sensitized (arrows) regions were found over the greater part of L1−S1, B, L2−S2 and L3−S3 segments, indicating that nucleolin altered the overall structure of NUBE. It is worth noting that some DNaseI-sensitive sites showed a particularly high protection against cleavage in the presence of nucleolin (Fig. 7C, heavy boxes). Interestingly, the original DNaseI sensitivity of hyperprotected sites proved to be much increased upon heat-denaturation of the probe (data not shown), suggesting that the single-strandedness of NUBE, a prerequisite for its specific recognition by nucleolin, allowed a secondary structure making these sites especially accessible for direct contacts with proteins.

DISCUSSION

We have previously shown that a human fibroblast nuclear protein (p102) formed a high-affinity complex with the 3′ terminal portion of MVMp single-stranded DNA (17). The present study led to the identification of p102 as nucleolin, on the basis of the ability of this protein: (i) to be uniquely picked out by MVM3′ in a human cDNA expression library; (ii) to comigrate with p102 in SDS-PAGE; (iii) to have DNA-binding properties that account for the observed p102 activity; (iv) to form a similar retarded complex with MVMp DNA as nuclear extracts in electrophoretic mobility shift assays.

Nucleolin is an ubiquitous multifunctional nucleolar protein involved in ribosome biogenesis (31, 36, 37). It has been suggested that nucleolin could modulate nucleolar chromatin condensation (19, 38) and regulate the synthesis and maturation

of ribosomal RNA (39,40). Indeed, the carboxy-terminal domain of nucleolin contains four closely related 80–90 amino-acid repeats that possess affinity for ribosomal RNA (31). In addition, nucleolin associates with nascent pre-ribosomal RNA *in vivo* (41). It has recently been shown that several sites of interaction with nucleolin are present in the 5′ external transcribed spacer (5′ETS), 18S and 28S regions of pre-ribosomal RNA (L.Ghisolfi, personal communication). The 5′ETS element involved has been characterized and appears to consist of a uridine-rich stem-loop structure which undergoes alterations upon nucleolin binding. Furthermore, nucleolin was reported to bind to single-stranded DNA (42), in particular to an A-T-rich region of the non-transcribed spacer of denatured ribosomal DNA *in vitro* (43). Nucleolin also associates with nucleolar chromatin during interphase and with nucleolus organizer regions of chromosomes (43, 20).

The South-western blotting experiments reported herein provided evidence that nucleolin specifically interacted with the viral (minus) strand of MVMp DNA *in vitro*. The complementary strand only showed a low level of non-specific binding, as did heat-denatured plasmid DNA. The greater part of the affinity of the viral DNA strand for nucleolin could be ascribed to a 222 nucleotide fragment (NUBE) located within the non-structural transcription unit of MVMp. The specificity of the association of nucleolin with NUBE was further ascertained by gel retardation assays which, unlike the South-western blotting procedure, avoid the prior denaturation of nuclear protein extracts. Interestingly, NUBE exhibited a striking secondary structure whose theoretical prediction was consistent with a characteristic pattern of DNaseI and nuclease S1 digestion. The responsiveness of this structure to purified nucleolin was apparent from protein-induced changes in the nuclease-sensitivity of multiple sites scattered over the greater part of the NUBE sequence.This feature contrasts with the narrow (usually 10 to 20 nt long) footprints generated by proteins that interact with double-stranded DNA in a sequence-specific way. It may be speculated that the high level of DNA strand folding allowed nucleolin to induce long-range conformational perturbations and/or to recognize most of the NUBE structure. The latter possibility would be consistent with the suppression of nucleolin binding after NUBE fragmentation and with the previously reported capacity of this protein for self aggregation (44).

Several eukaryotic single-stranded DNA binding proteins have been identified and appear to play important roles in the metabolism of nucleic acids *in vivo*. Calf thymus unwinding protein 1 (UP1) (45) and mouse myeloma helix-destabilizing-protein (HDP) (46) stimulate DNA polymerization through non-specific interactions with melted DNA at the replication fork (45, 47). Recently, protein H16 was found to specifically bind to the late strand of the early promoter of simian virus 40, and stimulate RNA polymerase II-driven *in vitro* transcription (48). Likewise, a regulatory function of nucleolin may also be considered with respect to the production of ribosomal RNA and nucleolar-replicating DNA viruses such as MVM. Most parvoviral DNA elements found to interact with host factors are located in promoter regions or terminal palindromic sequences involved in DNA replication. In addition, a downstream promoter element was recently identified and shown to be necessary for efficient transcription of MVMp structural genes from a promoter referred to as P38 (49). Similarly, the NUBE element characterized in the present study is located downstream from the P4 promoter of the MVMp non-structural transcription unit. Nucleolin specifically recognized the single-stranded genome of MVMp. Although it has a minus polarity, the viral DNA strand is thought not to be transcribed unless first converted to a duplex form (2). One may therefore speculate about a possible control of nucleolin over the conversion step of MVMp DNA replication. It should be stated, however, that the present *in vitro* study does not allow one to draw conclusions as to the physiological significance of the interaction of nucleolin with NUBE DNA. Although nucleolar localization as well as modulation by cell cycling and transformation are distinctive features of both nucleolin and MVMp replication, the direct demonstration of the role of nucleolin in the regulation of the parvoviral life-cycle awaits further *in vivo* investigations.

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