Quantitative RT-PCR on CYP1A1 Heterogeneous Nuclear RNA: A Surrogate for the In Vitro Transcription Run-On Assay

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ABSTRACT

A quantitative reverse transcription polymerase chain reaction (RT-PCR) assay was developed to amplify a region of the CYP1A1 heterogeneous nuclear RNA (hnRNA) transcript encompassing the first intron-exon boundary. The RT-PCR protocol uses a CYP1A1 recombinant RNA internal standard identical to the target hnRNA except for an engineered unique internal restriction site. Its inclusion enables normalization between reactions and a measurement of the absolute number of target hnRNA transcripts. Specificity for the hnRNA was achieved by using intron-directed primers in both the RT and the PCR. Nuclear run-on assays and the hnRNA RT-PCR assay detected an equivalent increase in transcription of *Cyp1a-1* in cultured murine Hepa 1c1c7 cells following exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The RT-PCR assay also revealed TCDD-dependent transcriptional activation of the *Cyp1a-1* gene in murine skin, a tissue unsuited to the nuclear run-on assay because of inherent difficulties associated with the isolation of nuclei. These examples demonstrate that the hnRNA RT-PCR assay is a facile surrogate for the nuclear run-on assay. Moreover, the sensitivity and design characteristics of the RT-PCR assay suggest the potential for its broad application in general transcriptional research.

INTRODUCTION

The predominant method used to examine the transcriptional status of a given gene is the in vitro nuclear run-on assay. However, it embodies several significant constraints that limit its usefulness. The assay is laborious, time-consuming, expensive and involves significant amounts of radioactivity. Most notably, it requires large numbers of isolated nuclei, restricting it to systems where adequate numbers of nuclei can be isolated. Furthermore, obtaining reproducible data is difficult particularly in situations where gene transcription is low. The advent of a more amenable method to examine gene transcription would be desirable.

Quantitative reverse transcription polymerase chain reaction (RT-PCR) amplification of cellular messenger RNA (mRNA) is increasingly used to study gene expression, especially when tissue samples are limiting. However, analyses of mRNA provide only an indirect account of gene transcription, since mRNA levels represent the sum of both transcriptional and post-transcriptional events (e.g., processing, degradation). Heterogeneous nuclear RNAs (hnRNA) are nascent, as yet unspliced transcripts. In principle, measuring hnRNA levels may prove useful in determining a gene's transcriptional state. In fact, several reports suggest that RT-PCR on hnRNA might serve as a complementary approach to the nuclear run-on assay, but adequate experimental support for this idea was never provided (3,6,11,19).

This report describes a simple, quantitative RT-PCR-based strategy to amplify hnRNA for use as a transcriptional index of any gene of interest. We used this strategy to investigate the transcriptional activation of *Cyp1a-1* in cultured murine Hepa 1c1c7 cells and in vivo in murine keratinocytes. Accumulation of the cytochrome P450 CYP1A1 protein in response to xenobiotics such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) is a consequence of transcriptional activation of the *Cyp1a-1* gene (8,18). The well-characterized transcriptional response of *Cyp1a-1* to TCDD in the Hepa cells (18) provided an excellent model in which to develop and validate the RT-PCR transcriptional assay. In turn, the keratinocytes provided a test case for the assay since *Cyp1a-1* induction occurs primarily in differentiating keratinocytes, from which it is virtually impossible to isolate nuclei (12). We consider this application of the RT-PCR strategy a facile surrogate for the in vitro transcription nuclear run-on assay offering several significant advantages over the run-on assay.

MATERIALS AND METHODS

Internal Standard Cloning Vectors

*Pst*I-digested pBluescript® II KS(+) (Stratagene, La Jolla, CA, USA) was tailed with adenosines using terminal deoxynucleotidyl transferase (TdT) and dATP from Boehringer Mannheim (Indianapolis, IN, USA) (Figure 1). Polyadenylated vectors were PCR-

amplified using an oligo(dT) primer harboring an *Xba*I restriction site (see Table 1). PCR products were digested with either *Sma*I or *Eco*RV to remove the polyadenosine sequence from either vector arm, and the vector circularized in the presence of T4 DNA ligase (Life

Technologies, Gaithersburg, MD, USA). DNA sequence analysis of individual clones using Sequenase® Version 2.0 (United States Biochemical, Cleveland, OH, USA) identified clones with 21–90 nucleotide polyadenosine cassettes in either orientation.

Synthesis of the *Cyp1a-1* Internal Standard Sequence

The 960-bp *Acc*I/*Msc*I fragment from pHAVcat (9), representing nucleotides -47 to +913 of the *Cyp1a-1* gene, was end-filled and subcloned into *Sma*I-cut pBluescript II KS(+). A partial *Bsa*HI digestion (*Bsa*HI cuts once within both the insert and vector) of a subclone containing the *Cyp1a-1* sequence in the correct orientation, and subsequent gel isolation of the 3.9-kb product, was followed by dephosphorylation of the DNA ends with calf intestinal phosphatase (Boehringer Mannheim) and ligated in the presence of 1 µg *Nco*I linkers (New England Biolabs, Beverly, MA, USA). Individual clones were screened for integration of an *Nco*I site into the insert (at position 356 in the *Cyp1a-1* gene sequence) by restriction analysis. The *Cyp1a-1* sequence, excised with *Eco*RI and *Bam*HI, was end-filled and introduced into *Sma*I cut pKST₇/A₇₀ to generate the RT-PCR internal standard plasmid p1A1NcoA⁺ (Figure 1).

In Vitro Transcription of the Internal Standard

Ten microliters of *Hind*III-linearized p1A1NcoA⁺ DNA (0.2 µg/µL) were added to a 10-µL volume of reaction mixture containing T7 transcription buffer, 1 mM of each ribonucleoside triphosphate (0.5 mM final concentration) and 2 units T7 RNA polymerase (all from Boehringer Mannheim). Transcription occurred at 30°C for 1 h. Following phenol/chloroform extraction and ethanol precipitation, the recombinant complementary RNA (rcRNA) was purified by oligo(dT)-Sepharose® (Pharmacia Biotech, Piscataway, NJ, USA) chromatography (2), quantified spectrophotometrically and stored at -80°C as single-use aliquots containing 10⁸ rcRNA molecules/µL.

Cell Culture and Total RNA Extraction

Hepa 1c1c7 cells were grown in 100-mm plates as previously described (13) and exposed to TCDD (5 nM, 1 h) or dimethyl sulfoxide (DMSO) (vehicle) for 1 h. For the RT-PCR, α-amanitin (Sigma Chemical, St. Louis,

Figure 1. Scheme for the synthesis of the internal standard plasmid. The left half depicts the strategy for adding the polyadenosine tails to pBluescript II KS(+), followed by PCR amplification using an oligo(dT) primer against the polyadenosine tails. Polyadenosines were removed from either vector arm using *Eco*RV (shown as pKST₇/A₇₀) or *Sma*I (not shown). The *Cyp1a-1* genomic sequence depicted on the right was subcloned into pKST₇/A₇₀ after introducing the *Nco*I site at position +356 to generate the internal standard rcRNA construct p1A1NcoA⁺.

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MO, USA) was added to the media at a final concentration of 2 µg/mL, 1 h post-TCDD treatment. The dorsa of outbred female SENCAR mice (National Cancer Institute, Frederick, MD, USA) were shaved 5–7 days prior to use. Mice were sacrificed, and the dorsa were excised at various times following topical treatment of the areas with acetone (200 µL) or TCDD (4 nmol in acetone). After removal of the fat pad, the skin pelts were cut into strips, quick-frozen in liquid nitrogen and ground with a mortar and pestle. RNA extraction from cultured cells and skin used the method of Chirgwin et al. (4), except that the samples were centrifuged at 55 000 rpm for 2.5 h in a TLS-55 rotor (Beckman Instruments, Fullerton, CA, USA). RNA was stored at -80°C.

Northern Analysis

Total RNA from mouse keratinocytes (20 µg per lane), or the internal standard rcRNA (1 µg), was electrophoresed in a 1.2% formaldehyde-agarose gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) by capillary transfer and UV-fixed in a Stratalinker® UV cross-linker (Stratagene). Membranes were prehybridized at 50°C for 3 h and hybridized at 50°C overnight with 10⁶ cpm/mL probe in 6× sodium chloride sodium phosphate EDTA (SSPE), 5× Denhardt's, 1% sodium dodecyl sulfate (SDS) and 100 µg/mL tRNA. The CYP1A1 probe was a ³²P-random primer labeled (Life Technologies) 700-bp *Cyp1a-1*-encoding *Pst*I fragment (10). A T4 Polynucleotide Kinase (New England Biolabs) ³²P-end-labeled RTase primer (see Table 1) served as a probe for the internal standard rcRNA transcript. Filters were washed in 1× standard sodium citrate (SSC) containing 0.1% SDS at RT for 30 min followed by 1× SSC containing 0.1% SDS at 42°C for 30 min and exposed to X-ray film (Kodak XAR-5; Eastman Kodak, Rochester, NY, USA).

Quantitative RT-PCR on hnRNA and Product Analysis

Reverse transcription was carried out using SuperScript™ II (Life Technologies) using conditions described by

Table 1. RT and PCR Primer Sequences

	Primer Sequence	Nucleotide* Position
Oligo(dT) primer	5'-GCTCTAGAGCTTTTTTTTTTTTTTTTTT-3'	Not Applicable
RTase primer	5'-TGCTTAATTCAGAGGGCTACAGATCC-3'	809-834
Forward primer	5'-GGTCCTAGAGAACACTCTTCACTTCAGTC-3'	678-700
Reverse primer	5'-TAATTGGTGTCCCTAAGGAACCC-3'	42-70

*Numbering refers to the *Cyp1a-1* structural gene.

the manufacturer. The RTase primer was annealed to a mixture containing 1–2 µg Hepa 1c1c7 cells or keratinocyte total RNA and 5 × 10⁶ copies of the internal standard rcRNA. Following a 1 h/37°C incubation, the reaction was terminated at 70°C for 15 min and diluted to 250 µL with water. The cDNA was either used immediately in the PCR or stored at -20°C. PCR was performed using 5 µL of the diluted

cDNA in a final reaction volume of 50 µL using a forward primer and a "nested" reverse primer specific for the *Cyp1a-1* gene's first exon and intron, respectively (Table 1). PCR was initiated by adding 25 µL of a 2× master mixture containing 20 mM Tris-HCl, pH 8.3 (at 25°C), 100 mM KCl, 5 mM MgCl₂, 0.002% gelatin, 400 µM each of four dNTPs (dATP, dCTP, dGTP, dTTP), 5 µCi dCTP (3000

Figure 2. Scheme for the RT-PCR assay. Depicted are the internal standard (p1A1NcoA⁺) and genomic target DNA sequences for *Cyp1a-1* and corresponding rcRNA and hnRNAs, respectively. A mixture of rcRNA and hnRNA was reverse-transcribed into cDNA using the RTase primer (nucleotides 809–834). PCR used a forward primer (42–70) and reverse primer (678–700) to amplify the cDNA. This was followed by *Nco*I digestion of the internal standard and fractionation of the products on a non-denaturing 5% polyacrylamide gel and visualized by autoradiography.

Ci/mmol; Du Pont NEN, Boston, MA, USA), 400 nM of both the forward and reverse primer and 1 unit AmpliTaq® DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) to the cDNA (25 µL) at 95°C. PCR was performed using the GeneAmp™ PCR System 9600 from Perkin-Elmer, cycling at 95°C/15 s (strand separation), 60°C/20 s (annealing) and 72°C/45 s (extension) for 30 cycles (except where indicated differently) with a final 10-min extension. Ten units of *Nco*I were added directly to the PCR tubes and incubated at 37°C for at least 1 h. Products were analyzed by fractionating 10 µL of the PCR on a nondenaturing 5% polyacrylamide/TBE (TBE = 45 mM Tris-borate, 1 mM EDTA, pH 8.0) gel followed by autoradiography of the dried gel. The absence of PCR products following the PCR on non-reverse-transcribed total RNA served as a routine check for contaminating genomic DNA.

In Vitro Transcription Nuclear Run-On Assay

Isolation of nuclei from Hepa 1c1c7 cells and the run-on assay were performed essentially as described previously (1,5). Briefly, 1.5×10^7 isolated nuclei (50 µL) were added to 100 µL 75 mM Tris-HCl, 7.5 mM MgCl₂, 225 mM KCl, 1.5 mM dithiothreitol,

1.5 mM MnCl₂, 3 µM UTP, 1.5 mM ATP, 1.5 mM CTP, 1.5 mM GTP and 100 µCi [α -³²P]UTP (3000 Ci/mmol), pH 7.9, and incubated for 30 min at 26°C. The reaction was stopped with 750 µL 0.5% SDS and 900 µL 100 mM Na acetate (pH 5.0). Radiolabeled RNA was prepared as described (16), and radiolabeled ribonucleotide incorporation was determined by TCA precipitation (5) using tRNA as carrier. Denatured, gel-purified insert DNA (2 µg) from p1A1NcoA⁺ and a β -actin clone (8) was immobilized onto Nytran® membranes (Schleicher & Schuell) by "slot-blotting". Blots were prehybridized overnight in 6× SSPE, 5× Denhardt's, 0.1% SDS, 0.1% Na pyrophosphate, 100 µg/mL tRNA, pH 7.6, at 52°C. Hybridizations were carried out in fresh hybridization solution containing 2×10^6 cpm radiolabeled RNA at 52°C for 72 h. Filters were washed twice in 2× SSC containing 0.1% SDS at room temperature for 30 min and once in 0.2× SSC containing 0.1% SDS at 65°C for 30–60 min. Filters were dried and exposed to X-ray film (Kodak XAR-5).

Data Quantitation

Autoradiographs were scanned using a Silverscanner II flatbed scanner with a transparency adaptor (La Cie, Beaverton, OR, USA), and quantitative analyses were performed on a Macintosh® Quadra 650 computer using the program NIH Image (Version 1.54). This program is written by Wayne Rasband and is available in the public domain. The hnRNA copy number is obtained by acquiring a densitometric value for both the target hnRNA and internal standard. Knowing the internal standard copy number, the signal ratios are used to determine the hnRNA copy number.

RESULTS

Quantitative RT-PCR on CYP1A1 hnRNA

Quantitative RT-PCR most frequently involves inclusion of an RNA internal standard. Plasmid p1A1NcoA⁺ encodes a portion of the *Cyp1a-1* gene

encompassing the first intron-exon boundary, corresponding to the region of interest in the target sequence (Figure 1). The poly(dA/dT) cassette located immediately downstream from the *Cyp1a-1* sequence permits the in vitro synthesis of polyadenylated transcripts from the T7 RNA polymerase promoter. These are readily purified by standard oligo(dT) chromatography (2) and quantitated, and the transcript copy number is calculated from the known sequence length. Chromatography also removes DNA template and any truncated transcripts lacking the poly(A) tail. Northern analysis performed with a [γ -³²P]ATP end-labeled oligonucleotide probe (RTase primer) confirmed that the internal standard rcRNA was full-length (results not shown).

The principle of quantitative RT-PCR and rationale behind the design and requirement for internal standards is well documented (15,17). To ensure reliable quantitation with the RT-PCR strategy (Figure 2), the PCR must not exceed the exponential phase of the amplification. Quantitation of RT-PCR products obtained after 25–40 cycles using a 100-fold range of internal standard rcRNA transcripts provided an empirical determination of the exponential phase (Figure 3). Amplification of 6×10^3 to 6×10^5 initial rcRNA copies remained exponential for at least 30 cycles. Based on product formation as a function of cycle number, the PCR efficiency was calculated to be 60% using the formula derived by Trapnell (14). Since the rcRNA internal standard and *Cyp1a-1* hnRNA target sequence are almost identical, RT-PCR amplification of the hnRNA target should occur with the same efficiency. This ensures that the PCR parameters that define exponential amplification of the internal standard also apply to the target sequence.

Cyp1a-1 Transcription Measured by the Run-On and RT-PCR Assays

Nuclei for the transcription run-on assay and total RNA for RT-PCR analyses were isolated from Hepa 1c1c7 cell cultures treated with TCDD under conditions that maximally induce *Cyp1a-1* transcription. The two methods detect quantitatively equivalent

Figure 3. RT-PCR product formation as a function of cycle number and template concentration. In vitro derived internal standard rcRNA transcripts were purified by oligo(dT) chromatography and used in RT-PCR at 6×10^3 (open squares), 6×10^4 (solid circles) and 6×10^5 (open circles) input molecules for 25–40 cycles. Undigested PCR products were gel-fractionated and visualized autoradiographically. Bands were quantified by densitometric analysis on scanned autoradiographs.

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increases in TCDD-induced *Cyp1a-1* gene expression (Figure 4). The minor difference in fold induction is not statistically significant according to a two-sample Student's *t* test. Whereas run-on data only provide information on relative transcriptional rates, the RT-PCR internal standard permits determination of the absolute number of hnRNA molecules per μg of total RNA. In fact, based on a total RNA recovery of 50 μg from 5×10^6 cells, we are detecting 1–2 and 8–10 copies per cell of the CYP1A1 hnRNA in untreated and TCDD-treated cells, respectively.

Since hnRNAs are transient intermediates, their concentration at any time reflects a steady-state equilibrium between transcription and processing (splicing). Therefore, changes in the hnRNA level may reflect alterations in transcription or the rate of splicing to mature transcripts. α -Amanitin is a potent inhibitor of RNA polymerase II (3). CYP1A1 hnRNA processing at the first intron-exon boundary was determined to have a $T_{1/2} = 34$ min in α -amanitin-treated cultures (Figure 5). Since CYP1A1 hnRNA levels remain constant for at least 4 h in the absence of α -amanitin (results not shown), the loss of transcript is not simply due to a spontaneous decrease in *Cyp1a-1* transcription. These results are consistent with a constant turnover of nascent transcripts and demonstrate that transcription and hnRNA processing can be readily dissociated in this assay. Experiments using α -amanitin concentrations ranging from 1–10 $\mu\text{g}/\text{mL}$ consistently gave the same $T_{1/2}$ value, suggesting that *Cyp1a-1* transcription was maximally suppressed even at the low α -amanitin concentration (results not shown). A previous report indicated that RNA polymerase II transcription is inhibited by 98% in the presence of 2 $\mu\text{g}/\text{mL}$ α -amanitin (3).

Cyp1a-1 Expression in Mouse Keratinocytes

The original objective behind the development of this RT-PCR assay stems from our desire to study *Cyp1a-1* transcription in skin. Previous investigation in the laboratory has demonstrated that the TCDD-mediated induction of *Cyp1a-1* occurs mostly in differentiating keratinocytes (12). Nor-

thern analysis reveals that steady-state CYP1A1 mRNA levels are dramatically elevated in murine skin within 1 h following topical administration of TCDD (Figure 6). The RT-PCR assay similarly detected an increase in CYP1A1 hnRNA content following TCDD application, which was consistent with a persistent increase in gene transcription. Therefore, the RT-PCR assay provides a tool to examine *Cyp1a-1* gene transcription in these cells as a function of their differentiation while simultaneously demonstrating its potential as a surrogate for the transcription run-on assay.

DISCUSSION

This report describes a quantitative RT-PCR assay for the analysis of

nascent CYP1A1 hnRNA transcripts. Specificity for CYP1A1 hnRNA is obtained by using an intron-directed primer in the RT reaction and a nested PCR primer (i.e., reverse primer) complementary to an upstream intronic sequence (Figure 2). Using a specific intronic primer, as opposed to oligo(dT) in the RT reaction, avoids amplification of mature mRNAs and the need to reverse-transcribe very long sequences. It also obviates possible complications arising from intron processing and removal prior to hnRNA polyadenylation. Quantitativeness of the assay is achieved by inclusion throughout the RT-PCR protocol of a CYP1A1 rcRNA internal standard that is nearly identical to the target hnRNA transcript. The sequence similarity should ensure that both RNAs are copied with equal efficiency during the RT reaction and during the exponential phase of the PCR. Consequently, absolute quantitation of CYP1A1 hnRNA sequences in the test RNA sample can be calculated simply from the ratio between the target and internal standard PCR products, irrespective of the efficiencies of the RT reaction or PCR. The internal standard also renders inter-sample comparisons more meaningful since it obviates the confounding effects of differences in RT and PCR efficiencies that may occur between samples.

Introduction of a unique *NcoI* re-

Figure 4. Comparison between the transcription run-on and RT-PCR assay. Hepa 1c1c7 cells treated with DMSO (vehicle) or 5 nM TCDD (in DMSO) for 1 h were used to isolate nuclei or total RNA. The top panel presents the results obtained by the run-on assay with nuclei and RT-PCR assay on hnRNA from the same batch of cells. The bottom panel presents quantitative analyses of the data obtained in three independent experiments. Run-on data and the RT-PCR data were normalized against β -actin and the internal standard PCR product, respectively. Error bars denote the standard error of the mean.

Figure 5. CYP1A1 hnRNA half-life. Hepa 1c1c7 cells were treated with 5 nM TCDD for 1 h prior to the addition of 2 $\mu\text{g}/\text{mL}$ α -amanitin. Total RNA was extracted thereafter at the indicated times and subjected to quantitative RT-PCR. Data from three independent experiments were normalized against the internal standard and represent the mean value $\pm$ standard error of the mean.

striction site into the CYP1A1 internal standard rcDNA provides a method whereby the PCR products corresponding to the internal standard and target hnRNA can be resolved from one another. However, accurate quantitation of the target transcript assumes that the internal standard is digested to completion following exposure to *Nco*I, since uncut internal standard co-migrates with the target sequence leading to overestimates of the target transcript. In our experience, based upon performing the PCR as replicate samples, incomplete digestion of the internal standard PCR product has not proven to be a problem. A second potential complica-

tion to quantitation is heteroduplex formation between internal standard and target sequence PCR products. Such products cannot be cut. This represents a legitimate concern only if the PCR is allowed to proceed beyond the exponential phase, when DNA templates can reanneal without replication. This issue is easily addressed by limiting the PCR to the exponential phase of the reaction (Figure 3). Others (15,17) report using a truncated internal standard that is distinguishable from the target sequence without the need for DNA digestion. The potential for an altered internal standard sequence to possess different RT and PCR efficiencies from

Figure 6. *Cyp1a-1* induction in mouse keratinocytes. Total RNA was extracted from the skins of control mice (lane 1) or mice treated for 1–4 h with either acetone (lanes 2–4) or 4 nmol TCDD (lanes 5–7). 20 µg of total RNA were fractionated on a 1.2% agarose-formaldehyde gel and stained with ethidium bromide (Panel B) prior to transfer to nitrocellulose for detection of CYP1A1 mRNA (Panel A). Quantitative RT-PCR on CYP1A1 hnRNA (Panel C) was performed on 1 µg total RNA.

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the target sequence, thereby compromising assay quantitation, renders this strategy less desirable.

RT-PCR and nuclear run-on assays of *Cyp1a-1* transcription in Hepa cultures gave quantitatively similar results in multiple analyses, each performed on a single batch of cells (Figure 4). This supports our contention that the assay is a potential surrogate for the in vitro transcription nuclear run-on assay. Variability between the RT-PCR experiments was less than 30%, suggesting that the assay should readily detect modest changes in transcription rate (e.g., 2-fold). The inherent difficulty of isolating nuclei that retain the true physiological transcription status renders run-on assays more susceptible to experimental variation.

Studies using the RT-PCR assay assume that changes in the amount of a given hnRNA sequence reflect trans-

criptional events instead of an altered processing rate. Transcription inhibitors such as α -amanitin can be used to dissociate these processes (Figure 5). If hnRNA processing rates are unaffected, then changes in the level of a given hnRNA can be directly attributed to a transcriptional event. Additionally, RT-PCR could be used to also quantify the corresponding mature mRNA levels. In this event, a proportional change in hnRNA and mRNA levels is consistent with a constant processing rate. Conversely, a disproportionate accumulation of either RNA species is indicative of a change in the rate of RNA processing. These relationships are only meaningful, however, if mRNA degradation is unaffected.

Detection of CYP1A1 hnRNA transcripts in murine keratinocytes by the RT-PCR assay paralleled the TCDD-induced increase in mRNA, consistent with a transcriptional res-

ponse (Figure 6). This system represents a practical example of where run-on assays are unfeasible due to inherent difficulties with the isolation of nuclei; and RT-PCR of hnRNA functions as a surrogate assay for assessment of the transcriptional activation of a gene. In principle, the assay could be adopted to analyze any hnRNA providing that genomic sequence information and suitable primer sites are available. In addition, the polyadenosine cassette-containing cloning vectors we generated will simplify the preparation of internal standard rcRNA sequences. By using suitable PCR primers, an internal standard can be prepared containing both a unique restriction site and appropriate restriction sites for insertion into the vector MCS.

The exquisite sensitivity of the RT-PCR assay renders it suitable even with small tissue samples comprising only tens of thousands of cells. The RT-PCR

studies reported here were performed on 1–2 µg total RNA, representing an estimated $1-2 \times 10^5$ cells. However, only a fraction of the cDNA—equivalent to 20 ng total RNA—was amplified by PCR. In situations where cell number or absolute RNA concentrations cannot be determined, quantitative genomic Southern analysis using a repetitive DNA sequence probe (e.g., Alu sequence) has been used to enumerate human cells with the intent of establishing absolute cellular transcript levels (14). Such an approach might facilitate the use of the hnRNA RT-PCR assay to study the transcriptional activation of genes in tissues of limited sample size, such as embryonic tissue or in the analyses of clinical biopsy samples. The hnRNA RT-PCR assay may also be a useful tool for addressing other issues. For instance, in hnRNA splicing studies, both the rate and the sequential order of intron splicing in a given gene could be determined without reliance on the traditional approaches of Northern analysis, S1 nuclease or RNase protection assays. Alternatively, expression of a reporter construct in transient transfection studies could be used to measure actual transcriptional events, without concern that RNA stability, translation, protein stability or enzyme activity of a reporter is affected by experimental manipulation.

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