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Recognition but no repair of abasic site in single-stranded DNA by human ribosomal uS3 protein residing within intact 40S subunit

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ABSTRACT

Isolated human ribosomal protein uS3 has extra-ribosomal functions including those related to base excision DNA repair, e.g. AP lyase activity that nicks double-stranded (ds) DNA 3′ to the abasic (AP) site. However, the ability of uS3 residing within ribosome to recognize and cleave damaged DNA has never been addressed. Here, we compare interactions of single-stranded (ss) DNA and dsDNA bearing AP site with human ribosome-bound uS3 and with the isolated protein, whose interactions with ssDNA were not yet studied. The AP lyase activity of free uS3 was much higher with ssDNA than with dsDNA, whereas ribosome-bound uS3 was completely deprived of this activity. Nevertheless, an exposed peptide of ribosome-bound uS3 located far away from the putative catalytic center previously suggested for isolated uS3 cross-linked to full-length uncleaved ssDNA, but not to dsDNA. In contrast, free uS3 cross-linked mainly to the 5′-part of the damaged DNA strand after its cleavage at the AP site. ChIP-seq analysis showed preferential uS3 binding to nucleolus-associated chromatin domains. We conclude that free and ribosome-bound uS3 proteins interact with AP sites differently, exhibiting their non-translational functions in DNA repair in and around the nucleolus and in regulation of DNA damage response in looped DNA structures, respectively.

INTRODUCTION

It is currently believed that many ribosomal proteins (RPs) not only play roles in translation as constituents of the ribosome but also execute extra-ribosomal functions implicated in various cellular processes besides protein synthesis (for review, see (1-3)). However, these additional functions have been discovered mainly for isolated RPs that are outside of ribosomes. To our knowledge, involvement of ribosome-bound RPs in extra-ribosomal functions has been suggested only for two eukaryotic RPs, RACK1 and L13a. The former has a very specific structure enabling its binding to other proteins such as protein kinases and membrane receptors, which can link the ribosome to signaling pathways (for review, see (1,4)). RP L13a regulates ceruloplasmin mRNA translation; in response to a specific signal, it is phosphorylated and leaves the ribosome (which is viewed as a depot for L13a when its regulatory function is not concerned) to bind to the 3′-untranslated region of the mRNA and stop its translation (5).

One of the most studied eukaryotic RPs is uS3, a key player in translation initiation on the small (40S) ribosomal subunit. When not bound to ribosomes, uS3 shows a number of activities unrelated to translation including those in the regulation of the genes controlled by NF-κB transcription factor and in DNA repair (reviewed in (6)). A number of issues concerning the latter implication of uS3 remain obscure, for example, it is unknown which kinds of DNA sequences or structural motifs preferably interact with uS3, and it is largely unclear when and where this ribosomal protein could contribute to DNA repair in the cell. It has been shown that mammalian uS3 binds damaged double-stranded (ds) DNA containing oxidative lesions and cleaves DNA at abasic (AP) sites (7–11); the ability of uS3 to cleave single-stranded (ss) DNA has not yet been examined. The chemistry of this reaction includes formation of a covalent Schiff base-type intermediate between the enzyme's catalytic amino group and C1′ of the AP site, followed by β-elimination of the 3′-phosphate. In its N-terminal part, uS3 contains a conserved fold known as K homology (KH) domain (see (12) and references therein) that interacts with single-stranded nucleic acids. The KH domain of uS3 has been suggested to mediate at least some of its extra-ribosomal functions (13,14). Probing the mRNA binding site of the human ribosome with derivatives of oligoribonu-

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cleotides bearing cross-linking moieties has revealed that they usually target uS3 even if not fixed in the ribosome by codon–anticodon interactions with cognate tRNA (15–17). Moreover, uS3 could covalently trap ssDNA bearing a photoactivated cross-linking group at the 5′-terminus (18). Initially, it has been proposed that this ability is due to the location of uS3 at the ribosomal mRNA entry channel where it might transiently bind unstructured RNAs as mRNA analogues (17). However, recently, using RNAs with the 3′-terminal ribose oxidized to dialdehyde, we have shown that these derivatives of unstructured RNAs cross-link to the ribosome-bound uS3 away from the mRNA binding site, and mapped the cross-linking site to a fragment of the KH domain, a large part of which is exposed to solvent (19). Thus, it is possible that uS3 can exercise its extra-ribosomal functions via the KH domain even when ribosome-bound. Considering the ability of the ribosome-bound uS3 to trap derivatives of ssDNA (18) and 3′-dialdehyde derivatives of unstructured RNAs (19), one could expect that ribosome-bound uS3 might be capable of cross-linking to AP sites in DNA, which partially exist in an aldehyde form (20). Thus, the question arises whether uS3 retains the enzymatic activities related to DNA repair within 40S ribosome.

In this study, we examined the ability of the human 40S ribosome to interact via uS3 with ssDNA and dsDNA oligonucleotides bearing various oxidative lesions including an AP site. Along the way, we re-examined the DNA repair activities of the free human uS3. We found that ribosome-bound uS3 is able to form covalent adduct with an AP site in ssDNA. However, unlike the isolated protein that cross-links to both ssDNA and dsDNA and cleaves them at AP site, ribosome-bound uS3 does not interact with this lesion in dsDNA and cannot cleave ssDNA at AP site. We showed that AP site is trapped by the same KH domain region in uS3 within the 40S ribosome that interacts with unstructured RNAs carrying the oxidized ribose, while the putative catalytic site previously reported for the isolated uS3 is hidden in the ribosome. Moreover, chromatin immunoprecipitation sequencing (ChIP-seq) analysis enabled us to reveal that uS3 binds preferentially to pericentromeric regions of chromosomes, where the protein binding sites to a significant extent overlap with or are adjacent to so-called nucleolus-associated chromatin regions (NADs) that interact with nucleoli. Our findings suggest that uS3 residing in the 40S ribosome might perform extra-ribosomal functions related to control of DNA quality whereas isolated uS3 protein apparently is involved in DNA repair in nucleolus and nucleolus-associated chromatin.

MATERIALS AND METHODS

Ribosomes and oligonucleotides

Oligodeoxyribonucleotide (23-mer) containing 8-oxoG, U or tetrahydrofuran and the complementary 23-mer were synthesized in-house from commercially available phosphoramidites (Glen Research, Sterling, VA, USA) and purified by reverse-phase HPLC followed by 20% polyacrylamide gel electrophoresis (PAGE) in the presence of 8 M urea. The modified strand was 5′-^{32}P-labeled using phage T4 polynucleotide kinase (Biosan, Novosibirsk, Russia) according to the manufacturer's instructions and desalted using NenSorb C_{18} resin (DuPont, Wilmington, DE, USA). Single-stranded AP site-containing oligomer (ssDNA-AP) was prepared by treatment of the respective dU-containing substrate with 2.5 U of *Escherichia coli* uracil–DNA glycosylase (New England Biolabs, Ipswich, MA, USA) per 100 pmol of the oligonucleotide for 30 min at 37°C in a buffer containing 20 mM Tris–HCl (pH 8.0), 1 mM EDTA, and 1 mM dithiothreitol, and used immediately.

DNA duplex containing AP site (dsDNA-AP) was obtained in two steps. At first, the 5′-labeled single stranded oligonucleotide containing a dU residue (45 μM) was annealed with the complementary strand (90 μM) in 6 μl of a buffer containing 10 mM HEPES–KOH (pH 7.5) and 1 mM EDTA for 2 min at 95°C. The mixture was then incubated for 5 min at 37°C and for 30 min at 25°C. Finally, the resulting DNA duplex was treated with uracil–DNA glycosylase as described above and the obtained dsDNA-AP was used immediately.

Nonaribonucleotide derivative oxidized at its 3′-terminal ribose was obtained as described (19). 40S ribosomal subunits were isolated from fresh human placenta according to (21) and stored in liquid nitrogen as small aliquots, each was thawed only once to use immediately. Prior to use, the subunits were re-activated by incubation in binding buffer A containing 20 mM HEPES–KOH (pH 7.5), 120 mM KCl, 13 mM $MgCl_2$ and 0.65 mM EDTA at 37°C for 10 min.

Recombinant human uS3

To produce recombinant uS3 (rec-uS3) containing an N-terminal His-tag, *E. coli* BL21 (DE3) transformed with the pET-15b vector containing human uS3 cDNA (kindly provided by Dr Alexey Malygin, SB RAS ICBFM) was grown in 0.4 l of LB medium containing 100 mg/l ampicillin for 12 h at 37°C. Protein synthesis was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM and the growth was continued at 18°C for 6 h. The cells were collected by centrifugation and resuspended in 10 ml of an ice-cold buffer containing 20 mM HEPES–KOH (pH 7.5), 20 mM imidazole, 150 mM NaCl, 0.5% Triton-X100, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 2 U/ml DNAse I, and 2 mM PMSF followed by incubation with 1 mg/ml lysozyme for 30 min on ice. Finally, the cells were sonicated on ice at 44 MHz, and the debris was separated by centrifugation at 7000×g for 20 min.

To purify rec-uS3, the cell lysate was loaded onto a column packed with 1 ml of Ni-NTA agarose (Sigma) pre-equilibrated with a solution containing 20 mM HEPES–KOH (pH 7.5), 20 mM imidazole, 50 mM NaCl and 2 mM PMSF. The column was washed with 10 ml of the same buffer but with 500 mM NaCl, and proteins were eluted with 10 ml of a solution containing 20 mM HEPES–KOH (pH 7.5), 400 mM imidazole, 50 mM NaCl and 2 mM PMSF. The resulting protein solution was dialyzed at 4°C for 12 h against the buffer containing 60 mM HEPES–KOH (pH 7.5), 100 mM KCl and 2 mM dithiothreitol using a membrane with 8000–15000 Da cut-off (Serva), diluted with an equal volume of glycerol, and stored at –20°C. Protein concentration in the solution was about 0.06 mg/ml (~2 μM).

Cross-linking of uS3 to DNA oligomers

Cross-linking of rec-uS3 to ssDNA-AP and dsDNA-AP was performed by incubation of the protein (1.2 μM) with 0.1 μM single-stranded or double-stranded substrate in 20 μl of buffer containing 25 mM HEPES–KOH (pH 7.5), 72 mM KCl, 4.5 mM $MgCl_2$, 0.6 mM DTT, 0.6 mM PSMF and 30% glycerol for 1 h at 25°C with the subsequent addition of $NaBH_3CN$ to 5 mM and incubation at 37°C for 1 h. Proteins were precipitated from the reaction mixture by addition of six volumes of acetone at –20°C, dissolved in the standard Laemmli sample buffer, incubated for 10 min at 80°C and resolved by 12% PAGE in the presence of sodium dodecyl sulfate (SDS) under standard conditions. The gels were dried and exposed to a BioRad Phosphorimager plate.

Cross-linking of ssDNA-AP or dsDNA-AP (1 μM) to 40S ribosomes (3 μM) was carried out by incubation of their mixture in 6 μl of buffer A at 25°C for 1 h with subsequent treatment with $NaBH_3CN$ as described above. The ribosomes were ethanol-precipitated, the pellets were resuspended in the Laemmli sample buffer and analyzed by SDS-PAGE. In some experiments, 40S ribosomes were purified after the cross-linking procedure by centrifugation in 10–30% sucrose gradient in a buffer containing 300 mM KCl, 3 mM $MgCl_2$, 0.15 mM EDTA, 6 mM 2-mercaptoethanol and 20 mM Tris–HCl, pH 7.5 (rotor SW-41, 24 000 rpm, 4°C, 17 h); the ribosomal fraction was ethanol-precipitated and analyzed by SDS-PAGE as described above. Quantification of the autoradiograms was performed using Molecular Imager FX Pro (Bio-Rad); mean values and standard deviations of relative intensities of the bands were calculated from three independent experiments.

Preparation of 40S ribosomes with uS3 cross-linked to 3′-dialdehyde derivative of nonaribonucleotide AAUAAAUUC was obtained by incubation of the ribosomes (1.8 μM) with the derivative (18 μM) in buffer A with subsequent treatment with $NaBH_3CN$ as described (19). After completion of the reaction, ribosomes were ethanol-precipitated, resuspended in buffer A and used immediately in experiments on cross-linking with ssDNA-AP as described above. In the control experiments, 40S ribosomes were treated in the same way with unmodified AAUAAAUUC or without it. Experiments on cross-linking of 3′-dialdehyde derivative of nonaribonucleotide AAUAAAUUC to rec-uS3 were performed similarly to those described above for its cross-linking to 40S ribosomes, but the components were incubated directly in the rec uS3 storage solution (see the previous section).

Identification of uS3 cross-linked to damaged DNA by immunoblotting was carried out as in (19) using specific antibodies against rat liver uS3 (S3e) prepared according to (22).

Cleavage of DNA AP sites by recombinant or ribosome-bound uS3

DNA substrates (0.5 μM) were incubated with variable amounts of rec-uS3 or 40S ribosomes in 10 μl of buffer containing 30 mM HEPES–KOH (pH 7.5), 50 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 0.01 mg/ml BSA and 0.05% Triton X-100 at 37°C for 1 h. The reaction was stopped by adding an equal volume of formamide-containing gel-loading solution; then the mixture was heated for 1 min at 95°C and analyzed by denaturing urea–PAGE in a 20% gel.

ChIP-seq experiment for uS3

ChIP-seq experiment for uS3 was carried out as described (23) with moderate modifications. The experiment was performed in three biological replicates with ∼2.5 × 10^7 HEK293 cells as an input material per replicate using formaldehyde as cross-linking reagent. The chromatin was resuspended in modified LB3 buffer (0.5% *N*-lauroylsarcosine was changed to 0.5% SDS) (23), 25 μl of the buffer per 10^6 cells. Chromatin shearing was performed using CovarisS2 System as described in the manufacturer's protocol for Applied Biosystems SOLiD 4 System SOLiD ChIP-seq Kit Guide. Triton-X100% was added to the sheared chromatin up to the final concentration of 1%, and the chromatin was cleared by centrifugation at 20 000×*g* for 10 min at 4°C to remove the cell debris. To prepare the input control, 50 μl of the cleared sheared chromatin were used. For the immunoprecipitation step, the cleared sheared chromatin (625 μl) was diluted 5-fold in the modified LB3 buffer (without SDS) (23) supplemented with 1% Triton-X100 to decrease SDS concentration to 0.1%. For each replicate immunoprecipitation, 15 μl of specific antibodies against rat liver uS3 (see above) were conjugated to 50 μl of Protein G Dynabeads (Thermo Fischer Scientific). In the control experiment, all procedures were carried out with Protein G Dynabeads without conjugated antibodies. uS3 with bound DNA-fragments was eluted from the beads, formaldehyde cross-links were reversed by heating, DNA fragments were recovered by phenol–chloroform extraction, precipitated by ethanol with GlycoBlue (Thermo Fischer Scientific) and dissolved in 20 μl of nuclease-free water (Ambion, Thermo Fischer Scientific). DNA-libraries were prepared from the samples using the 5500 SOLiD Fragment Library Core Kit (Life Technologies) according to the manufacturer's instructions, and sequenced on SOLiD 5500xl platform (Life Technologies) in SB RAS Genomics Core Facility (ICBFM, SB RAS). The resulting read length was 50 bp. To minimize sequencing errors, color-space reads were subjected to error correction using SOLiD Accuracy Enhancement Tool v.2.4 (Life Technologies). The corrected data were analyzed using CLC GW 9.5 (Qiagen) with the default parameters. After adapter and quality trimming, SOLiD reads were mapped to hg38 reference genome with the Ensemble annotation GRCh38.82 ignoring non-specific matches. Peaks for experiment and control samples versus the input sample were found by Transcription factor ChIP-seq tool (CLC GW). Peaks of the experimental samples that did not overlap with those of the control sample (without antibodies) were used in the further analysis. Chromosomal ideograms were prepared using Genome Decoration Page (www.ncbi.nlm.nih.gov/genome/tools/gdp). The ChIP-seq read data reported in this study were deposited in GenBank under the study accession PRJNA310826 and the experiment accessions SRX2312326–SRX2312330.

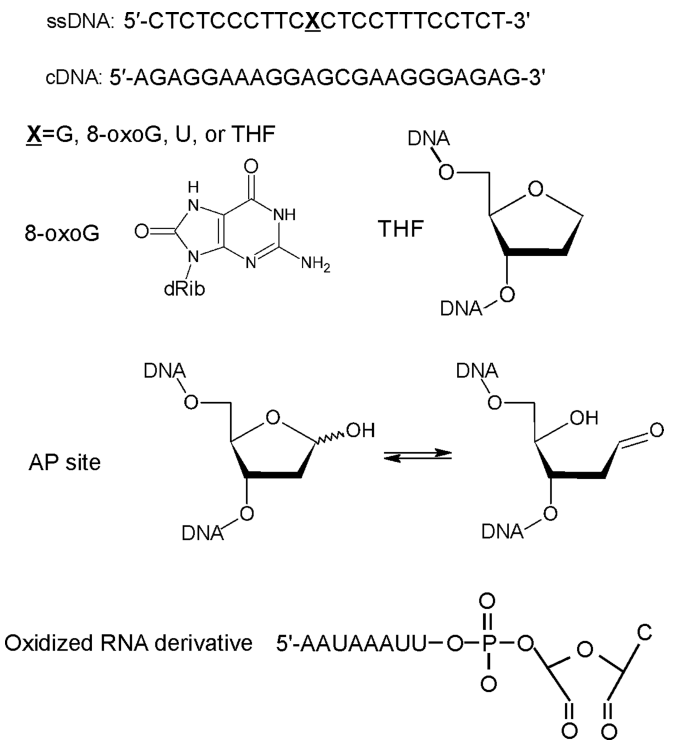

Figure 1. Nucleic acids and their derivatives used in this study. ssDNA, single stranded oligodeoxyribonucleotide (23-mer); cDNA, 23-mer complementary to the ssDNA. THF, tetrahydrofuran; 8-oxoG, 8-oxoguanine. AP site is shown in equilibrium between the hydroxyl and aldehyde forms.

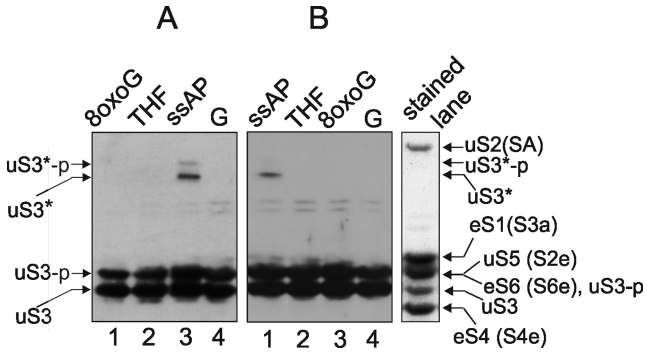

Figure 2. Identification of cross-links of ssDNA derivatives to 40S ribosome-bound uS3. Western blot analysis of 40S ribosomal proteins separated in SDS-PAGE with antibodies against mammalian uS3. Lanes 8oxoG, THF, ssAP and G correspond to the experiments with 40S ribosomes incubated with ssDNAs bearing residue of 8-oxoG, THF, AP site or unmodified G (Figure 1). Bands related to unphosphorylated and phosphorylated uS3 (uS3-p) are marked. The respective bands of cross-linked uS3 are indicated by asterisks; assignment of these bands to unphosphorylated and phosphorylated forms of uS3 is based on the data of our previous report (19). Panels A and B correspond to proteins isolated from 40S ribosomes after cross-linking procedure directly and after purification by centrifugation in sucrose gradient, respectively. 'Stained lane' shows total protein of 40S ribosomes separated in SDS-PAGE and stained by Coomassie Brilliant Blue G-250, ribosomal proteins (except uS3) corresponding to the bands are marked.

RESULTS

Ribosome-bound human uS3 can be trapped by AP site in ssDNA

We started to examine potential extra-ribosomal properties of the ribosome-bound human uS3 by checking whether it can be reductively cross-linked to an internal AP site within ssDNA (Figure 1). Treatment with a mild reducing agent such as $NaBH_3CN$ converts the Schiff base protein–DNA conjugate to a non-hydrolysable amine, thus stably tagging the protein with the strand of DNA that contained the AP site. Figure 2 shows the immunoblot of 40S ribosomal proteins after cross-linking of 40S ribosomes to 23-mer ssDNA bearing various lesions, including an AP site, with antibodies against uS3. Without cross-linking, uS3 is visualized by the specific antibodies as a double band since in human 40S ribosomes this protein is found in two forms, unphosphorylated and phosphorylated, the latter migrating slower (a detailed verification of two uS3 forms in the 40S subunit was presented in our previous report, see (19)). Both bands in the doublet have similar intensities, reflecting an approximately equal content of uS3 forms in the ribosome. Relative to the mixture of 40S ribosomal proteins, as revealed by Coomassie Blue staining, the doublet of bands corresponding to non-cross-linked uS3 migrated near two upper strong bands in the middle part of the Coomassie stained gel containing RPs eS1 (S3a), uS5 (S2e), RACK1 and eS6 (S6e) so that location of the slower migrating band of the doublet (corresponding to phosphorylated uS3) coincides with the faster migrating strong band. The doublet of bands of DNA-tagged uS3 was observed only with ssDNA-AP; these bands are seen above those of the unmodified protein since the attached oligonucleotide notably slows down protein migration during electrophoresis (Figure 2A, lane 3). No cross-linking to ssDNA containing other lesions, namely 8-oxoG (the most abundant oxidized purine) and THF (a non-aldehydic AP site analogue resistant to β-elimination) (Figure 1), was detected.

To ensure that the cross-linking of ssDNA-AP to uS3 occurred within the 40S ribosome rather than to the protein dissociated from it, we performed cross-linking with 40S subunits, then purified the subunits by centrifugation in a sucrose gradient, and analyzed proteins as described above. The double band corresponding to DNA-tagged uS3 was again revealed (Figure 2B, lane 1). Thus, uS3 within the human 40S ribosomal subunit is able to form a covalent adduct with an AP site in ssDNA. In the lane corresponding to the purified 40S ribosomes (Figure 2B, lane 1), the ratio of the DNA-tagged and unmodified uS3 was somewhat lower than it was before the sucrose gradient purification of ribosomes (Figure 2A, lane 3), which could be caused by partial dissociation of the DNA-tagged uS3 from the ribosomes during purification. Noteworthy, of two bands corresponding to DNA-tagged uS3, the upper one (presumably representing the phosphorylated protein) was much weaker than the lower one (see Figure 2); in the experiment with purified ribosomes, where the amount of DNA-tagged uS3 was lower than in non-purified ribosomes, the upper band was barely visible (Figure 2B, lane 1). Therefore, unphosphorylated uS3 recognizes AP sites in ssDNA much more efficiently than the phosphorylated protein.

To inquire whether any other RPs besides uS3 interact with ssDNA-AP, cross-linking of 40S ribosomes to ^{32}P-

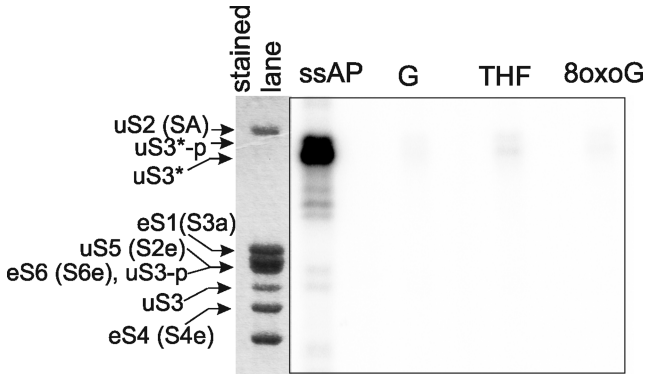

Figure 3. SDS-PAGE analysis of 40S ribosomal proteins cross-linked to 5′-^{32}P-labeled ssDNA derivatives (designations are the same as in Figure 2). Autoradiogram of the gel. 'Stained lane' is the same as in Figure 2. The results of urea-PAGE analysis of the respective reaction mixtures of the ssDNA derivatives with 40S ribosomes are presented in Figure 5B.

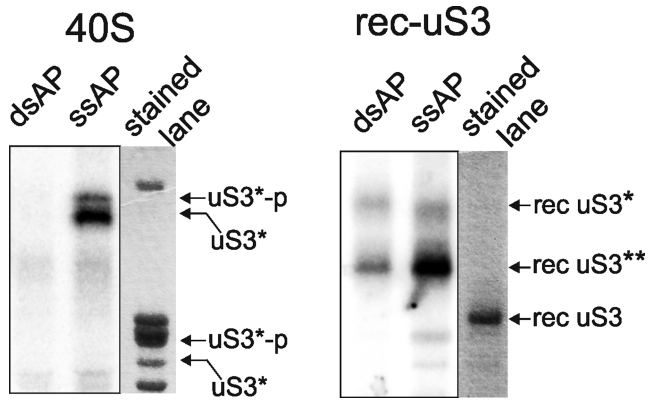

Figure 4. SDS-PAGE analysis of cross-linking of 5′-^{32}P-labeled ssDNA-AP and dsDNA-AP to the recombinant human uS3 and 40S ribosome-bound uS3. Designations ssAP and dsAP indicate ssDNA-AP and dsDNA-AP, respectively; other designations are the same as in Figures 2 and 3. Autoradiogram of the gel. Weak bands seen below the bands of cross-linked uS3 are not reproducible and are not discussed in the text. Quantification of the results presented in the right panel showed that the intensity of the band of rec uS3** in the lane *ssAP* is (3 ± 1)-fold of that in the lane *dsAP* and (4 ± 1)-fold of the intensity of the band of rec uS3* in the lane *ssAP*.

labeled ssDNA was performed; labeled ssDNA containing 8-oxoG or THF instead of the AP site (Figure 1) were used as well. The tagged proteins was observed as a doublet of radioactive bands only with ssDNA-AP (Figure 3), and the location of these bands exactly corresponded to those of the DNA-tagged uS3 revealed by immunoblotting (Figure 2). No reproducible visible bands that might be assigned to DNA-tagged proteins other than uS3 were seen in the gel. Therefore, uS3 is the only 40S protein capable of forming a covalent adduct with AP sites in ssDNA.

Ribosome-bound and isolated uS3 interact with AP site differently

As the next step, we have compared the abilities of ribosome-bound and isolated rec-uS3 to form covalent adducts with AP sites in ssDNA and dsDNA. Figure 4 shows that the patterns of cross-linking of AP site-containing oligodeoxyribonucleotides to ribosome-bound uS3 are significantly different from those occurring with rec-uS3. First, rec-uS3 could be cross-linked to both ssDNA-AP and dsDNA-AP, although cross-linking to the latter was much less efficient than to the former (Figure 4, right panel), while the ribosome-bound protein formed adducts only with ssDNA-AP (Figure 4, left panel). Second, uS3 cross-linked to ssDNA-AP within the 40S ribosome appeared as the closely spaced doublet of bands, likely representing two forms of the protein (Figure 4, left panel) mentioned above. In contrast, with rec-uS3 two radioactive products considerably differing in their electrophoretic mobility were observed (Figure 4, right panel). Based on our data on the relative mobilities of uS3 cross-linked to various ^{32}P-labeled oligonucleotides (19), we can attribute the lower band to rec-uS3 cross-linked to a 10-mer oligonucleotide remaining attached to the protein after the cleavage at the AP site. Cross-link of this kind arises because the cleavage of AP site by β-elimination occurs in the covalent intermediate, which then has to undergo hydrolysis to release the 5′-stretch of DNA, and this step is slow. The upper band evidently corresponds to the protein cross-linked to the uncleaved 23-mer ssDNA-AP, as the location of this band is close to that of the double band of uS3 cross-linked to ssDNA-AP within the 40S ribosome (Figures 2 and 3). One can notice that rec-uS3 cross-linked to the full-length ssDNA migrates slightly slower than the cross-linked protein from 40S ribosomes, which is expected because the recombinant protein is 20 residues longer due to a His-tag and a linker. Thus, ribosome-bound uS3, in contrast to free rec-uS3, can form covalent adduct only with ssDNA-AP, and this adduct appears to contain only the full-length ssDNA. Therefore, it seems that ribosome-bound uS3 is unable to cleave the DNA at AP site as rec-uS3 does, or at least the AP lyase activity of the ribosome-bound protein is much lower in comparison with the free protein. The results presented in Figure 3 also show that rec-uS3 strongly prefers to cross-link to 10-mer oligonucleotide remaining after cleavage of ssDNA-AP at the AP site than to the full-length uncleaved DNA, and that the cross-link to cleaved dsDNA-AP is much weaker than that to ssDNA-AP.

To find out more definitely whether ribosome-bound uS3 retains the AP lyase activity inherent to the free protein, we examined the ability of free rec-uS3 and ribosome-bound uS3 to cleave ssDNA-AP and dsDNA-AP using the respective ^{32}P-labeled DNA oligomers. Figure 5 shows that rec-uS3, in agreement with the cross-linking data above, is able to cleave both ssDNA-AP and dsDNA-AP at the AP site, and that ssDNA can be almost completely digested at this lesion when the protein is added in sufficient amounts. The lanes corresponding to rec-uS3 in Figure 5A show a significant portion of radioactivity trapped in the upper bands near the wells, which could be caused by inability of proteins of the size of uS3 with cross-linked DNA-AP to enter 20% urea-PAG used here as well as by the formation of insoluble protein–DNA-AP aggregates taking place when recombinant ribosomal proteins are mixed with nucleic acids (e.g. see (24)). In the respective experiments with 40S subunits, radioactive bands similar to those mentioned above

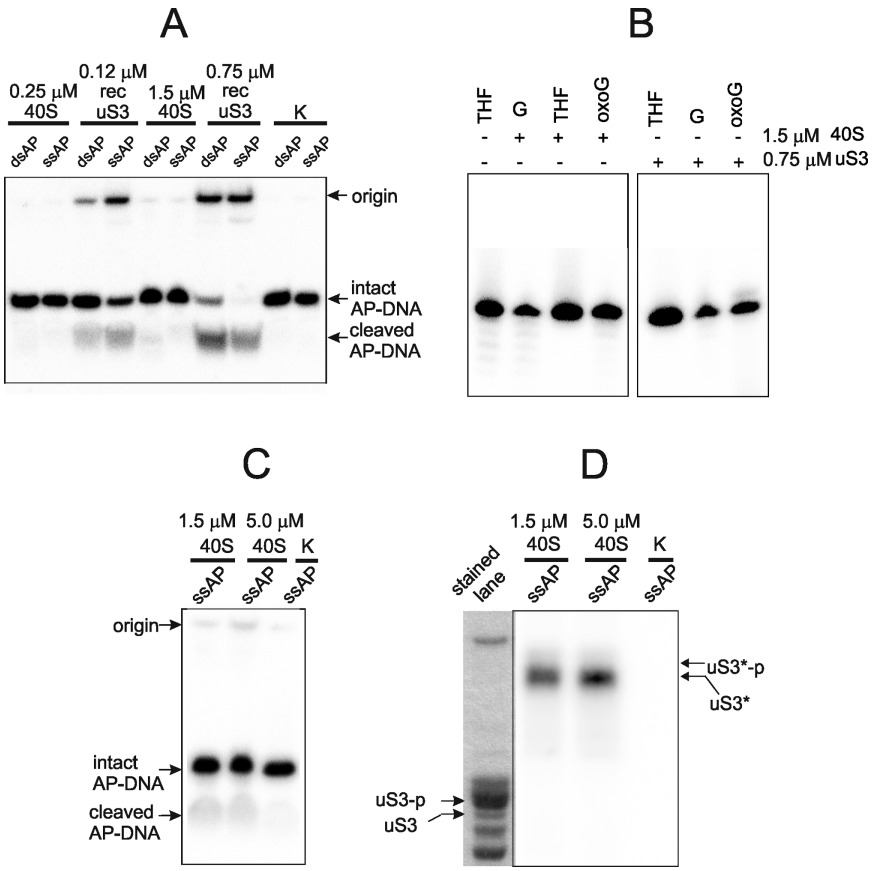

Figure 5. Urea-PAGE analysis of cleavage of 5′-^{32}P-labeled DNA derivatives by rec-uS3 and by 40S ribosomes taken at different concentrations (indicated above or on the side of the lanes); panels **A** and **C** correspond to the experiments carried out with different preparations of 40S ribosomes. Panel **D** presents SDS-PAGE analysis of cross-linking of the labeled ssDNA-AP to ribosome-bound uS3 in aliquots from the reaction mixtures used for examination of DNA-AP cleavage in the panel C. Note that intensities of bands of cross-linked uS3 in the panels C and D could not be directly compared with each other because of different exposure times. Designations are the same as in the previous Figures. Autoradiograms of the gels. Lanes 'K' correspond to the control experiments with ssDNA-AP and dsDNA-AP incubated without rec-uS3 and 40S ribosomes.

are much weaker (at the lowest 40S concentration, these bands are hardly visible in Figure 5A but they could be clearly seen at higher exposure not shown here) indicating that radioactivity in the respective bands with rec-uS3 corresponds mainly to protein–DNA-AP aggregates. Undamaged, 8-oxoG- or THF-containing DNA was not cleaved by either 40S ribosomes or free rec-uS3, and the patterns with these DNA derivatives were not noticeably affected by the presence of uS3 or 40S subunits (Figure 5B). Mirroring better cross-linking yield, rec-uS3 cleaved the AP site in ssDNA much more efficiently than the same lesion in dsDNA (Figure 5A). Quantification of the results showed that at 0.12 μM of rec-uS3, the amount of undigested dsAP was (2.8 ± 0.8)-fold more than that obtained with ssAP, whereas at 0.75 μM of rec-uS3, undigested ssAP was undetectable. Significant differences in the substrate properties of ssDNA-AP and dsDNA-AP are reported here for the first time, since, to our knowledge, the earlier studies (8–11) utilized only dsDNA. In contrast to rec-uS3, incubation of AP site-containing ssDNA and dsDNA with 40S ribosomes did not lead to substantial DNA cleavage, and even at the highest available 40S ribosomes concentration, it was barely detectable; the results with different ribosome preparations were nearly identical (compare results in the panels A and C in Figure 5). Thus, ribosome-bound uS3 loses the ability to cleave DNA at AP sites inherent to the isolated protein.

Ribosome-bound uS3 is cross-linked to AP site-containing DNA through the KH domain

Previously, we have found that unstructured RNAs with the 3′-terminal ribose oxidized to dialdehyde can be efficiently cross-linked to peptide 55–64 in the KH domain of the ribosome-bound human uS3, and Lys62 is the only residue capable of interaction with aldehydes in this peptide (19). It is reasonable to suggest that AP site-containing DNA could be cross-linked to the same uS3 fragment through the aldehyde moiety. To test this hypothesis, we examined the ability of ssDNA-AP to interact with 40S subunits whose peptide 55–64 was preblocked by cross-linking to an dialdehyde-derivatized oligoribonucleotide (Figure 1). According to our previous results, incubation of 40S ribosomes with such derivative of AAUAAAUUC yields ribosome preparations with 70–80% of uS3 molecules tagged with the RNA 9-mer at Lys62. Figure 6A shows that blocking Lys62 of the ribosome-bound uS3 with the 9-mer RNA drastically decreased the yield of cross-linking of ssDNA-AP to the protein in 40S ribosomes. The effect was not observed with 40S

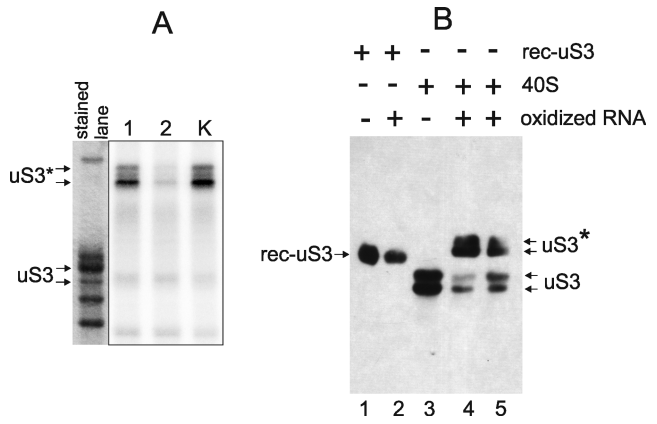

Figure 6. Cross-linking of 3′-dialdehyde derivative of AAUAAAUUC to ribosome-bound uS3 (**A**) and rec-uS3 (**B**) and the effect of the derivative cross-linked to ribosome-bound uS3 on its ability to trap ssDNA-AP (A). Panel A, autoradiogram of the gel after SDS-PAGE analysis of proteins isolated from 40S subunits incubated with 5′-^{32}P-labeled ssDNA-AP without the nonaribonucleotide derivative (1), from the subunits preincubated with this derivative (2) and from the subunits preincubated with unmodified nonaribonucleotide (K). Panel B, western blot analysis of rec-uS3 (lanes 1 and 2) and of 40S ribosomes (lanes 4 and 5) incubated with the derivative of AAUAAAUUC; lanes 1, 2 and 5, incubation in rec-uS3 storage solution; lane 4, incubation in buffer A. Cross-links to the ribosome-bound uS3 are seen in both solutions albeit in rec-uS3 storage solution their yield is somewhat lower than that in buffer A. Lanes 1 and 3 correspond to control rec uS3 and 40S ribosomes incubated without the oligoribonucleotide derivative, respectively. Quantification of the results from the panel A showed that the intensity of the uS3* bands in the lane 2 is (0.24 ± 0.04) and (0.21 ± 0.05) of that in the lanes 1 and K, respectively.

ribosomes that were preincubated under the conditions of the 9-mer derivative cross-linking but without the derivative or with the unmodified 9-mer instead of its dialdehyde derivative (lanes 1 and K, respectively). Thus, cross-linked RNA 9-mer prevented the ribosome-bound uS3 from reacting with damaged DNA, implying that site of cross-linking to ssDNA-AP in the protein coincides with or is located close to Lys62. Interestingly, free rec-uS3 was not modified by the RNA derivative at all (Figure 6B), suggesting possible differences between free and ribosome-bound uS3 in their mode of interaction with single-stranded nucleic acids.

uS3 binds preferentially to NADs in pericentromeric regions of chromosomes *in vivo*

To identify the genome-wide uS3 binding regions on DNA *in vivo*, we carried out the ChIP-seq analysis in HEK293 cells. This method reveals binding sites of both free and ribosome-bound uS3 since the protein in both forms could be cross-linked to DNA by formaldehyde, and the conditions of chromatin preparation after the cross-linking before immunoprecipitation are disruptive for ribosomal particles. In particular, SDS-containing buffer was utilized to dissolve nuclei, and chromatin was sheared by sonication. Therefore, immunoprecipitation involved DNA fragments cross-linked to free uS3 as well as those that were cross-linked to uS3 when it resided in ribosomal particles. ChIP and control samples and input DNA were sequenced, yielding an average of 15 million uniquely mapped reads on version hg38 of the human genome using CLC GW.

We observed 229 peaks (see Supplementary Table S1), and the peak distribution analysis of ChIP-seq data clearly revealed that uS3 prefers to bind to pericentromeric chromosome regions. These regions of chromosomes 1, 2, 4, 7, 10, 16, 17 and 20 were predominantly implicated in the binding (Figure 7). Besides, in chromosomes 7, 10, 16 and 20 the ChIP-seq peaks overlapped with or were adjacent to so-called NADs (nucleolus-associated chromatin regions) (Figure 7) that interact with nucleoli (25,26). A comparison of our results presented in Supplementary Table S1 with data on chromosome locations corresponding to NADs in HeLa cells showed that the genome coordinates of the most of peaks identified here by ChIP-seq were close to NADs regions identified in (25) by means of high-throughput sequencing techniques. It is noteworthy that the ChIP-seq peaks were presumably located in intergenic regions, although 12 of them are mapped to locations corresponding to genes (see Supplementary Table S1).

Further analysis showed that the majority of identified peaks are located in DNAse I hypersensitive regions, where chromatin is not condensed and DNA is more accessible to DNAse I cleavage and to binding of DNA-binding proteins such as transcription factors (27–30). These regions are considered as possible regulation sites (29,30) that might serve as markers of many types of *cis*-regulatory elements such as promoters, enhancers, silencers and insulators.

DISCUSSION

In this study, we explored the activities of human ribosome-bound uS3 and free recombinant uS3 towards AP sites in DNA. The results show that uS3 within 40S ribosome is able to form a covalent adduct with ssDNA-AP but cannot cleave it, and the uS3 region implicated in the formation of such adduct is the same as had been identified earlier as recognizing dialdehyde derivatives of unstructured RNAs. The unphosphorylated form of ribosome-bound uS3 is more active in cross-linking to ssDNA-AP. Free rec-uS3 can form covalent intermediates both with ssDNA-AP and dsDNA-AP with the following cleavage, consistent with the literature data on its AP lyase activity, albeit with a preference for ssDNA-AP. However, free rec-uS3 does not recognize dialdehyde RNA derivatives and does not prefer to cross-link to uncleaved ssDNA-AP as the ribosome-bound protein does. Thus, our findings demonstrate that ribosome-bound uS3 almost completely loses the AP lyase activity and give indications for different extra-ribosomal functions of free and ribosome-bound uS3. Below we discuss possible reasons for the loss of the uS3 AP lyase activity within the 40S ribosome, and possible functional implications of the ability of ribosome-bound uS3 to form a covalent adduct with ssDNA-AP without its cleavage; cellular events, in which recognition and cleavage of ssDNA- and dsDNA-AP sites by free uS3 might occur, are considered as well.

Why ribosome-bound uS3 lacks the AP lyase activity and does not trap AP site-containing dsDNA?

The structure of the active site of human uS3 responsible for its enzymatic activities possibly involved in DNA repair, in particular, for the AP lyase activity, remains unknown.

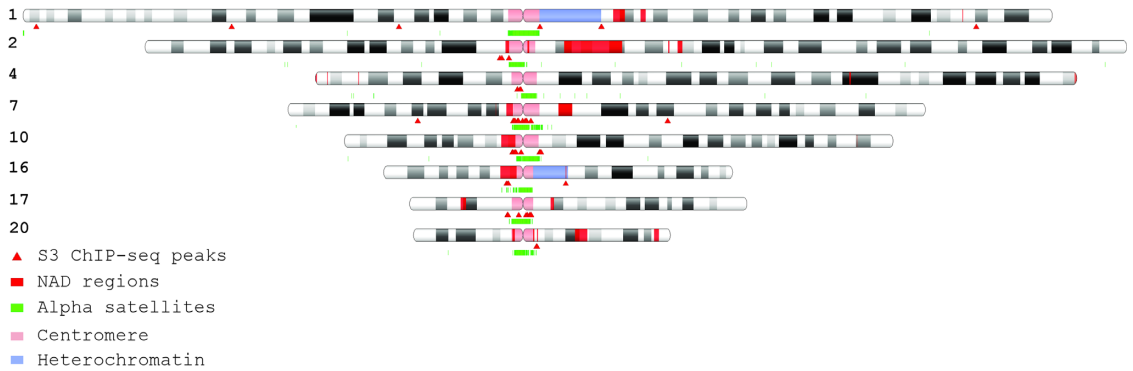

Figure 7. Distribution pattern of uS3 ChIP-seq peaks on chromosome ideograms (hg38). Only chromosomes that contain more than five peaks are presented. The positions of the peaks are shown by red triangles. Chromosomal regions are color coded as shown below the ideogram.

However, assumptions about the protein fragments forming its catalytic center can be made from indirect data. In particular, it has been reported that a single mutation K132A drastically reduces the affinity of human uS3 for AP site-containing dsDNA yet does not affect the protein ability to cleave DNA at the AP site, indicating that Lys132 is probably involved in the recognition of AP sites within dsDNA (10). Two other amino acid residues, Asp154 and Lys200, have been suggested to be responsible for catalytic activity of yeast uS3 based on the effect of mutations at these residues on the enzymatic activity towards AP sites in dsDNAs (31). These residues are conserved, occupying positions 154 and 201, respectively, in the human uS3 sequence (see (6)), and might be responsible for the catalytic activity of human uS3.

Analysis of the available structures of the mammalian 40S ribosomal subunit deposited in Protein Data Bank (e.g. PDB ID: 4KZX, (32)) shows that all mentioned above putative catalytic residues of human uS3 reside in the conserved C-terminal domain adjacent to the unstructured C-terminal tail located far away from Lys62 in the KH domain responsible for cross-linking with single-stranded nucleic acids including AP sites (Figure 8). Moreover, the analysis reveals that the C-terminal domain regions where Lys132, Asp154 and Lys201 are found are not easily accessible for DNA binding. In particular, Lys201 is located behind RP uS17 and likely interacts with it; Lys132 and Asp154 are not implicated in the intra-ribosomal interactions but are considerably shielded from solution by fragments of uS3 itself. We infer that ribosome-bound uS3 lacks both AP lyase activity and ability to be cross-linked to AP site-containing dsDNAs most probably because the putative active site(s) responsible for these reactions in the free protein are hidden in the 40S subunit. The structure of full-length free uS3 is currently not known; nevertheless, it is reasonable to assume that free and ribosome-bound uS3 adopt quite different shapes due to multiple interactions of uS3 with the 18S rRNA and neighboring proteins in the ribosome. The suggested uS3 target for ssDNA-AP site cross-linking, Lys62, is exposed in the KH domain at the edge of a narrow groove between the head and the body of the 40S subunit, which for all that could fit ssDNA. Why does ribosome-bound uS3 prefer to interact with AP sites in ssDNAs rather than in dsDNAs? One can assume that accommodation of dsDNA requires a wider groove, or that AP site in dsDNA is sterically less accessible for the reaction than in ssDNA.

In principle, a possibility that KH domain may also contribute to AP site recognition by free uS3 could not be excluded. Lys62 in human uS3 lies in a short turn connecting helices αB and αC of the KH domain. In all available structures of non-ribosomal KH domain-containing proteins (33–38), this turn binds the backbone of the interacting DNA or RNA. Despite the KH domain is considered a single-stranded nucleic acids binding motif (34,39), the structures show that the isolated domain can bind hairpin RNA (40). However, in order to get access to the reactive C1′ of the AP site in dsDNA, the protein likely has to undergo a conformational rearrangement or induce rearrangement in the DNA molecule (41). This might not be a problem for the free protein but prove impossible in the ribosome-bound uS3, as part of the KH domain is involved in the intra-ribosomal interactions, restricting its conformational flexibility. However, the reduced flexibility of the KH domain of uS3 within 40S subunit does not prevent its interaction with AP site in ssDNA and with dialdehyde RNA derivatives. Thus, one can assume that structural features of KH domain in free and ribosome-bound uS3 proteins are considerably dissimilar, and they mainly interact with AP sites by different protein fragments. Our findings suggest that KH domain structure of ribosome-bound uS3 is much more suitable for interacting with aldehyde groups in single-stranded nucleic acids than that of isolated protein and likely reflect different modes of single-stranded nucleic acids binding by isolated and ribosome-bound human uS3 proteins. Apparently, free uS3 interacts with AP sites mainly by its catalytic center responsible for the AP lyase activity, whereas the ribosome-bound protein traps them by its KH domain located far away from the catalytic center.

Functional assignments of capability of ribosome-bound uS3 of cross-linking to AP site-containing ssDNAs and of AP lyase activity of free uS3 towards these DNAs

The ability of ribosome-bound human uS3 to interact with ssDNA-AP sites without their cleavage evidently does not immediately result in DNA repair. Thus, the questions arise as to where and when interactions between AP sites in ss-

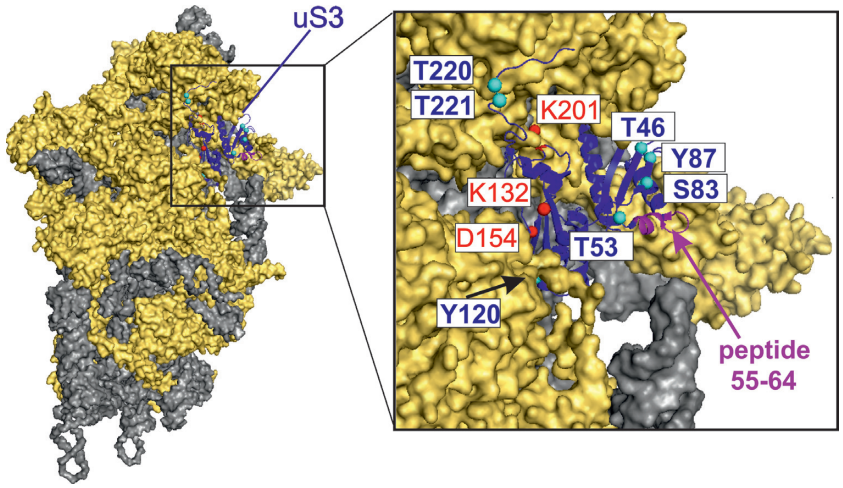

Figure 8. Positioning of uS3 on the mammalian 40S ribosomal subunit (PDB accession number 4KZX) (32). General view from the solvent side and zoomed structure of the 40S subunit region containing uS3, which is shown in dark blue. Segment 55–64 of uS3 interacting with single-stranded nucleic acids bearing aldehyde groups is shown in magenta; amino acid residues belonging to the putative catalytic center previously suggested for isolated uS3 are presented as red spheres, and phosphorylation sites found in (19) are shown as cyan spheres, one of which (Y120) is almost hidden in the ribosome structure (indicated by black arrow).

DNA and ribosome-bound uS3 may occur in a cell, and to what consequences.

It is well known that, even under normal conditions, tens of thousands of AP sites spontaneously arise in DNA of each human cell every day (e.g. see (42,43)). Our ChIP-seq data mapped many of the sites of uS3 binding to DNA to pericentromeric domains and nucleolus-associated chromatin, which is enriched in the centromeric and pericentromeric DNA (26,44). These chromosomal domains contain a large fraction of alpha satellite repeats, which are prone to form loops with a significant exposure of ssDNA (45–47). Moreover, the amount of AP sites in alpha satellite DNA is greatly increased in comparison with regular DNA (45). When we mapped the repetitive reads on the human genome, the frequency of alpha satellites was about 30% higher than in the control without the antibodies, which indicates that uS3 could also be associated with alpha satellite DNA. This suggests that the protein might participate in the DNA repair or in signaling for damage in these chromatin regions.

It should be noted that the ChIP-seq approach doesn't tell whether free or ribosome-bound uS3 is trapped, neither does ChIP-seq discriminate between protein bound to ss- and dsDNA. Therefore, we cannot assign the observed uS3 interactions in the genome to particular forms of the protein and the DNA; nevertheless, one can expect that at least a fraction of these interactions relates to the ribosome-bound protein.

What could be functional implications of the interactions of ribosomal or pre-ribosomal particles with ssDNA mediated by uS3? Assembly of 40S subunits takes place in the nucleolus with following maturation of the particles in the nucleus; therefore, these particles can bind ssDNA regions in or near nucleolus. Under conditions of oxidative stress, when the content of AP sites in DNA would be much higher than usual, trapping of ribosome-bound uS3 on ssDNA in the loops in pericentromeric repeats becomes quite probable. An obstacle of this size would likely arrest DNA topoisomerases and helicases operating in the nucleolus to prevent illegitimate recombination between the repeated sequences (48,49) and present an unmistakable signal for DNA damage response systems, possibly attracting proteins responsible for DNA–protein cross-link repair and signaling. In this regard, an interesting possibility would be to activate the Fanconi anemia (FANC) pathway, which responds to the aldehyde-induced DNA–protein cross-links (50) and is active in the nucleolus resolving R-loops formed during transcription of repetitive rDNA units (51). It is known that uS3 undergoes phosphorylation during 40S subunit assembly in the nucleolus with subsequent dephosphorylation in the mature ribosome (52). Therefore, our finding that the phosphorylated form of ribosome-bound uS3 traps ssDNA-AP much less efficiently allows us to assume that need for damage signaling might trigger dephosphorylation of uS3 activating the capability of the ribosome particle to cross-link to DNA. In our previous study (19), we found phosphorylated amino acid residues in four peptides located in different parts of ribosome-bound uS3 and identified these residues as T46/T53, S83/Y87, Y120, T220 and T221 (Figure 8). Unfortunately, this wide scattering of phosphorylation sites over the uS3 structure makes it hard to hypothesize which uS3 site(s) should become dephosphorylated to enable the ribosomal particle to trap ssDNA.

It is worth mention that RNA has its own quality control to exclude damaged templates from translation. For example, apurinic/apyrimidinic endonuclease 1 (APEX1) interacts with rRNA as well as with proteins involved in the ribosome assembly and RNA maturation, and is able to cleave rRNA at AP sites (for review, see (53)). Recently, it has been shown that 8-oxoG stalls mRNA translation on 80S ribosomes, which leads to degradation of the damaged mRNA via no-go decay, one of the mRNA quality-control processes (54). Therefore, we can speculate that uS3 is able to interact with AP sites not only in DNA but in RNA

too. In the latter case the protein might be involved in preventing aberrant translation of abasic mRNAs, e.g. translation might be stalled via cross-linking of AP sites to the ribosome-bound uS3. Examination of this hypothesis is a task for our future studies.

Isolated uS3, which has an AP lyase activity, has been earlier considered as a possible participant of base excision DNA repair (BER) (7–11). However, it is largely unclear whether uS3 replaces canonical BER enzymes or supplements their action; moreover, it is still unknown where and how the putative uS3 repair activity shows up in a cell. It is unlikely that human uS3 appreciably contributes to global nuclear DNA repair in human cells, because the nucleus possesses a full complement of canonical BER enzymes, and the major fraction of endogenous uS3 is ribosome-bound. However, the mode of DNA repair in the nucleolus-associated chromatin domains is probably different from the canonical pathway, since some BER enzymes are excluded from the nucleolus, while others actively redistribute between nucleoli and the nucleoplasm in response to genotoxic stimuli (55–58). Thus, free uS3 might be specifically involved in the repair in the nucleolus and nucleolus-associated chromatin.

Overall, the results of this study suggest that human rp uS3 could be involved in two different modes of non-translational interactions with AP sites in DNA, with specific functions of the isolated and ribosome-bound proteins. One of these functions is based on well-known AP lyase activity of uS3; here we found that it is present in free uS3 but not in the ribosome-bound protein, and that the protein prefers ssDNA rather than dsDNA as a substrate. Another non-translation function inherent to ribosome-bound uS3 but not to the free protein relates to the ability to trap, via the KH domain, AP sites in ssDNA without their cleavage. The free uS3 could be implicated in DNA repair in nucleolus and nucleolus-associated chromatin while ribosome-bound uS3 might be involved in signaling for damage caused by oxidative stress. Establishing the cellular mechanisms mediating these interactions of free and ribosome-bound uS3 with AP sites in DNA would require methods to distinguish between free and ribosome-bound uS3 *in situ*, which are presently absent, and this could be the next line of investigation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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