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Characterization of RNA in Saliva

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Background: **We have previously shown that human mRNAs are present in saliva and can be used as biomarkers of oral cancer. In this study, we analyzed the integrity, sources, and stability of salivary RNA.**

Methods: **We measured the integrity of salivary RNA with reverse transcription followed by PCR (RT-PCR) or RT-quantitative PCR (RT-qPCR). To study RNA entry sites into the oral cavity, we used RT-PCR analysis of salivary RNA from the 3 major salivary glands, gingival crevice fluid, and desquamated oral epithelial cells. We measured stability of the salivary β-actin mRNA by RT-qPCR of salivary RNA incubated at room temperature for different periods of time. We measured RNA association with other macromolecules by filtering saliva through pores of different sizes before performing RT-qPCR. To assess RNA–macromolecule interaction, we incubated saliva with Triton X-100 for different periods of time before performing RT-qPCR.**

Results: **In most cases, we detected partial- to full-length salivary mRNAs and smaller amounts of middle and 3′ gene amplicons compared with the 5′. RNA was present in all oral fluids examined. Endogenous salivary β-actin mRNA degraded more slowly than exogenous β-actin mRNA, with half-lives of 12.2 and 0.4 min, respectively ($P < 0.001$). Salivary RNA could not pass through 0.22 or 0.45 μm pores. Incubation of saliva with Triton X-100 accelerated degradation of salivary RNA.**

Conclusions: **Saliva harbors both full-length and partially degraded forms of mRNA. RNA enters the oral cavity from different sources, and association with macromolecules may protect salivary RNA from degradation.**

Saliva is an important component for speech, digestion of food, and protection from microorganisms. In addition, biomarkers for viral, bacterial, and fungal parasitic infections and for both systemic and nonsystematic diseases have been found in saliva *(1–3)*. These markers can be various molecular species ranging from proteins and antibodies to DNA and viral RNA.

Recently, human RNA obtained from cell-free saliva was shown to be a biomarker for oral cancer *(4, 5)*. By means of expression-based microarray analysis and reverse transcription-quantitative PCR (RT-qPCR),[6] we were the first to show that cell-free saliva from healthy individuals contains more than 3000 species of mRNA *(6)*. We also found 17 mRNAs that were present in higher amounts in patients with oral cancer than in healthy persons *(4)*. We developed a prediction model based on salivary mRNA concentrations of 4 of these genes and showed that they have 91% sensitivity and specificity for oral cancer detection.

Little is known about the molecular nature and properties of human salivary RNA *(6, 7)*. Saliva is known to contain ribonucleases from various sources, and this fact may explain the lack of analysis of human salivary RNA *(8–11)*. It is not clear how RNA and ribonucleases can coexist in saliva. One possible explanation is that endogenous salivary RNA is protected from degradation, as was shown for plasma RNA *(12, 13)*. In addition to human RNAs, viral RNAs can also be detected in saliva *(14–16)*. The mechanism of viral RNA stability in saliva is also not well understood.

RNA can enter the oral cavity through various routes, including saliva secretions from the 3 major salivary glands (the parotid, submandibular, and sublingual glands) and minor glands, gingival crevice fluid (GCF), and desquamated oral epithelial cells. Like many micro- and macromolecules, RNA in salivary gland secretions could originate from acinar cells or by circulation

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[6] Nonstandard abbreviations: RT-qPCR, reverse transcription-quantitative PCR; GCF, gingival crevice fluid; hβ- and mβ-actin, human-specific and mouse-specific β-actin, respectively; and NSCT, normal salivary core transcriptome.

(1, 3, 17, 18). GCF is also a likely source of RNA, because many blood cells and their cellular components are released into the oral cavity from GCF *(19)*. Frequent turnover of epithelial cells in the oral cavity could be another source of RNA. Finally, microwounds inside the oral cavity could release the RNA directly from the blood.

We report the first characterization of the origin, integrity, and stability of human salivary RNA.

Materials and Methods

PARTICIPANTS

All of the healthy volunteers included in this study signed the UCLA Institutional Review Board–approved consent form. The mean age of the volunteers was 31 years (range, 26–43 years). The volunteers had no history of malignancy, immunodeficiencies, autoimmune disorders, hepatitis, or HIV infection.

SALIVA AND BLOOD COLLECTION AND RNA ISOLATION

The unstimulated supernatant phase of the whole saliva was collected as described previously *(4)*. Blood was collected in BD Vacutainer Tubes containing clot activator (BD Biosciences) and centrifuged at 1000*g* for 10 min, after which the serum was removed for RNA isolation.

Oral fluids from the 3 major salivary glands, GCF, and oral epithelial cells were collected within a time span of 2 days. Samples were stored on ice during the collection, and Superase Inhibitor (Ambion) was added to samples immediately after the collection, to a final concentration of 100 units/mL. Samples were then stored in −80 °C, or RNA was isolated immediately. Stratified saliva secretions were stimulated by citric acid. Parotid saliva was collected as described by Lashley *(20)*, using a Lashley-like cup that was fabricated by the UCLA Life Science Machine Shop. Submandibular and sublingual saliva collections were obtained by use of a Wolff collector according to the published protocol *(21, 22)*. GCF was collected with the paper strip sampling technique with minor modifications *(23)*. Briefly, 5 sterile paper strips (Periopaper) were gently inserted into the mesiolabial or distolabial gingival sulcus between the upper anterior teeth within 2 mm. To prevent saliva contamination during GCF collection, oral fluid was first removed with cotton rolls at the sampling site and the site was dried with a gentle stream of air. After 90 s of GCF isolation, the strips were placed in microcentrifuge tubes containing 500 μL of phosphate-buffered saline containing Triton X-100. When blood contamination was noticed on the strip during the GCF sampling, the paper strip was discarded. Oral epithelial cells were collected by use of a Cytology Brush (Medical Packaging). The buccal mucosa was brushed gently 5 times after the field was dried by use of dental gauze. The Cytology Brush was then place in a microcentrifuge tube containing 2 mL of phosphate-buffered saline containing Triton X-100 for 30 min. The oral epithelial cells were centrifuged at 14 000*g* for 10 min at 4 °C.

The volume of whole saliva collected from each participant, shown in Fig. 2, ranged from 2 to 4 mL. The volume of parotid saliva collected was 1.0–1.5 mL, the volume of submandibular saliva was 0.50–0.70 mL, and the volume for the sublingual collection was 0.03–0.05 mL.

Supernatant phases of whole saliva, gland-specific saliva, and GCF were obtained by centrifugation at 2600*g* for 15 min at 4 °C. The GCF strips were processed by inversion of the microcentrifuge tubes for 30 min at room temperature. The strips were then removed from the buffer solution.

We isolated salivary and serum RNA with a QIAamp Viral RNA Kit (Qiagen) as described previously *(4)* and RNA from oral epithelial cells with an RNeasy Mini Kit (Qiagen); we quantified RNA with the Ribogreen Kit (Molecular Probes).

RT-PCR AND RT-qPCR

We performed RT-PCR with the GeneAmp RNA PCR Kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, we used 3 μL of purified RNA in a 20-μL (total volume) reverse transcription reaction. Except for the experiments shown in panels D and E of Fig. 1, random hexamers were used during the reverse transcription step. Of this 20-μL reverse transcription product, 3 μL was used for the PCR or qPCR reactions. RT-PCR reactions were carried out in the PTC-200 Thermal Cycler (MJ Research), and qPCR was performed with SYBR Green Supermix (Bio-Rad) in the ICycler thermocycler (Bio-Rad). Triplicate measurements were done per sample for all qPCR reactions performed.

MEASUREMENT OF DIFFERENT PARTS OF INTERLEUKIN 8 AND β-ACTIN mRNAs BY RT-qPCR

A plasmid that contains full-length human interleukin 8 (*IL8*)[7] cDNA, which was used as a DNA calibrator for the *IL8* qPCR reactions in Fig. 1E, was purchased from Origen Technologies, Inc. For β-actin DNA calibrator, β-actin cDNA was amplified by use of 5′ forward primer (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue6/) and a reverse primer, 5′-TCAAGTTGGGGGACAAAAAG-3′, which ends at nucleotide 1694 of β-actin cDNA. The gel-extracted PCR product was inserted in the pCR2.1-TOPO vector by use of TOPO TA cloning reagents according to the manufacturer's instructions (Invitrogen). The insert was confirmed by sequencing and PCR. Reverse transcription was performed with specific reverse primers at 42 °C for 45 min. We generated calibration curves by use of serial dilutions

[7] Human genes: *IL8*, interleukin 8; *RPS9*, ribosomal protein S9; *RPS9P2*, ribosomal protein S9 pseudogene 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; and *SAT*, spermidine/spermine N1-acetyltransferase.

of the plasmid DNA. The amounts of RNA from other regions were expressed relative to the 5′ region.

MICROARRAY

U133 plus 2.0 microarray chips (Affymetrix) were used, and the experimental procedure was described previously *(4)*. The dchip program was used to analyze the data *(24)*.

TIME-COURSE ANALYSIS OF SALIVARY RNA

The supernatant phases of saliva samples from 3 participants were used. At the beginning of the incubation, 2 μg of purified total RNA from mouse cell line ES-LW1 was added per 1 mL of saliva supernatant. Incubation took place at room temperature for up to 20 min. At each time point, 300 μL of saliva was removed and incubated with a 4-fold excess (by volume) of lysis buffer at room temperature until the time course was completed.

SALIVA AND SERUM FILTRATION

Supernatant phases of saliva samples from 5 participants and sera from 4 participants were passed through 0.22 μm (Millipore) and 0.45 μm (Osmonics) syringe filters, and equal volumes of filtered samples were used for RNA isolation and RT-qPCR.

INCUBATION OF SALIVA WITH TRITON X-100

We added Triton X-100 (Sigma) to saliva to a final concentration of 10 mL/L and incubated the samples at room temperature for up to 20 min. When RNA was isolated from these samples, we added water and Triton X-100 to Triton-added and water-added samples, respectively, to balance the chemical compositions between samples.

PRIMERS

Primers that generate short amplicons were described previously *(4–6)*. Primers that were not described elsewhere are shown in Table 1 of the online Data Supplement. All primers were designed using the PRIMER3 software and were synthesized commercially (Sigma-Genosys) *(25)*. Human-specific β-actin (hβ-actin) and mouse-specific β-actin (mβ-actin) primers were checked for their target specificities by use of both human and mouse RNA. The middle/3′ primers for β-actin and 5′ primers for *IL8* are the same as the ones used in Fig. 1B. The amplification regions for the *IL8* 5′, middle, and 3′ primers are nucleotides 358–445, 694–920, and 1356–1472 of *IL8* mRNA, respectively. The amplification regions for β-actin 5′, middle, and middle/3′ primers are nucleotides 209–341, 785–969, and 1006–1159 of β-actin mRNA, respectively. Note that the ribosomal protein S9 (*RPS9*) primers that yield short and long PCR primers can also recognize a ribosomal protein S9 pseudogene 2 (*RPS9P2*) mRNA and yield the same size products. It is therefore possible that the *RPS9* PCR products in panels B and C of Fig. 1 are produced from both *RPS9* and *RPS9P2* mRNAs.

Results

INTEGRITY OF SALIVARY RNA

Previous expression-based microarray analysis showed that 185 different transcripts were consistently detected in the supernatants of 10 healthy human saliva samples *(6)*. We refer to these transcripts as the normal salivary core transcriptome (NSCT). As a first step to understand these NSCT mRNAs, we evaluated the integrity of 5 NSCT mRNAs from 8 healthy participants. The genes that we selected were β-actin, *RPS9*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *IL8*, and spermidine/spermine N1-acetyltransferase (*SAT*) mRNAs. β-Actin, *RPS9*, and *GAPDH* are cellular housekeeping genes, and their transcripts are present at high concentrations in saliva *(6)*. *IL8* and *SAT* were used as oral cancer biomarkers in our previous study *(4)*. To first validate the presence of these mRNAs in the saliva samples, we performed RT-PCR using primers that yield short PCR amplicons that range from 88 to 154 bp (Fig. 1A). These 5 genes, their full-length mRNAs, the expected sizes of the PCR amplicons, and the locations of these amplicons on the mRNA (in parentheses) are listed in Fig. 1A. In all participants, we were able to successfully detect short PCR amplicons for all 5 mRNAs (Fig. 1B). Initially for participant 3, no amplicons or only small amounts were detected, likely because of the small amount of starting RNA material in the saliva. We detected all 5 mRNAs (Fig. 1B, lane 3*) in participant 3 by use of a 5-fold higher amount of RNA and 5 additional PCR cycles compared with the other samples. These data confirm that the NSCT mRNAs are present in the saliva of healthy persons.

We next sought to determine whether saliva contains any full-length mRNA by performing RT-PCR using primer pairs for which the amplicons nearly span their respective full-length mRNAs (see Fig. 1A, long amplicons). As shown in Fig. 1C, *SAT* PCR amplicons could be seen in 4 of 8 participants. β-Actin, *RPS9*, and *IL8* amplicons were detected in the saliva of 2 participants. Amplicons for *GAPDH* mRNA, on the other hand, were not detected in any participants. These data suggest that most of the salivary RNA exists in partially degraded forms; however, some transcripts appear to remain intact.

To further characterize the integrity of salivary mRNA, we evaluated the relative abundances of amplicons that target different regions in both the β-actin and *IL8* transcripts. We chose to analyze β-actin and *IL8* mRNAs by RT-qPCR because their full-length mRNAs are the longest among the 5 genes tested in this study and, therefore, should enable easy identification of any possible degradation patterns. Such an analysis has been done previously for the study of maternal and placental RNA in maternal blood samples *(26)*. As shown in Fig. 1D, compared with the 5′ region of the β-actin mRNA, we detected ~50% of the middle region amplicon ($P < 0.001$), whereas the middle/3′ region was present in similar but slightly lower amounts ($P = 0.075$). Analysis of *IL8* salivary mRNA showed that we detected ~7-fold less of

A

Gene name	cDNA length (bp)	Short amplicons (bp)	Long amplicons (bp)
β-actin	1793	153 (m/3′)	1513
RPS9	585	144 (3′)	565
GAPDH	1283	154 (3′)	1193
IL8	1666	88 (5′)	1451
SAT	1060	99 (3′)	908

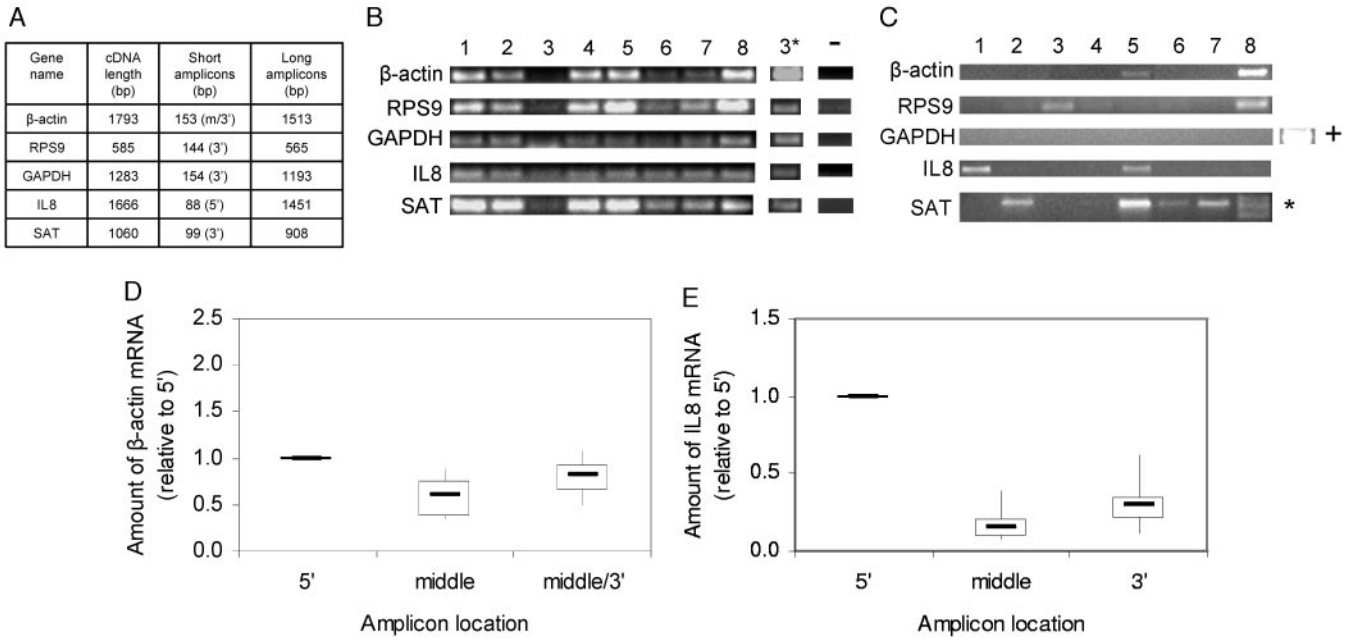

Fig. 1. Integrity measurement of salivary RNA.

(*A*), list of 5 NSCT genes, their cDNA length, and expected sizes of PCR amplicons shown in *panels B* and *C*. The *3′* and *5′* in parentheses indicate the location of amplicons on the gene. The *m/3′* in parentheses for β-actin indicates that the amplification region is located between the middle and the 3′ end of the β-actin mRNA. (*B*), RT-PCR of salivary RNA with primers that yield short PCR amplicons. For RNA isolation, 560 μL of the supernatant phase of saliva was used. PCR was done for 40 cycles. *Lanes 1–8* represent salivary RNA from 8 different participants. *Lane 3** contains 5-fold more RNA starting material than *lane 3*, and PCR was done for 45 cycles instead of 40 cycles. *Lane* (–) represents a negative control, for which an equal volume of water was used during the PCR instead of the reverse transcription products. (*C*), salivary RNA from the same participants as in *panel B* was used for RT-PCR (45 cycles) that yielded long PCR amplicons. The *inset* indicated by (+) next to the *GAPDH* gel image shows the result of the positive control, in which RT-PCR was performed with RNA isolated from the MCF7 cell line. *SAT* product for participant 8 (*lane 8*) gave triplicate bands, none of which matched the expected PCR size (*). (*D* and *E*), RT-qPCR of salivary RNA from 8 participants with β-actin (*D*) and *IL8* (*E*) primers that target different regions of β-actin and *IL8* mRNAs as indicated. *Thick horizontal lines* indicate the medians. The *boxes* represent the interval between the 25th and 75th percentiles. Maximum and minimum values are indicated by the *vertical lines*.

the middle amplicon ($P < 0.001$) and ~3.5-fold less of the 3′ amplicon ($P < 0.001$) than the 5′ amplicon (Fig. E). Together, these data suggest that the amounts of middle and 3′ region β-actin and *IL8* mRNAs are lower than the amounts of 5′ region salivary β-actin and *IL8* mRNAs.

ASSESSING THE ENTRY SITES OF RNA INTO THE ORAL CAVITY

To determine where RNA enters the saliva of the oral cavity, we measured the presence of RNA in stratified oral fluids from the 3 major salivary glands, GCF, and desquamated oral epithelial cells. β-Actin mRNA RT-PCR was performed on RNA isolated from 6 participants. Although individual variations exist, we detected β-actin mRNA in all 3 major salivary glands, GCF, and desquamated oral epithelial cells (Fig. 2A), suggesting that RNA enters the oral cavity from different sites.

We also measured the complexity of human mRNAs in these segregated fluids. RNA was isolated from stratified fluids and oral epithelial cells of 1 individual, and the number of human mRNAs was measured by use of the gene expression-based microarray (U133 Plus 2.0; Affymetrix). As shown in Fig. 2B, whereas 6570 transcripts were detected in the whole saliva, the assay detected 4778, 1831, 1543, 2689, and 3142 transcripts in the parotid saliva, sublingual saliva, submandibular saliva, GCF, and oral epithelial cells, respectively. For these genes, analysis of the number of NSCTs found in each site revealed 183 NSCTs in the whole saliva; 184, 132, and 76 NSCTs in the parotid, submandibular, and sublingual saliva, respectively; 163 NSCTs in the oral epithelial cells; and 162 NSCT in the GCF. These data show the presence of a substantial number of mRNAs in different oral fluids.

MEASUREMENT OF SALIVARY RNA STABILITY

Because human saliva is known to contain various ribonucleases *(8, 27)*, we reasoned that salivary RNAs are detectable because they are protected from degradation. To test this, we performed a time-course analysis in which the saliva was incubated at room temperature for different periods of time followed by RT-qPCR analysis of β-actin mRNA in the isolated RNA. As a control for degradation of nonsalivary RNA, we added purified total RNA from an ES-LW1 mouse cell line to the human saliva at the beginning of the incubation and monitored the stability of mβ-actin mRNA. Because the β-actin primers used in the experiments reported in Figs. 1 and 2 recognize both human and mouse β-actin mRNAs (data not shown), we used species-specific primers for this analysis (hβ-actin and mβ-actin primers). As shown in Fig. 3A, within 1 min of incubation, <10% of mβ-actin mRNA was detected. In contrast, the salivary hβ-actin mRNA concen-

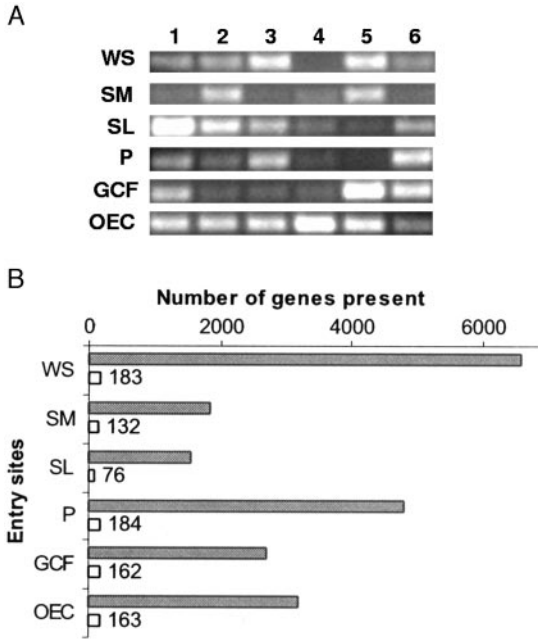

Fig. 2. Measurement of RNA from different oral fluids.

WS, supernatant of whole saliva; *SM*, submandibular saliva; *SL*, sublingual saliva; *P*, parotid saliva; *OEC*, oral epithelial cells. (*A*), RT-PCR of oral fluids obtained from 6 participants (*lanes 1–6*) with β-actin primers. Oral fluids were collected as described in the *Materials and Methods*. PCR was performed for 45 cycles. (*B*), RNA in oral fluid samples from 1 participant was analyzed by U133 plus 2.0 expression-based microarray (Affymetrix). ▨, total number of genes present in each site; □, numbers of NSCT genes.

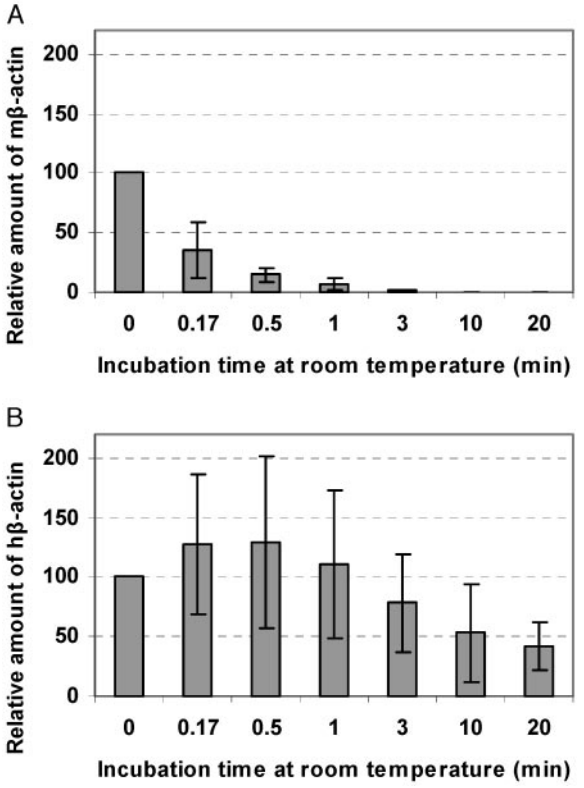

Fig. 3. Stability of salivary and nonsalivary RNA in saliva.

(*A*), at time 0, 2 μg of mouse ES-LW1 cell line total RNA was added per 1 mL of the supernatant phase of human saliva, and the samples were incubated at room temperature for up to 20 min. At each time point, 300 μL of saliva was removed for RT-qPCR to measure mβ-actin mRNA. The amount of RNA quantified at each time point was normalized to the amount measured at time 0. Saliva samples from 3 participants were used. *Error bars* represent SD of samples from these participant tested in 1 RT-qPCR reaction. (*B*), the same samples as in *A* were used, but the qPCR was performed with hβ-actin primers to measure the hβ-actin mRNA.

tration did not decrease significantly, and even after 20 min of incubation, we recovered >40% of hβ-actin mRNA (Fig. 3B). The half-lives of mouse cellular β-actin and human salivary β-actin mRNAs were 0.4 and 12.2 min, respectively (P <0.001). These results suggest that the degradation of salivary RNA is relatively slow compared with the degradation of exogenous RNA and indicates that there is a mechanism by which salivary RNAs are protected from degradation.

ASSESSING THE ROLE OF RNA–MACROMOLECULE ASSOCIATION IN SALIVARY RNA STABILITY

The observation that salivary RNAs are protected from degradation led us to hypothesize that association with certain macromolecules leads to the relative stability of salivary RNAs, a phenomenon known as particle-associated RNA, which has been observed for plasma RNA *(12, 13, 28)*. To test this, we filtered saliva through 0.22 and 0.45 μm size pores and measured the amount of β-actin mRNA in the filtered fractions by RT-qPCR. To exclude the possibility that these filters bind nonspecifically to RNA, we filtered purified human total RNA through different size pores and recovered >80% of the β-actin mRNA (see Fig. 1a in the online Data Supplement). The amount of filtered RNA is expressed as a percentage of the amount of unfiltered RNA. We found that the amount of salivary β-actin mRNA recovered from 0.22 and 0.45 μm pores was <5% (see Fig. 1b in the online Data Supplement). In addition to the saliva samples, we also measured RNA–macromolecule associations in serum and found that ~8% and <1% of β-actin mRNA could be detected in serum filtered through 0.45 and 0.22 μm pores, respectively (see Fig. 1c in the online Data Supplement). These data suggest that both saliva and serum RNAs, similar to plasma RNAs, are associated with macromolecules.

To test whether this RNA–macromolecule association is important for salivary RNA stability, we disrupted the RNA–macromolecule interaction with a detergent, Triton X-100 (10 mL/L). The saliva was incubated at room temperature for various periods of time for up to 20 min, and the stability of β-actin mRNA was measured by RT-qPCR analysis. As a control, saliva was incubated with water instead of Triton X-100, and the RNA stability of these samples was measured as well. As shown in Fig. 4, saliva mixed with water showed no markedly reduced amount of salivary β-actin mRNA for up to 20 min. However, incubation of the saliva with Triton X-100 dramatically accelerated the disappearance of the amount of salivary β-actin mRNA. After 10 min, <5% of β-actin

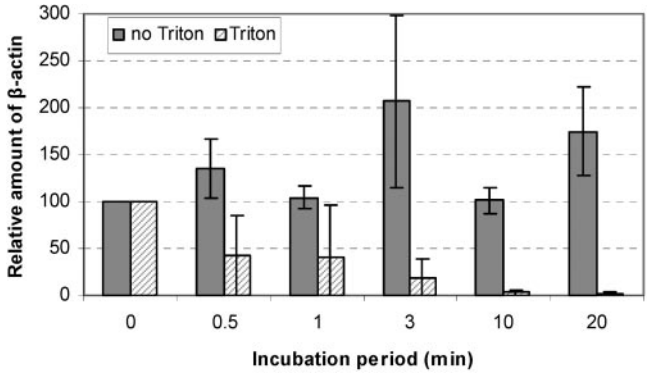

Fig. 4. Stability of salivary RNA after incubation with Triton X-100.

At time 0, saliva supernatant was mixed with either Triton X-100 or water (*no Triton*) and incubated for up to 20 min at room temperature. For RT-qPCR, salivary β-actin was measured. The amount of RNA quantified at each time point was normalized to the amount of RNA at time 0. Saliva samples from 4 participants were used. *Error bars* represent SD for the samples from these participants tested during 2 qPCR reactions.

mRNA could be detected, suggesting that most of the salivary RNA was degraded. These data indicate that there is a significant difference in salivary RNA stability between Triton X-100- and water-incubated saliva samples ($P < 0.001$), which strongly suggests that the RNA–macromolecule interaction is important for salivary RNA stability.

Discussion

We previously showed the presence of human mRNA in saliva *(4, 6)*. In this study, we presented the first characterization of salivary RNA. For 4 of the 5 genes analyzed, we found long PCR amplicons from at least 1 participant, suggesting that some salivary transcripts are present in full-length forms. In addition, many of these salivary RNAs are likely to contain a poly(A) tail because our microarray (Fig. 2B) data were generated with oligo(dT)-based cDNA synthesis followed by amplification *(4, 6)*. Our data suggest that different persons have different concentrations and species of long RNAs, possibly because saliva harbors different microorganisms that have different amounts and types of endo- and exoribonucleases, which affect the stability and length of salivary RNA. Alternatively, but not mutually exclusively, there are at least 2 known types of human nucleases that are active in saliva *(8, 11)*, and different individuals may have different amounts of these nucleases. The presence of partially degraded RNA suggests that endonucleases rather than exonucleases are primarily involved in the degradation of salivary RNA, because exonucleases are usually processive, degrading the target RNA substrates completely or giving rise to significant degradation on one end of the mRNA, as has been shown for plasma RNA *(26)*. On the other hand, endonucleases tend to produce partially degraded products when their concentrations are limited, with no preferential degradation at either end *(29)*. Consistent with this idea, the data in panels D and E of Fig. 1 show that although the assay detected lower amounts of 3′ regions compared with the 5′ regions, it detected even lower amounts of the middle region, which suggests that there is no preferential degradation from either end. We have shown that RNA enters the saliva of the oral cavity from various sources; it is therefore likely that the degradation we observed in the whole saliva mixture is the outcome of different nucleolytic activities.

One explanation of how RNAs enter the saliva is cell death. Cell lysis at the salivary ducts, gingival pockets, or the oral epithelium can lead to the release of RNA into the saliva. It is also possible that RNAs are actively secreted. These RNAs could originate from secreting cells or they could be produced elsewhere in the body, travel through the circulatory system, and be secreted into the saliva *(3, 17, 18)*. It has been reported that a breast cancer marker protein, c-erb-B-2, also known as HER-2/neu, is found in stratified saliva fractions *(17)*. In addition, it is known for GCF that different types of cells and their cellular components are released into the oral cavity *(19)*. These cells include cells in gingival pockets and blood cells, including leukocytes and erythrocytes.

We showed that salivary hβ-actin mRNA is protected from degradation, whereas exogenous mβ-actin mRNA is rapidly degraded. We also showed that salivary RNA is associated with macromolecules because it could not be filtered through 0.22 and 0.45 μm pores. Disruption of these macromolecules by Triton X-100 caused rapid degradation of salivary β-actin mRNA, suggesting that this RNA–macromolecule interaction is important for salivary RNA stability. This finding could be similar to what was reported in plasma. Plasma also contains nucleases, but studies have shown that endogenous plasma RNA is stable for more than 1 day *(12, 13)*. It has been also shown that plasma RNA cannot be filtered through 0.22 and 0.45 μm pore sizes, which suggests that plasma RNA is associated with macromolecules *(13, 28)*. Together, these data suggest that RNA degradation in the body fluids may be prevented by (a) protective mechanism(s). It is our future goal to test whether some of these RNA-associated complexes in saliva and serum are from the same origin.

What are these macromolecules that protect RNA from degradation? Saliva contains mucin proteins consisting mostly of MUC5B and MUC7. MUC5B forms oligomers that are highly glycosylated *(30)*, and it is possible that in saliva, some RNA is associated with these oligomers. However, this cannot be the only type of RNA–macromolecule complex because we detected RNA in the saliva from the parotid gland that lacks MUC5B. It has been speculated that in plasma, RNA-associated macromolecules are apoptotic bodies *(13, 28)*. Indeed, incubation of RNA-containing apoptotic bodies with serum protected RNA from degradation *(31)*. In addition, it appears that DNA in the blood originates from apoptosis or necrosis *(32)*. Therefore, it is a possibility that some of these salivary RNA are present in apoptotic bodies.

In conclusion, we showed that RNAs in saliva, which exist in partial to full-length forms, are protected from degradation by their association with macromolecules and that these RNAs originate from many different sources. These findings, together with our previous findings, further demonstrate that salivary RNA shows stability, complexity, and consistency and can be used as a biomarker for oral cancer and possibly other diseases.

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