Research Report

Assessment of RNA Quality by Semi-Quantitative RT-PCR of Multiple Regions of a Long Ubiquitous mRNA

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ABSTRACT

A simple method to assess the degree of degradation present in a total RNA preparation from cells or tissues is based on the increasing probability of RNA cleavage with increasing length of an RNA molecule. Under ideal conditions, reverse transcription of a particular mRNA species with oligo-dT as the primer generates a population of cDNAs, terminating at the 5¢ end of the mRNA if all template RNA molecules are intact, or at the first cleavage site 5¢ to the polyA if some template RNAs are partially degraded. Consequently, for cellular RNA preparations with some degradation, the 5¢ end of an mRNA is represented in the cDNA population to a lesser extent than the 3¢ end of the mRNA. We describe a sensitive assay of mRNA quality that compares the relative PCR amplification of 5¢ and 3¢ regions of a long and ubiquitous mRNA following oligo-dT-primed reverse transcription.

INTRODUCTION

The preparation of total cellular RNA in which mRNA is uncleaved by nucleases during purification is a starting point for many research projects. For example, the creation of a library containing primarily full-length cDNA inserts depends, first of all, on the absence of degraded mRNAs in the RNA preparation to be reverse transcribed. Such a library of full-length cDNAs can be essential for cloning schemes that depend on the expression of active proteins from the cDNA library. A library containing full-length cDNAs also eliminates the tedious rounds of screening and assembly required to acquire a full-length cDNA sequence from partial clones.

Assessing the quality of RNA preparations by denaturing gel electrophoretic analysis of 18S and 28S rRNAs is not sufficiently rigorous for many applications. Simple detection of one or more mRNA species by RT-PCR, with its great sensitivity, also may not be appropriate because it is not clear what fraction of molecules of a given mRNA are uncleaved. Thus, we find that methods such as the Gene Checker™ kit (Invitrogen, Carlsbad, CA, USA) or the multiplex PCR method of Watzinger and Lion (7), which assess RNA quality by comparing PCR amplification of large and/or rare mRNAs with short and/or abundant mRNAs, give positive results (amplification of all primer pairs) with RNA preparations that are actually significantly degraded. In this report we present an RT-PCR protocol that assesses the relative quality of RNA preparations isolated from cells and organs by semi-quantitative analysis of the relative degradation of a single long mRNA species.

Overview

This RT-PCR assay measures the quality of an RNA preparation by comparing the fraction of molecules of the ubiquitous, approximately 10 kb fatty acid synthase (FAS) mRNA that extends 2, 6, 8 or 9 kb from the polyA tail (Figure 1). First, the mRNAs in a total cellular RNA preparation are reverse transcribed using oligo-dT as the primer. The oligo-dT-primed cDNA population will contain cDNAs that extend from the 3′ end to the 5′ mRNA cap structure unless an mRNA molecule has been cleaved. In this case, the truncated cDNAs would extend to the cleavage site nearest the polyA. The 5′ end of each mRNA species will therefore be underrepresented in the cDNA population to a degree corresponding to the extent of degradation of the RNA preparation. Second, several pairs of PCR primers are used to amplify short regions spaced at intervals along the FAS cDNA. The relative yields of the amplified fragments from regions distal and proximal to the 3′ end of the mRNA are used as a relative measure of the fraction of intact FAS mRNA in the RNA preparation.

This method has allowed us to determine which RNA preparations from chicken endoderm are suitable for construction of a cDNA library with maximal full-length cDNAs. It is applicable to RNA preparations of any species, provided suitable primers are designed for multiple regions of a long mRNA of

Table 1. Oligonucleotide Primer Pairs for the FAS RT-PCR Assay

2 kb	5′-ACCTTCTCTGACACTGACACGTGGC-3′	1854-1878	
	5′-TTGATGGGTCTCATTCCTTTGTGGC-3′	2402-2377	549 bp
6 kb	5′-AAAGGAGATTCCAGCATCGTGCAGC-3′	5520-5544	
	5′-GGAGTCAAACTAGTTATCCATGGCC-3′	5942-5918	423 bp
8 kb	5′-TCTCTGTTATGTCACTCTCAGTGCC-3′	7976-8000	
	5′-GCTTTACACGATGGCTCCTTGAAGG-3′	8315-8291	340 bp
9 kb	5′-AGCTTCTGAGCCACTTGCACCAACC-3′	9069-9093	
	5′-TGATATGGTCACAGAGGACGATCGG-3′	9326-9302	258 bp

that species. If RNA is to be prepared from multiple tissues, a ubiquitous mRNA, such as FAS mRNA, should be chosen. If RNA from a single tissue is to be assayed, the RNA may be specific to that tissue. We designed this method to test the quality of chicken RNA preparations, but PCR primers could also be generated for the FAS mRNA sequences available in GenBank® for human (Accession No. NM_004104) as well as rat (Accession No. X62888).

MATERIALS AND METHODS

RNA Isolation

RNA was prepared from chick embryo tissues by a modification of the method of Chomczynski and Sacchi (3) using Trizol™ reagent (Life Technologies, Gaithersburg, MD, USA) according to manufacturer's instructions. Chick gut RNA was isolated from pooled liver, stomach, pancreas and intestine of stage 28 (4) *Gallus gallus* embryos. Chick endoderm RNA was isolated from the anterior endoderm dissected from stage 12 to stage 15 embryos.

Reverse Transcription

Approximately 0.2 µg of total RNA was co-precipitated with 20 µg of glycogen as a carrier. Pellets were washed with 75% ethanol and air-dried. The RNA was then dissolved in either 5 µL of 15 µM random hexamer (dN_6; Amersham Pharmacia Biotech, Piscataway, NJ, USA) or in 5 µL of 2 µM anchored oligo-dT primer. This anchored primer (CDSIII/3′ PCR primer; Clontech Laboratories, Palo Alto, CA, USA) has a mixture of all possible dinucleotide combinations at its 3′ end, which results in annealing and priming only at the 5′ end of the polyA tract. Following heating for 2 min at 70°C and chilling on ice, 5 µL containing 100 mM Tris-HCl, pH 8.3, 150 mM KCl, 6 mM $MgCl_2$, 4 µM dithiothreitol, 2 mM each of dCTP, dGTP, dATP and dTTP, 0.5 µCi of ^{32}P-dCTP, 40 U of Recombinant RNasin® (Promega, Madison, WI, USA) and 200 U of SuperScript™ II MMLV reverse transcriptase (Life Technologies) were added, and the reaction was incubated for 1 h at 42°C under paraffin oil. (Reactions containing dN_6 were incubated for 10 min at room temperature before the 42°C incubation.) The reaction was then diluted to 50 µL before use in PCRs.

PCRs

PCRs were performed with *Taq* DNA polymerase (Promega) according to the manufacturer's instructions. Each 25 µL reaction contained 0.5 µL of the diluted reverse transcription reaction (the product from approximately 2 ng of total RNA), and 5 pmoles of each oligonucleotide primer. A small amount of ^{32}P-dCTP was included in each reaction to permit visualization and quantification of the products following separation by PAGE. We performed 25 cycles of the PCR, with denaturation at 94°C for 1 min, annealing at 58°C for 1 min and DNA synthesis at 72°C for 1 min in a RoboCycler® (Stratagene, La Jolla, CA, USA).

Oligonucleotide Primers

Primer pairs were designed to amplify short regions of the *G. gallus* fatty acid synthase mRNA sequence (GenBank Accession No. J03860) (5,8). Each primer pair resulted in amplification of a fragment of approximately 250–550 bp. Initial tests of primer pairs distributed at 1–2 kb intervals across the mRNA sequence resulted in the choice of four pairs for subsequent experiments, based on their relatively equal efficiency of amplification of dN_6 primed cDNA and their location (Figure 1). The sequence of each primer and its location relative to the 3′ end of the mRNA are shown in Table 1.

These oligonucleotides (all 25-mers) were based on an alignment of the 9345 nucleotide FAS sequence J03860 and a partial FAS sequence (2) J04485 in GenBank to avoid regions of ambiguity. Primer locations are given relative to the polyA addition site.

Quantification of PCR Products

Aliquots of each PCR were subjected to PAGE in a 5% acrylamide, 0.12% bisacrylamide gel in Tris-borate buffer (100 mM Tris base, 100 mM boric acid, 2 mM EDTA, pH 8.3). Gels were fixed in 10% methanol, 10% acetic acid, dried and exposed for 16–72 h to a PhosphorImager® plate (Molecular Dynamics, Santa Clara, CA, USA). The relative amount of product in each band was quantified from the scanned plate with ImageQuant™ software (Molecular Dynamics).

RESULTS AND DISCUSSION

The assay requires an endogenous tester mRNA (e.g., FAS mRNA) and a set of PCR primer pairs that amplifies short regions of the mRNA spaced along its length. One primer pair near the polyA region will amplify a fragment effectively even for RNA preparations with moderate degradation and is used as a baseline for the amount of FAS mRNA present. Primer pairs were positioned at increasing distances from the polyA detect degradation with increasing sensitivity. We chose FAS mRNA to measure mRNA quality because it is an exceedingly long mRNA (1) (and so is a sensitive measure), ubiquitously expressed (6) (so generally useful) and nearly all of its sequence is known for *G. gallus* (5,8) (so optimal

Table 2. Comparison of RNA Preparations by FAS RT-PCR

Distance of primer pair from poly A	Chick gut RNA preparation						Fragment length
	1	2	1+ RNasin	2+ RNasin	3	3,dN_6 primed	
2 kb	1.0	1.0	1.0	1.0	1.0	1.0	549 bp
6 kb	0.87	1.2	1.3	1.4	1.3	1.3	423 bp
8 kb	0.42	0.46	0.54	0.58	0.42	1.1	340 bp
9 kb	0.17	0.21	0.38	0.36	0.32	1.1	259 bp

Reverse transcription of chick gut stage 28 RNA followed by PCR amplification of primers specific for different regions of the FAS mRNA (see Figure 1). Results are from the quantitation of the bands in Figure 2. Numbers in columns represent relative molar ratios of products for each primer pair to the product of the primer pair at 2 kb.

PCR primers can be designed).

We initially tested seven primer pairs distributed at roughly 1 kb intervals along the 9.5 kb published sequence of the mRNA for chicken FAS. To identify primer pairs that amplified the FAS mRNA regions with equal efficiency, we used random hexamer primers (dN_6) to synthesize a cDNA population from total chick embryo gut RNA in which all regions of each mRNA should be represented equally. Four primer pairs at 2, 6, 8 and 9 kb from the polyA tail (Figure 1) were selected because they span the FAS mRNA and generate approximately equimolar amounts of PCR products (Figure 2 and Table 2) with a single set of PCR conditions (see Materials and Methods). The PCR cycle number was chosen so that no component of the reaction, other than the amount of target present, was limiting. Initial tests of 25–45 cycles established that PCR amplification of the cDNA product was well below the point of PCR plateau after 25 cycles (data not shown). The amplified fragments were radiolabeled by including ^{32}P-dCTP in the PCR, resolved by PAGE and quantified by phosphorimaging.

Figure 2 shows the results of a typical experiment comparing several different preparations of embryonic chick gut RNA reverse transcribed with oligo-dT primer and PCR-amplified with the four FAS primer pairs. All three RNA preparations were high quality, as judged by the nearly equal amplification of targets at 2 and 6 kb (molar ratios near 1 in Table 2), which indicates that all of the FAS cDNA synthesized for 2 kb continued to 6 kb. This means that there was no detectable cleavage by nucleases anywhere in a 4 kb region, and, therefore, the vast majority of mRNA molecules in each RNA preparation were intact. Thus, any of these preparations would be suitable for the synthesis of a cDNA library.

Significantly less PCR product was obtained for the regions 8 and 9 kb from the 3′ end of the mRNA using oligo-dT-primed cDNA from each RNA preparation. Including the RNase inhibitor RNasin in the reverse transcription reaction increased the amount of 5′ end product, indicating the action of contaminating nuclease activity during cDNA synthesis. However, even in the presence of RNasin, the oligo-dT-primed cDNA had decreased amplification of the targets at 8 and 9 kb (molar ratios below 0.6), whereas the amplified products from the randomly primed cDNA did not. This decrease represents a sharp increase in termina-

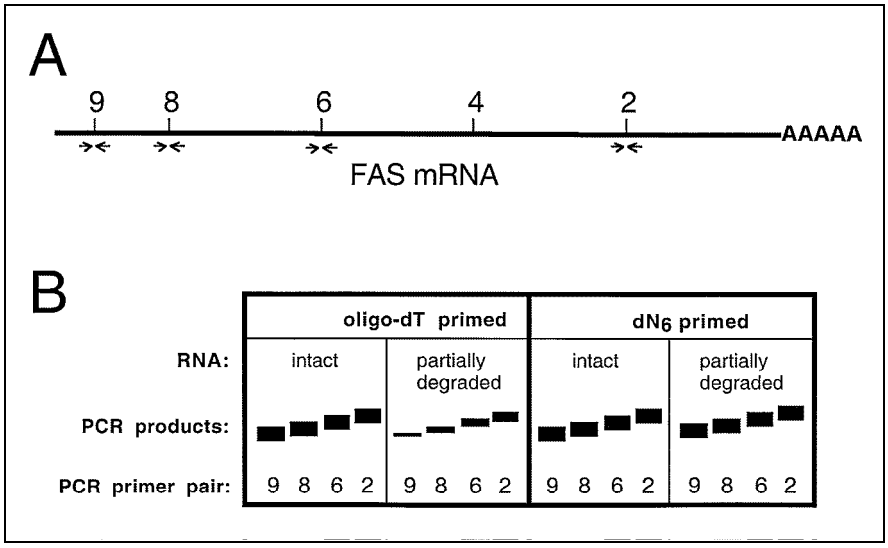

Figure 1. Schematic representation of the FAS mRNA target (A) and typical results (B). (A) The four PCR primer pairs (double arrows) are located approximately 2, 6, 8 and 9 kb upstream of the polyA tail of the FAS mRNA. See Materials and Methods for the exact location and sequence of each primer. (B) Typical results for PCR amplification of FAS mRNA target sites with either oligo-dT-primed or dN_6-primed cDNA templates from intact or partially degraded RNA preparations. When primed with oligo-dT, cDNA synthesis with reverse transcriptase will proceed from the polyA tract to the 5′ end of the message, or to the first nuclease-cleaved site 5′ to the polyA. The amount of cDNA target for each primer pair, and therefore, the yield of each PCR product (indicated by the thickness of the bands) will decrease with increased distance from the polyA in proportion to the degree of degradation. In contrast, cDNA primed with dN_6 will contain fragments representing the entire length of the cDNA equally, and the relative intensity of the bands from each primer pair will be unchanged for intact or partially degraded RNA. Also, the ratio of the amount of the amplified products from the 6, 8 and 9 kb regions to that of the product from the 2 kb region is a measure of the extent of degradation for comparison among different RNA preparations.

Table 3. The FAS RT-PCR Assay Reveals Significant Degradation in Endoderm RNA Preparations

Distance of primer pair from poly A	Chick endoderm RNA preparation											
	a	b	b[2]	c	d	f	f[2]	g	g[2]	gut1	gut1[2]	gut1[3]
2 kb	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
6 kb	0.56	0.64	0.82	0.43	0.51	0.81	0.75	1.1	1.2	1.6	1.3	1.3
8 kb	0.14	0.19	0.22	0.16	0.11	0.22	0.22	0.38	0.35	0.74	0.48	0.54
9 kb	0.06	0.08	0.08	0.06	0.06	0.10	0.08	0.14	0.17	0.34	0.23	0.38

Numbers in columns represent relative molar amounts of products for each primer pair amplified from oligo dT primed cDNA from each RNA preparation. All reverse transcription reactions included RNasin. Data are from Figure 3, except that ([2]) a duplicate experiment is shown to demonstrate reproducibility for RNA samples b, f, g, and gut1, and ([3]) the gut1 + RNasin results from Figure 2 and Table 2 are also included for comparison.

tion of the cDNA after 6 kb, but would not be expected if the RNA cleavage sites were randomly distributed. This pattern of termination may be due to a combination of the presence of nuclease hypersensitive sites in the area between 9 and 6 kb and secondary structures that increase the probability of terminating cDNA synthesis. Even though the FAS tester mRNA gives an abrupt decrease for amplified fragments past 6 kb, the relative quality of different RNA preparations can still be determined by comparing the ratios of 5′ and 3′ PCR fragments amplified from oligo-dT-primed cDNA. The enhancement by RNasin, for example, is most readily detected by the increased yields of the 8 and 9 kb fragments (Figure 2 and Table 2).

Preparation of RNA from tissues of early embryos often involves tedious dissections from many embryos to obtain sufficient RNA for subsequent analysis or cDNA cloning. The time required for such preparations greatly increases the probability that some nuclease degradation will take place. Therefore, we have used the FAS RT-PCR assay to determine which of several small-scale endoderm RNA preparations from embryonic chicks has the highest fraction of intact RNA, and so would be best for constructing a cDNA library with full-length inserts (Figure 3). Even though analysis by denaturing agarose gel electrophoresis indicated that these RNA preparations were equivalent (data not shown), the RT-PCR analysis detected clear differences in the extent of degradation. Table 3 shows the quantified results of the assay for six different endoderm RNA preparations and one high-quality RNA preparation from the gut of an older embryo for comparison. The reproducibility of the assay is demonstrated by the consistent results between duplicate assays for several of the RNA preparations. All of the chicken endoderm RNA preparations were more degraded than any of the three RNA preparations from stage 28 chick gut and therefore would not be suitable for construction of cDNA libraries having a high fraction of full-length cDNA inserts.

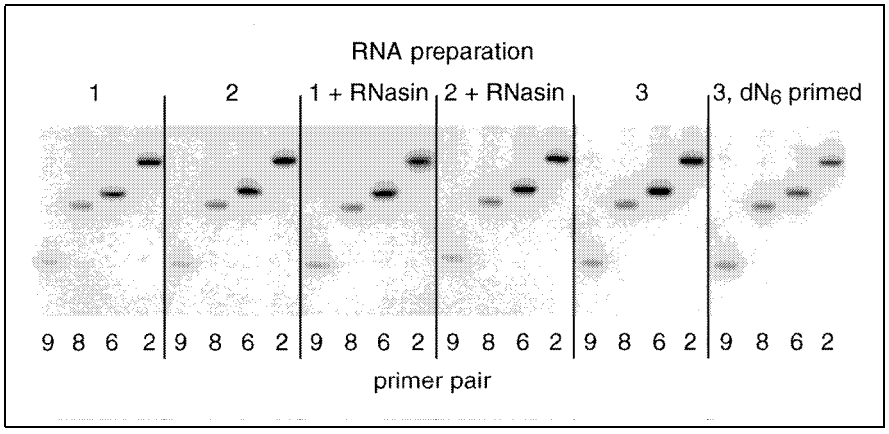

Figure 2. PCR products of a typical FAS RT-PCR assay. The relative quality of three different preparations of chick gut RNA was assessed following oligo-dT-primed reverse transcription as described in Materials and Methods. Parallel reactions with or without RNasin were performed for RNA samples 1 and 2. The results of PCR following dN_6-primed reverse transcription of sample 3 are also shown. Results are shown for primer pairs 9, 8, 6 or 2 kb from the 3′ end of FAS mRNA. Quantification of these assays is given in Table 2.

The degradation is manifested in two ways in this assay. First, the ratios of the PCR products' primer pairs positioned at 9, 8 and 6 kb to the product of the primer pair at 2 kb are lower for all of the endoderm RNA preparations than for the RNA preparations from the gut of older embryos. Second, the decrease in the ratios is most pronounced for the primer pairs at 9 kb, which reflects the increasing probability of terminating elongation of the cDNA at an RNase-cleaved site with increasing distance from the polyA. The ratio of the products of the 9 kb primer pair to the 2 kb primer pair for the gut RNA preparations was between 1/3 and 1/4, while the same ratio in the endoderm RNAs ranges from 1/17 (for preparations a, c and d) to 1/6 (for preparation g) (Table 3). Of the six endoderm RNA preparations, g is the best and a, c and d are the worst. By analyzing the PCR products of four primer pairs from each reverse transcription reaction, one can be confident that the results are meaningful.

Analysis of chick endoderm cDNA libraries validated the FAS RT-PCR

Table 4. The FAS RT-PCR Assay Predicts cDNA Library Quality

Distance of primer pair from polyA	RNA preparation 1	RNA preparation 2
2 kb	1.0	1.0
6 kb	0.35	1.6
8 kb	0.11	0.60
9 kb	0.02	0.35
average clone size:	0.9 kb	2.0 kb
fraction full length:	5/16	10/15

cDNA libraries were prepared from two different chicken endoderm RNA preparations by an RT-PCR method (SMART™ PCR cDNA library construction kit; Clontech Laboratories). Part of each oligo-dT-primed reverse transcription reaction used to create a library was assayed by the FAS RT-PCR method. The relative molar amount of PCR product for each primer pair is shown and the average clone size of each cDNA library and the fraction of identified cDNA clones that were full length.

assay (Table 4). An initial library made from RNA deemed intact by the ratio of 28S to 18S rRNAs that were analyzed by denaturing gel electrophoresis had cDNA inserts of average size of only 0.9 kb. Sequence analyses of 20 randomly chosen clones showed that of 16 cDNA clones identifiable by database homology, only five were full length. Subsequent FAS RT-PCR analysis of an aliquot of the oligo-dT-primed cDNA used to construct the library gave low molar ratios of the amplified fragments from 6, 8 and 9 kb (Table 4, RNA preparation 1), indicating that the RNA preparation was significantly degraded. A second library was prepared from RNA with high molar ratios (Table 4, RNA preparation 2). The average size of the cDNA inserts for this library was 2 kb, and 10 of 15 cDNAs identifiable by database homology were full length. These results demonstrate that the FAS RT-PCR assay can effectively identify RNA preparations suitable for constructing libraries of full-length cDNAs.

This is a useful method to check the quality of very small preparations of total cellular RNA. For example, each FAS RT-PCR assay (four primer pairs) of Figures 2 and 3 required an amount of cDNA arising from the reverse transcription of 8 ng of total RNA (roughly equivalent to 0.3 ng of mRNA). Tests of the first-strand cDNA subsequently used to create libraries (Table 4) used less than 1% of the cDNA transcribed from 1 µg of total RNA. Greater sensitivity could be obtained easily by increasing the specific activity of the ^{32}P-dCTP in the PCRs. This method is also useful to assay for possible degradation following various treatments of the RNA, for example, after oligo-dT cellulose chromatography to isolate mRNA or after DNase treatment.

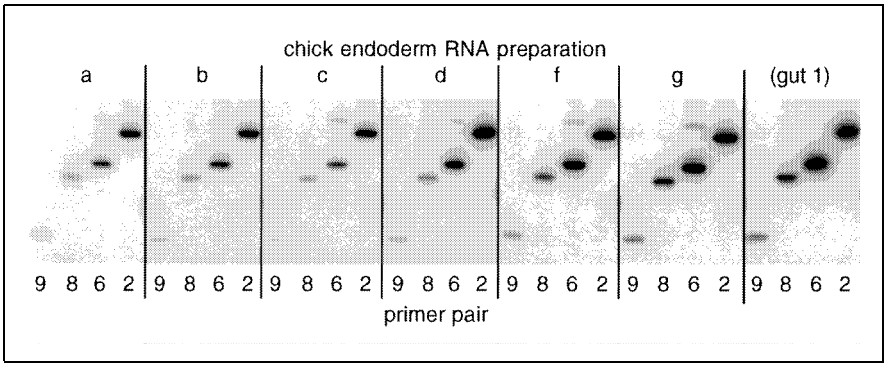

Figure 3. The relative quality of six different chicken endoderm RNA preparations compared to a stage 28 gut RNA preparation of high quality. Oligo-dT-primed reverse transcription followed by PCR was performed as described in Materials and Methods. RNasin was included in all reverse transcription reactions. Results are shown for primer pairs 9, 8, 6 or 2 kb from the 3′ end of FAS mRNA. Quantification of these assays is given in Table 2.

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