Global Analysis of mRNA Decay in *Halobacterium salinarum* NRC-1 at Single-Gene Resolution Using DNA Microarrays^{†‡}

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RNA degradation is an important factor in the regulation of gene expression. It allows organisms to quickly respond to changing environmental conditions by adapting the expression of individual genes. The stability of individual mRNAs within an organism varies considerably, contributing to differential amounts of proteins expressed. In this study we used DNA microarrays to analyze mRNA degradation in exponentially growing cultures of the extremely halophilic euryarchaeon *Halobacterium salinarum* NRC-1 on a global level. We determined mRNA half-lives for 1,717 open reading frames, 620 of which are part of known or predicted operons. Under the tested conditions transcript stabilities ranged from 5 min to more than 18 min, with 79% of the evaluated mRNAs showing half-lives between 8 and 12 min. The overall mean half-life was 10 min, which is considerably longer than the ones found in the other prokaryotes investigated thus far. As previously observed in *Escherichia coli* and *Saccharomyces cerevisiae*, we could not detect a significant correlation between transcript length and transcript stability, but there was a relationship between gene function and transcript stability. Genes that are known or predicted to be transcribed in operons exhibited similar mRNA half-lives. These results provide initial insights into mRNA turnover in a euryarchaeon. Moreover, our model organism, *H. salinarum* NRC-1, is one of just two archaea sequenced to date that are missing the core subunits of the archaeal exosome. This complex orthologous to the RNA degrading exosome of eukarya is found in all other archaeal genomes sequenced thus far.

Fast decay of mRNA allows quick adaptation of organisms to changes in the environment by altering the expression of selected genes. The half-lives of individual transcripts or even transcript segments within an organism show considerable variations contributing to differential gene expression. The stabilities of several bacterial transcripts vary in response to external factors (reviewed in reference 46); the stabilities of eukaryotic transcripts can vary in response to cellular stimuli and differentiation stage (reviewed, for example, in reference 42), thus contributing to regulated gene expression. Microarray technology allows the study of mRNA half-lives of organisms on a global level. Up to now such studies have been performed for the bacterial model organisms *Escherichia coli* (6, 45) and *Bacillus subtilis* (18) and the eukaryotic model organism *Saccharomyces cerevisiae* (49), as well as two species of the hyperthermophilic crenarchaeon *Sulfolobus* (2). In all of these organisms a wide range of stabilities was found for individual mRNAs. Most *E. coli* and *B. subtilis* mRNAs (80%) exhibited half-lives of 3 to 8 min (6, 18). A study of the effect of the *Staphylococcus aureus* virulence factor regulator SarA on log-phase mRNA half-lives in this organism revealed that 90% of mRNAs expressed during log-phase growth had half-lives below 5 min (41). In the two *Sulfolobus* species the median

half-life was found to be about 5 min (2), whereas the median half-life in *S. cerevisiae* was 20 min (49). In *E. coli* and *S. cerevisiae* the decay rates for most mRNAs encoding proteins of related biological function were similar.

The mechanisms of mRNA decay have been extensively studied in bacteria and eukarya, and models for the turnover of the majority of mRNAs emerged (reviewed in references 5, 14, 24, 39, and 40). Not only have the mechanisms of mRNA processing and degradation been found to differ in bacteria and eukaryotes, even *E. coli* and *B. subtilis* show marked differences in regard to their mRNA degrading machinery (14). In *E. coli* decay of most mRNAs is initiated by endoribonuclease RNase E, which organizes a large protein complex, the degradosome (12). RNase E-based degradosome complexes are also found in other gram-negative bacteria, such as *Rhodobacter capsulatus* and *Pseudomonas syringae*, as well as the gram-positive *Streptomyces coelicolor*. All four characterized bacterial degradosomes contain 3' to 5' exoribonucleases and RNA helicases in addition to RNase E, but the individual proteins vary among the different bacterial species (12, 20, 25, 36). The set of RNases identified in *B. subtilis* is very different from that found in *E. coli*; most notably, an RNase E homolog is missing (23). Eukarya also contain a large protein complex mediating mRNA turnover, the exosome. This complex is composed of 3' to 5' exoribonucleases and additional RNA-binding proteins, RNA helicases, and associated protein factors (1, 29). Both the degradosome and the exosome contain RNase PH domain proteins.

Much less is known about mechanisms of mRNA decay in archaea. Archaeal mRNAs are not capped; some mRNAs were reported to carry short poly(A) tails (9, 10, 47). A number of RNases with functions in the processing and maturation of

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stable RNAs have been identified (26, 28, 32, 43). A few years ago the half-lives of some mRNAs were determined in *Sulfolobus solfataricus* and *Haloferax mediterranei* after the blocking of transcription with the antibiotic agent actinomycin D (8, 19), and recently a global study on mRNA half-lives in two *Sulfolobus* species was performed (2). In these archaea half-lives of individual transcripts showed large variations, ranging between 4 and 80 min in *H. mediterranei* and between 2 and more than 20 min in the two *Sulfolobus* species. Furthermore, it was shown that the half-lives of certain mRNA species are affected by salt concentration or growth phase in *H. mediterranei* (19), providing the first evidence that mRNA decay is an important step in gene regulation in archaea. Based on a bioinformatics analysis of sequenced archaeal genomes, the existence of an exosome-like complex was predicted (22). This in the meantime experimentally proven complex (11, 15, 16, 38) shows similarity in composition to the eukaryotic exosome but also contains some archaea-specific proteins. As the degradosome and the eukaryotic exosome, it includes RNase PH domain proteins. Besides the exoribonucleolytic activity the archaeal exosome also exhibits polyadenylation activity (27, 34, 48). Remarkably, genes encoding the counterparts of the core subunits of this exosome are not present in the genomes of *Halobacterium salinarum* NRC-1 and *Methanocaldococcus jannaschii* (22). Recent findings revealed that halophilic archaea, as well as methanogenic archaea, which are missing the exosome do not show polyadenylation of mRNAs, whereas hyperthermophilic and methanogenic archaea encoding the complete exosome show polyadenylation (34, 35; M. Brenneis, O. Hering, C. Longe, and J. Soppa, unpublished data). This suggests that the mRNA degrading machinery of these halophilic and methanogenic archaea is significantly different from that of other members of the archaea.

In the present study we used DNA microarrays covering the genome of *H. salinarum* NRC-1 for the first global analysis of mRNA stability in an euryarchaeon. *Halobacterium* species are obligate halophiles that grow at extreme high salt concentrations (4 M and higher) and accumulate high intracellular salt concentrations. *H. salinarum* NRC-1 harbors one large chromosome and two minichromosomes of a total of 2,571 kbp (31). The two minichromosomes are substantially less GC-rich than the large chromosome. The comparison of the genome of *H. salinarum* NRC-1 to other completed genome sequences showed closest similarity to *Archaeoglobus fulgidus* and *M. jannaschii*. The *H. salinarum* NRC-1 predicted proteins are more similar to those of *B. subtilis* than to other bacteria and display a large number of unique homologues with *Deinococcus radiodurans*, suggesting that *H. salinarum* NRC-1 may have acquired a number of genes from bacteria by lateral transfer (31).

Our work with an extremely halophilic euryarchaeon and similar studies on the gram-negative bacterium *E. coli* (6, 45), the gram-positive bacterium *B. subtilis* (18), and the yeast *S. cerevisiae* (49), as well as the hyperthermophilic crenarchaeon *Sulfolobus* (2), allow us to compare mRNA turnover in the different groups of microorganisms.

MATERIALS AND METHODS

Strain and growth conditions. *H. salinarum* NRC-1 obtained from the American Type Culture Collection (ATCC), Manassas, VA (reference no. ATCC 700922) was grown in *Halobacterium* growth medium prepared as described by

the ATCC (ATCC medium 2185). Cultures inoculated from exponentially growing precultures were grown aerobically at 37°C with shaking to mid-exponential-growth phase (an optical density at 600 nm of ~0.8) with doubling times of 5.5 to 8 h. For inhibition of transcription, the antibiotic agent actinomycin D was added to final concentrations of 50, 100, or 200 µg/ml for the *in vivo* labeling of RNA and 200 µg/ml for the determination of mRNA half-lives.

In vivo labeling of RNA. Radiolabeled uridine ([5,6-³H], 32 Ci/mmol; GE Healthcare, Chalfont St. Giles, United Kingdom) was added to exponentially growing cultures to a final concentration of 105 µCi/ml at time point zero. The addition of actinomycin D followed 12 min later. Samples of 20 µl were collected at several time points after the addition of uridine and were mixed with 500 µl of unlabeled cells. Cold trichloroacetic acid (TCA) was added to a final concentration of 11.5% (wt/vol). After incubation on ice for 5 min, the samples were centrifuged, and the TCA-precipitated counts were quantified in a liquid scintillation counter (Beckman Coulter, Fullerton, CA).

RNA isolation for determination of mRNA half-lives. Culture samples (20 ml) were taken immediately after the addition of actinomycin D (time point 0 min) and 10, 15, and 20 min later. The samples were directly mixed with 5 ml of ice-cold medium in precooled 50-ml centrifuge tubes and centrifuged at 8,000 rpm for 5 min at 4°C (Sorvall RC5C, SS34 rotor). Total RNA was prepared from cells harvested at each time point by using the RNeasy Midi kit (QIAGEN, Hilden, Germany) including a DNase I treatment according to the manufacturer's instructions. For normalization of the data, an *in vitro*-transcribed *E. coli rpoS* fragment (stationary-phase alternative sigma factor S) of 633 nucleotides was added at a concentration of 35 ng per 10⁹ cells to each sample of harvested cells before the RNA isolation as an internal standard. The isolated RNA was used for microarray and Northern blot analysis, as well as for quantitative real-time reverse transcription-PCR (RT-PCR).

Microarray analysis. The *H. salinarum* NRC-1 DNA microarrays used in the present study were produced in cooperation with D. Oesterheldt (Max Planck Institute [MPI] Martinsried, Germany). Generation of the microarrays will be described elsewhere (C. Lange, A. Zaigler, M. Hammelmann, and J. Soppa, unpublished data). The microarrays are comprised of PCR products of about 500 nucleotides in length, representing 2,586 of the 2,839 protein-encoding genes and 48 DNA sequences that are in the meantime reduced to spurious open reading frames (ORFs) in the complete genome sequence of *H. salinarum* strain R1 (<http://www.halolex.mpg.de/public/>).

In order to minimize biological noise, RNA preparations from three cultures grown under identical conditions were pooled to equal parts for cDNA synthesis. The synthesis of labeled cDNA was performed as described by Zaigler et al. (50). Portions (21 µg) of RNA were pooled, the RNA concentration was measured, and equal amounts of RNA from the samples that were directly compared were labeled fluorescently by using 400 U of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and 0.6 µg of random hexamer primers (oligonucleotides NNNNNT, NNNNNA, NNNNNG, and NNNNNC mixed equimolar to 1 mg/ml; Sigma Aldrich, St. Louis, MO). The reference sample (time point zero) was labeled with Cy3-dUTP (GE Healthcare), and subsequent time point samples were labeled with Cy5-dUTP (GE Healthcare). Cy5- and Cy3-labeled cDNAs were combined and then concentrated with a Microcon-YM30 concentrator (Millipore, Billerica, MA). Afterward, the cDNA was mixed with hybridization buffer (3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2.5× Denhardt, 4 µg of yeast tRNA [Roche, Basel, Switzerland]). The sample was then denatured for 2 min at 98°C before 0.08% sodium dodecyl sulfate was added. Hybridization was performed at 68°C overnight. After hybridization, the slides were washed four times at room temperature, i.e., twice with 0.5× SSC plus 0.01% sodium dodecyl sulfate and twice with 0.06× SSC. Slides were then dried by centrifugation for 5 min at 500 rpm and room temperature. Microarrays were scanned with an Axon Instruments GenePix 4000A scanner, and the data were collected with the GenePixPro 3.0 software (Molecular Devices, Sunnyvale, CA).

Analysis of microarray data. Images produced by the Axon scanner were analyzed by using the GenePixPro 3.0 software package. A 633-nucleotide-long *in vitro*-transcribed *E. coli rpoS* fragment was used to determine the normalization coefficient. The data spots that were damaged physically, as well as spots that did not have a median signal intensity (minus the background intensity) at time point zero ≥ 1000 , a signal/background ratio at time point zero ≥ 3 , and a positive signal intensity at the later time points were excluded from further analysis. The half-life of each mRNA was determined by using the decay law $t_{1/2} = [(x_2 - x_1)/\log_2(y_1/y_2)]$, with x_1 and x_2 being the time points the samples were taken (x_1 always = 0, x_2 = 10, 15, or 20 min) and y_1 and y_2 being the signal intensities at time points x_1 and x_2 , respectively.

Starting from the initial multi-set X_0 of measured values an iterative outlier-elimination-heuristic with $X_{n+1} := \{x \in X_n \mid x - \bar{X}_n < k_n \cdot S_{X_n}\}$ was used, where

$\bar{X}_n$ and S_{X_n} are the arithmetic mean and the sample standard deviation of X_n , respectively. Good results were obtained by using X_3 with parameters $k_0 := k_1 := 1.4$ and $k_2 := 1.3$.

Only ORFs for which results were obtained in at least six of the nine microarray experiments and for which the sample standard deviation of the half-life between the single experiments lay between zero and 33.34% of the obtained average half-life either right from the beginning or after using the above-described iterative outlier-elimination-heuristic were considered reliable regarding the corresponding mRNA half-life. All half-life and normalization calculations were carried out by using Microsoft Excel.

Northern blot analysis. To verify the microarray data, we performed Northern blot analyses for six chosen genes (OE1304F, OE4165R, OE4511R, OE4735R, OE4736R, and OE4743R). A total of 10 μ g of isolated total RNA per lane was evaluated on a 1% (wt/vol) agarose 2.2 M formaldehyde gel and then transferred to a Biotrans B membrane (Pall, East Hills, NY) by vacuum pressure blotting (Vacuum Blotter; Appligene [now MP Biomedicals], Irvine, CA) according to the manufacturer's instructions.

The specific primer for the 16S rRNA (5'-CCACCGTCTACCTAATCGAC CG-3') was radiolabeled with [γ - 32 P]ATP at the 5' end, and specific DNA fragments of the chosen genes (in the case of OE4743R the fragment covers the whole gene [228 nucleotides]; in the case of the other genes same probes as on microarrays were used) were radiolabeled with [α - 32 P]dCTP using nick translation (Nick Translation Kit; GE Healthcare). Products were purified on microcolumns (ProbeQuant G-50; GE Healthcare). Aliquots of 0.25×10^6 to 2×10^6 cpm were used per hybridization reaction. The signals were quantified by using a phosphorimaging system (Molecular Imager FX; Bio-Rad, Hercules, CA) and the appropriate software (Quantity One; Bio-Rad). Evaluation of the Northern blot analyses was done after normalization to the amount of 16S rRNA loaded on the gel by using the formulas described above and Microsoft Excel.

Quantitative real-time RT-PCR analysis. In addition to Northern blot analysis, we also performed quantitative real-time RT-PCR for four chosen genes (OE1304F, OE4735R, OE4736R, and OE7003R) to validate the microarray results. A portion (10 ng) of total RNA was used in the One-Step RT-PCR kit (QIAGEN), according to the manufacturer's instructions. The same primer as for the microarrays was used for the amplification of PCR products, which was monitored with SYBR green using the Rotor-Gene 3000 real-time PCR cycler (LTF, Wasserburg, Germany). The internal standard *rpoS* was used to normalize the data, and the relative expression of the tested mRNAs was calculated according to the method of Pfaffl (33). The determination of mRNA half-lives was performed by using the formulas described above and Microsoft Excel.

Accession number. The microarray data were deposited in ArrayExpress (<http://www.ebi.ac.uk/arrayexpress>) under accession number E-MEXP-1088.

RESULTS AND DISCUSSION

Inhibition of transcription by actinomycin D. To investigate the mRNA half-lives in *H. salinarum* NRC-1 on a genome-wide level, we measured the mRNA transcript abundance at different time points after blocking transcription. To block transcription, we used the antibiotic agent actinomycin D, which was previously shown to arrest transcription in human cells (37, 44), as well as in the archaea *H. mediterranei* (19) and *S. solfataricus* (8). The first step of our analysis was to show that this antibiotic, which inhibits transcription by intercalative binding to double-stranded DNA (reference 13 and references therein) and thereby impeding RNA elongation, also effectively and quickly blocks transcription in our model organism, *H. salinarum* NRC-1.

We performed experiments similar to those Jäger et al. (19) used to show the effectiveness of this antibiotic in *H. mediterranei* by adding 3 H-radiolabeled uridine to cultures in the mid-exponential-growth phase (optical density at 600 nm of ~ 0.8) at time point zero. Twelve minutes later we added 50, 100, or 200 μ g of actinomycin D/ml to the cultures. As a control we used a culture without actinomycin D. RNA synthesis was monitored by quantifying TCA-precipitable tritium in a scintillation counter. As shown in Fig. 1 the antibiotic agent acti-

FIG. 1. 3 H-radiolabeled uridine was added to cultures in mid-exponential growth phase at time point zero. After 12 min 50 μ g (□), 100 μ g (△), or 200 μ g (×) of actinomycin D/ml was added, no inhibitor was added to a control culture (◇). RNA synthesis was monitored by quantifying TCA-precipitable tritium in a scintillation counter. The arrow indicates the time point of actinomycin D addition.

nomycin D blocks de novo synthesis of mRNA in *H. salinarum* NRC-1 quantitatively and immediately after the addition of the drug at concentrations of 100 and 200 μ g/ml. This allows for an accurate determination of mRNA half-lives in this organism. As a consequence of the immediate stop of transcription by the addition of actinomycin D, we used the time point of drug addition as the 0-min time point in all further experiments.

Half-lives as determined by microarrays. For the determination of mRNA half-lives using DNA microarrays, we performed three independent time course experiments with pooled RNA of three different cultures each and measured the transcript abundance at 0, 10, 15, and 20 min after the addition of actinomycin D. To estimate mRNA decay rates, we normalized the Cy3 and Cy5 fluorescence for each array using an internal standard and removed ambiguous spots from the data set based on the criteria described in Materials and Methods. By this approach we evaluated half-lives for 1,717 mRNAs, including 33 ORFs that have been in the meantime annotated as spurious, suggesting that at least these 33 spurious ORFs are transcribed genes.

We observed a wide range of mRNA half-lives (Fig. 2A). The most rapidly decaying mRNAs we found had half-lives of 5 or 6 min (32 genes). A total of 44% of all mRNAs in this evaluation had half-lives of 8 or 9 min, and 79% had half-lives of between 8 and 12 min. A total of 30 mRNAs showed half-lives of 18 min or more. We did not observe transcripts with half-lives of <5 min in our analysis. Such mRNAs would have been characterized by a large signal intensity difference between the signals at the time point of actinomycin D addition and 10 min later, the first time point taken after addition. However, such transcripts were not observed and therefore could only be hidden among those with missing data, if transcripts that are that unstable exist in *H. salinarum* NRC-1 at all.

The mean half-life of all mRNAs in our experiments was 10.1 min; the median half-life was 9.4 min. The distribution of half-lives among chromosome- and plasmid-encoded mRNAs differed significantly. About 83% of the 441 plasmid-encoded mRNAs had half-lives of 8 or 9 min, whereas the mRNA half-lives of the 1,276 analyzed chromosomal genes were more

FIG. 2. (A) Half-lives for 1,717 mRNAs were evaluated by DNA microarray analysis. A total of 44% of all mRNAs in this analysis had half-lives of 8 or 9 min, and 79% had half-lives of between 8 and 12 min. Fewer than 2% of the mRNAs had half-lives of 18 min or more. The mean overall half-life was 10.1 min. (B) About 83% of the plasmid-encoded mRNAs had half-lives of 8 or 9 min ($\square$); the mRNA half-lives of chromosomal genes were more widespread ($\blacksquare$). The mean half-life of chromosome-encoded mRNAs was 10.6 min, whereas the mean half-life of plasmid-encoded mRNAs was 8.7 min.

widespread. Here, only 31% of mRNAs showed half-lives of 8 or 9 min (Fig. 2B). This also led to a slight difference in the mean half-life when chromosome- and plasmid-encoded mRNAs were analyzed separately. Although the mean half-life of chromosome-encoded mRNAs was 10.6 min, the mean half-life of plasmid-encoded mRNAs was 8.7 min. The observed difference in transcript stability might reside in the significant difference in the GC content of the large chromosome compared to the two minichromosomes with an ~10% higher GC content for the large chromosome (67.9% versus 57.9% and 59.2% [31]). A correlation between GC content and transcript stability was not observed in *E. coli* (6) and *S. cerevisiae* (49), but differences between organisms in this regard can probably be expected.

Previous studies on global mRNA half-lives in prokaryotes suggested that transcriptomes of prokaryotes exhibit average transcript stabilities of around 5 min (2, 6, 18, 41, 45). The half-lives we obtained for *H. salinarum* NRC-1 by using DNA microarrays, with a mean half-life of 10 min, are considerably longer than the ones obtained for the faster-growing bacteria *E. coli* and *B. subtilis* (6, 18, 45). The mean half-life is furthermore about twice as long as found in the two crenarchaeota, *S.*

solfataricus and *S. acidocaldarius* (2), which have doubling times similar to that of *Halobacterium* (4 to 6 h) and about half as long as in the eukaryote *S. cerevisiae* (49). Although in *E. coli* tripling of the doubling time through changing the growth medium did not lead to an overall change in transcript stability (6), it is possible that the higher transcript stability in *H. salinarum* NRC-1 compared to *E. coli* and *B. subtilis* is due to the considerably longer doubling time of our model organism. The greater instability of *Sulfolobus* transcripts compared to the ones in *H. salinarum* NRC-1 despite similar doubling times of these organisms could suggest an influence of the growth temperature on mRNA stability. Whereas the optimal growth temperature of *H. salinarum* NRC-1 is around 42°C, the hyperthermophilic crenarchaeon *Sulfolobus* grows best at 75°C, a temperature at which RNA is highly unstable by itself. The high internal ion concentration in halophiles, on the other hand, may lead to a stabilization of RNA molecules. A further difference in regard to the mechanisms of RNA processing between the two investigated archaea *Halobacterium* and *Sulfolobus* is the lack of the core subunits of the exosome in *Halobacterium* (22), suggesting that this organism has an RNA-degrading machinery that significantly differs from those analyzed in other organisms. It is tempting to speculate that during its evolution *H. salinarum* NRC-1 has typically experienced slower changes in environmental conditions than the four previously characterized prokaryotic species and that this has led to the considerably greater transcript stability compared to *Sulfolobus*, *E. coli*, *B. subtilis*, and *S. aureus*.

In any case, *H. salinarum* NRC-1 is the only prokaryotic organism investigated thus far that has an average transcript stability significantly higher than 5 min, showing that transcripts in prokaryotes are not generally as unstable as seen thus far.

mRNA stability in relation to gene function. Some of the previous studies on global mRNA decay showed that transcripts encoding proteins with related functions often decay at similar rates (6, 45, 49). To investigate this issue in *H. salinarum* NRC-1, we compared the stability of mRNAs belonging to different function classes as used in the Halolex database (<http://www.halolex.mpg.de/public/>). In 11 of the 25 functional groups present in our analysis at least one mRNA with a half-life of 18 min or longer was detected, and in more than half of the functional groups (13 of 25) unstable transcripts with half-lives of 5 or 6 min were found. Interestingly, the only two transcripts of the function class “motility” that were present in the evaluation (*flgA1* and *flgA2*) both showed half-lives of 13 min, which is above the median overall half-life (data not shown).

To investigate whether there are functional groups that were either over- or under-represented under the conditions used here, we calculated the analyzed genes as a percentage of all genes of each functional group present on the microarrays. Overall, we evaluated half-lives for 65% of the microarray covered genes. For the function classes “central intermediary metabolism” (CIM), “lipid metabolism” (LIP), “miscellaneous” (MIS), “nucleotide metabolism” (NUM), “signal transduction” (SIG), and “translation” (TL) the analysis covered between 71% of genes for TL and 88% for NUM, showing that most genes of these functional groups were expressed under the tested conditions. In the case of CIM, LIP, NUM, SIG, and

FIG. 3. Distribution of mRNA half-lives according to functional classes in *H. salinarum* NRC-1 as annotated in the Halolex database. The percentages of mRNAs ranging from 0 to 40% were plotted against the half-lives ranging from 5 to ≥ 18 min. The numbers of genes present per function class are indicated on the right of each diagram. Panels: -, spurious ORF; AA, amino acid metabolism; CHY, conserved, hypothetical protein; CIM, central intermediary metabolism; COM, coenzyme metabolism; CP, cellular processes; EM, energy metabolism; HY, hypothetical protein; ISH, transposases and ISH-encoded proteins; LIP, lipid metabolism; MIS, miscellaneous; NOF, no function assigned to experimentally identified protein; NUM, nucleotide metabolism; REG, gene regulation; RRR, replication, repair, recombination; SIG, signal transduction; TC, transcription; TL, translation; TP, small molecule transport.

TL this is presumably due to the important role many genes of these functional groups play in growing cells. The high representation of MIS in this analysis, on the other hand, probably resides in the diversity of genes in this function class. The only function class of which clearly fewer genes were expressed was “hypothetical protein” (HY) with 58% of mRNAs in the analysis. That just HY of all function classes in this evaluation had such a low rate of evaluable mRNA half-lives suggests that not all genes annotated in this group are transcribed. Another reason for this relatively low number of expressed genes could be that especially in this group there are many genes unique to *H. salinarum* NRC-1 that are just needed under certain conditions.

We could not find any significant difference in the average mRNA half-lives of the different function classes, but an investigation of the distribution of mRNA half-lives in the individual groups revealed obvious differences. Figure 3 shows this distribution as a percentage of all analyzed mRNAs of each function class for function classes with more than 10 genes present in the analysis. Whereas the distribution of half-lives in nine of these groups was similar to the average half-life distribution of all mRNAs, with 28% of mRNAs having half-lives between 5 and 8 min, 50% having a half-life of 9 to 11 min, and the remaining 22% having half-lives of 12 min and longer, transcript stability in the other function classes differed from this distribution.

As shown in Fig. 3, the function classes “energy metabolism” (EM; 59% of analyzed mRNAs), “transcription” (TC; 67%), TL (50%), and SIG (36%) were overrepresented among the unstable transcripts (half-life of 5 to 8 min). Furthermore, there were very few mRNAs in these functional groups that had half-lives longer than 15 min. In the SIG class the most stable mRNA we found was *htr17*, with a half-life of just 12 min. Notably, all six mRNAs of the function class TL present

in the analysis that take part in gene regulation (*eif5a*, *eif2ba*, *infB*, *pelA*, translation initiation factor aIF-2 β -chain, and ribosome anti-association protein [initiation factor aIF6]) had half-lives above the overall average of 10 min (11 to 15 min). In the function class TC it is striking that eight TATA-box binding protein (TBP) mRNAs were present, all with half-lives of 8 or 9 min, whereas just one transcription initiation factor (*tbfH*) could be found (see Table S1 in the supplemental material). Specifically investigating the missing *tbf* genes revealed that just one of these (*tbfF*) was highly expressed. Reliable data for *tbfF* could not be obtained due to saturation of some data spots. For the other six *tbf* genes the signal intensities were too low to obtain reliable data, suggesting that these are not expressed under the conditions we used to perform our experiments. These results support the suggestion that differential use of TFBs, together with the use of alternative TBP-TFB pairs, may be a mechanism for gene regulation in *H. salinarum* NRC-1 (3).

Although the function classes “amino acid metabolism” (AA) and NUM were not overrepresented among the unstable transcripts, 83 and 89%, respectively, of mRNAs belonging to these functional groups had half-lives below or around the overall mean (5 to 11 min). In case of the AA class no mRNAs had half-lives of more than 15 min, and in the NUM class there was just *trxB3* (21 min) with a half-life of more than 14 min, suggesting that also the transcripts of these two function classes are generally unstable.

The obvious instability of transcripts belonging to the function classes EM, TC, TL, and SIG could be explained by the important role genes for energy metabolism, transcription, translation, and signal transduction play in adjustment to changing environmental conditions. Our results for *H. salinarum* NRC-1 are similar to the ones found in *Sulfolobus*, where the categories TL and “energy production and conversion,” as

well as “amino acid transport and metabolism,” were also overrepresented in the group of the shortest half-lives (2). Although the function class TC was not overrepresented in any half-life group in *Sulfolobus*, the components of the basal transcription machinery displayed significantly shorter half-lives than average, while mRNAs coding for transcriptional regulators were generally long-lived (2). We did not make a similar observation for transcripts of transcriptional regulators in *Halobacterium*, but the data we obtained in regard to the transcript stability of translational regulators correlates well with the data for *Haloferax volcanii*. In that organism a shift from amino acid-based medium to glucose-based medium led to a fast up- or downregulation of transcripts for transporters, translation apparatus, and enzymes, whereas changes in the concentration of transcripts for regulators were much slower (50).

In contrast to the above-mentioned function classes, mRNAs encoding genes of the function class CIM seem to be generally long-lived in *H. salinarum* NRC-1. A total of 43% of mRNAs in this group had half-lives of 12 min or more, and the shortest half-life was 7 min for *pyc* and *mdh* (Fig. 3). A similar observation could be made for “gene regulation” (REG) transcripts, none of which had half-lives shorter than 8 min. The function class “coenzyme metabolism” (COM) was not overrepresented among the stable mRNAs, but it was (with just 12% of mRNAs having half-lives below the average) underrepresented among the unstable transcripts, suggesting that transcripts of this functional group also are generally stable.

Interestingly, we found half-lives for 10 different cell division control protein *cdc6* homologues, 2 of which are annotated as the nonfunctional central part of *cdc6* (*orc3*) and the nonfunctional N-terminal part (*orc2*), respectively, showing for the first time that all of these *cdc6* homologues are actually expressed in *Halobacterium* (see Table S2 in the supplemental material). For three more proteins annotated as *cdc6* homologues (one is just annotated as nonfunctional N-terminal part) we did not obtain mRNA half-lives, suggesting that these three are not expressed under the culture conditions we used in these experiments. Half-lives for the 10 analyzed mRNAs ranged between 7 and 10 min with just one exception. This exception was *orc7*, with a half-life of 17 min, notably the only of the three chromosomal *cdc6* homologues (*orc6*, *orc7*, and *orc8*) for which a function in autonomous replication could be demonstrated thus far (7). The second chromosomal *cdc6* homologue located near a potential replication origin (*orc8*) is also expressed in *Halobacterium* (half-life of 9 min), although no function in autonomous replication has yet been shown for this protein (7). Notably *orc6*, the one chromosomal *cdc6* homologue not located at a potential replication origin based on GC-skew analysis (21), did not emerge in our analysis.

Furthermore, we detected 25 out of 28 mRNAs encoding proteins involved in gas-vesicle formation (*gvpMLKJIHGFEDACNO*) in this analysis (see Table 3 in the supplemental material; missing are *gvpC1*, *gvpJ1*, and *gvpM2*). These 28 *gvp* genes are clustered in two related regions with an identical arrangement of the 14 distinct *gvp* genes. Half-lives of all evaluated *gvp* mRNAs were at 8 or 9 min, which is—with the exception of *gvpA*—comparable to *gvp* transcript half-lives determined in *H. mediterranei*. In this organism the transcript stability of *gvpA* was considerably higher than that of the other

FIG. 4. mRNA half-lives of all evaluated mRNAs in relation to their transcript length for monocistronic genes (●) and polycistronic operons (□). In case of the polycistronic messages the shown half-life is the average half-life of the individual segments of each message.

transcripts (19). Since the mRNA stability of the individual segments was very similar in our study, the greater abundance of the *gvpA* transcript compared to the *gvpAC* transcript observed earlier (17) seems to be due to differences in transcription rates rather than to differences in transcript stability as seen in *H. mediterranei* (19).

Half-lives of individual segments of polycistronic transcripts. Although genes that are part of the same polycistronic transcripts often show similar mRNA half-lives there are polycistronic messages for which different stabilities of individual segments have been shown (see, for example, references 19 and 30). These segmental differences in mRNA stability, which can be due to stabilizing structures between the segments, have an important regulatory function in gene expression on the posttranscriptional level (39).

To investigate whether the individual segments of polycistronic messages in *H. salinarum* NRC-1 exhibit similar half-lives or not, we first had to predict operons in *Halobacterium* since just a few have been experimentally verified. We applied the gene gap method, which makes use of the relatively short gap between genes in operons compared to the gap between adjacent genes that are not part of the same operon. Applying the gene gap method to the annotated genome of *Halobacterium*, we found 402 stretches of genes that are encoded on the same strand with an intergenic distance of less than 10 nucleotides. For 190 of the 402 putative operons we obtained half-lives for at least two mRNAs in the evaluation. A total of 125 of these are comprised of only two ORFs, whereas the other 65 consist of 3 to 25 ORFs. To investigate whether the individual segments of a putative operon decay at similar rates, we calculated the average half-life and standard deviation for each operon with two or more mRNAs present in our analysis by using the half-lives of the individual segments. Just 2 of the 125 two ORF operons showed a standard deviation of the mean half-life of the polycistronic transcript larger than 33.34%, which was our cutoff for a reproduced mRNA half-life in the microarray analysis (see Materials and Methods). For one of these two operons (OE4736R and OE4735R) this difference in stability of the individual segments calculated from the microarray experiments could not be seen using Northern blot or quantitative real-time RT-PCR analysis (see below). Of the 65 “more than two ORFs” polycistronic messages, none showed a standard deviation of the individual segments of the mean

FIG. 5. Northern blots for six mRNAs representing a wide range of mRNA half-lives. The 16S rRNA hybridizations shown below the Northern blots were used as a control of the total RNA loaded on the gels. The half-lives evaluated by Northern blot are shown in Table 1.

half-life of the polycistronic transcript larger than 33.34%. Hence, individual segments of polycistronic messages in *H. salinarum* NRC-1 decay at similar rates.

Half-life in relation to transcript length. In previous genomewide mRNA stability analyses, findings in regard to a correlation between mRNA half-life and transcript length differed significantly. While in *E. coli* (6) and *S. cerevisiae* (49) no strong correlation could be found, mRNA half-life is negatively correlated with transcript length in *Sulfolobus* (2). To test for a possible correlation in *H. salinarum* NRC-1, we divided the 1,097 monocistronic mRNAs, as well as the 190 polycistronic transcripts that were represented with at least two genes in the evaluation (see above), into four different groups depending on their transcript length. The groups ranged from 0 to 500 nucleotides (348 transcripts), 501 to 1,000 nucleotides (399 transcripts), 1,001 to 1,500 nucleotides (290 transcripts), and longer than 1,500 nucleotides (250 transcripts). All groups had similar mean half-lives of 10 or 11 min. Plotting the mRNA half-lives against the transcript lengths clearly showed a much wider distribution of half-lives for monocistronic than for polycistronic transcripts (Fig. 4), where especially the longer transcripts did not have half-lives of more than 15 min. The average half-lives for the mono- and polycistronic transcripts separately, however, were alike: 10 min for each of the two groups. This suggests that there is, in contrast to *Sulfolobus*, no obvious correlation between half-life and ORF length.

Validation of the microarray data by Northern blot and quantitative real-time RT-PCR. To test the robustness of the data obtained by microarray analysis, we performed Northern blot analyses for six transcripts representing a wide range of half-lives (Fig. 5 and Table 1). For three of these, as well as one other transcript, we also performed quantitative real-time RT-PCR (Table 1). For five of the seven transcripts we found very similar half-lives using DNA microarrays and Northern blot analysis or quantitative real-time RT-PCR (Table 1). One of the exceptions is OE4735R (*rps7*), which is probably organized in an operon together with OE4736R (*rps12*; intergenic distance of four nucleotides). The mRNA half-life estimated for *rps7* using microarray technology is about twice as long as the half-life obtained by Northern blot analysis. It is also twice as long as the mRNA half-life of *rps12* obtained by both methods. An explanation for the discrepancy between microarray and Northern blot might be cross-hybridization to a transcript with a longer half-life, which occurred only during the microarray but not during the Northern blot hybridization due to different hybridization conditions used in the two methods. However, a homology search with the nucleotide sequence of *rps7* and the whole genome sequence of *H. salinarum* NRC-1 did not reveal a possible candidate to support this explanation. By performing quantitative real-time RT-PCR for both transcripts of this operon we could show that the shorter mRNA half-life for *rps7* detected by Northern blot is the accurate one (Table 1). Different results by microarray and Northern blot were also obtained for the transcript of OE4743R (*rpoH*), which is the first gene of an operon containing six different genes. In this case short, stable products accumulate in the Northern blot analyses during the time course resulting in an overall half-life of about 20 min (Fig. 5). The half-life of the full-length polycistronic transcript on the other hand is with 6 min similar to that of the microarray analysis, suggesting that the accumulating stable products do not hybridize on the microarray.

In addition to OE4735R and OE4736R, we also performed quantitative real-time RT-PCR for transcripts of OE1304F and OE7003R. In the case of OE7003R the mRNA half-life of 13 min fits perfectly with the microarray data, which shows that this mRNA is relatively stable. For OE1304F, on the other hand, the mRNA half-life obtained by using quantitative real-time RT-PCR was just 5 min. Because microarray and North-

TABLE 1. Comparison of microarray, Northern blot, and quantitative real-time RT-PCR results for seven different mRNAs representing a variety of half-lives found in *H. salinarum* NRC-1

Halolex database gene no.	Gene ^a	Function class ^b	Protein name or description	Mean half-life (min) $\pm$ SD as determined by:		
				Microarray	Northern blotting	Real-time RT-PCR
OE1304F	<i>xthA</i>	RRR	DNA (apurinic or apyrimidinic site) lyase (EC 4.2.99.18)	11 $\pm$ 3.4	9 $\pm$ 2.7	5 $\pm$ 1.1
OE4165R	<i>pcn</i>	NOF	DNA-directed DNA polymerase sliding clamp homolog	7 $\pm$ 1.2	6 $\pm$ 0.7	
OE4511R	–	HY	Hypothetical protein	8 $\pm$ 1.5	9 $\pm$ 1.1	
OE4735R	<i>rps7</i>	TL	Ribosomal protein S7	13 $\pm$ 3.9	6 $\pm$ 0.5	5 $\pm$ 1.2
OE4736R	<i>rps12</i>	TL	Ribosomal protein S12	6 $\pm$ 0.9	6 $\pm$ 0.5	6 $\pm$ 1.4
OE4743R	<i>rpoH</i>	TC	DNA-directed RNA polymerase (EC 2.7.7.6) chain H	7 $\pm$ 1.5	6 $\pm$ 1.2 ^c	
OE7003R	–	CHY	Conserved hypothetical protein	14 $\pm$ 4.2		13 $\pm$ 4.1

^a –, No gene name annotated.

^b Function classes are as defined in the text and in the legend to Fig. 3.

^c Half-life as determined for the polycistronic message; taking into account the different shorter products (Fig. 5), the half-life extends to 20 $\pm$ 6.1 min.

ern blot analyses showed a half-life of about 10 min (Table 1), we believe the longer half-life to be the accurate one in this case. For four more mRNAs we were not able to perform Northern blot analyses, probably due to a low expression of the corresponding genes.

The discrepancies we observed between microarray and Northern blot analyses are in contrast to some previous studies, where the two methods were found to be in congruence (4, 50), but they are not uncommon for genomewide studies of mRNA decay (18) and just illustrate the fact that the microarray analysis has a much higher sensitivity than the Northern blot analysis. Nevertheless, in the majority of cases, representing half-lives under, around, and above the overall mean half-life, the results of the two methods were very similar (Table 1). Together with the results obtained by using quantitative real-time RT-PCR, this confirms the data we obtained by microarray analysis.

Conclusion. Our results for *H. salinarum* NRC-1 provide initial insight into mRNA turnover in a euryarchaeon and in one of the two archaea known to date that are missing the core subunits of the archaeal exosome, a complex found in almost all archaeal genomes sequenced up to now. Moreover, these results are the first to show that transcriptomes of prokaryotes can be more stable than the mean half-life of around 5 min previously observed. Determination of transcript half-lives of more archaeal and bacterial species is necessary to determine whether *Halobacterium* is an exception with regard to transcript stability in prokaryotes or whether this is an additional case in which the biodiversity is greater than anticipated after the characterization of *E. coli*.

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