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Isolation of DNA-free RNA, DNA, and proteins by cesium trifluoroacetate centrifugation

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Abstract

The ability to simultaneously isolate intact DNA-free RNA, genomic DNA, and proteins from a biological specimen can be useful in cloning genes and analyzing gene expression. Equilibrium density gradient centrifugation with CsCl is a useful tool for fractionating, quantitatively separating, and characterizing RNA, DNA, and the total quota of proteins, respectively, based on differences in their buoyant densities. In the present study we have reexamined the rarely used cesium salt, cesium trifluoroacetate, for the same purpose. A significant advantage of CsTFA lies in the fact that, unlike in CsCl, RNA can be recovered from a single, soluble fraction of the CsTFA gradient. Furthermore, unlike CsCl, CsTFA is freely soluble in ethanol so that co-precipitation of the salt in the recovered RNA upon alcohol precipitation does not take place. Hence, the RNA is recovered with minimum manipulations. The one-step separation of cellular macromolecule classes free of each other in small amount of starting materials provides a major advantage over other methods currently in use.

Quantitative and representative RNA isolation in a single step from small amounts of tissue, whole cells, or subcellular organelles requires a procedure in which proteins and nucleic acids are each separated in intact form. Such a goal is difficult to achieve because ribonucleases are very stable and active enzymes that require no cofactors. Only cesium salt centrifugation methods can serve this purpose since they result in suppression of ribonuclease degradation and do not otherwise physically alter RNA. Moreover, they permit quantitative recovery of RNA and are applicable to very small tissue samples, indeed. In our initially published protocol [1], the biological specimens were lysed in Tris–HCl buffer and Sarkosyl, but later another lysing alternative was formulated [2] by using high concentration of guanidine chloride or guanidine thiocyanate. Both these lysing conditions have been favored to instantly and effectively inactivate cellular RNAses by denaturing them. The RNA is then fractionated from other cellular macromolecules in a number of different ways. In the cesium chloride ultra-centrifugation procedure, based on differences in their corresponding buoyant densities, one can quantitatively separate, from each other, the major cellular macromolecules, i.e., RNA, DNA, and protein, respectively [1]. In the CsCl method, the buoyant density of RNA in saturated CsCl solution exceeds the density of this salt at saturation; hence, the RNA is pelleted out in CsCl gradient and total RNA is separated from other cellular macromolecules, such as DNA and proteins. Meanwhile, the cellular DNA (chromosomal and mitochondrial) with an average buoyant density of 1.68–1.73 g/cm^3, and the proteins with their buoyant density of about 1.2 g/cm^3, respectively, can easily be separated in a carefully constructed stepwise CsCl gradient [1]. In the CsCl method, RNA is isolated as a solid form that precipitates out from the salt gradient. There have been reports showing that cesium trifluoroacetate (CsTFA), due to its higher aqueous solubility, can attain a higher density than CsCl [3,4]. This property makes it especially suitable for simultaneous purification of RNA, DNA, and proteins, while RNA remains soluble in a CsTFA gradient formed by centrifugation, instead of in precipitated form as in a CsCl salt gradient. By using the

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same approach as we did with CsCl centrifugation [1] and exploiting the properties of CsTFA, we have optimized CsTFA centrifugation for purification of RNA, DNA, and proteins, in particular RNA.

As an illustration we have used the method to study the fate of transcripts upon induction and to compare the composition of mature, fully processed RNA with intron-containing RNA in the total RNA isolated from intact cells.

Materials and methods

The CsTFA stepwise gradient. Routinely, a stepwise CsTFA gradient was performed in 3-ml Beckman conical polyallomer centrifuge tubes. The bottom layer (cushion) of 300 μl of a density of 1.99 g/cm^3 CsTFA was overlayered with 1.5 ml CsTFA at a density of 1.7 g/cm^3. The lysed cells or tissues were adjusted with CsTFA to a density of 1.5 g/cm^3 by mixing equal volumes of the lysed sample and CsTFA at a density of 1.99 g/cm [5,6]. The sample was placed atop the intermediate layer and centrifuged in a Beckman ultracentrifuge, SW 40.1 swinging bucket rotor, overnight (20 h) at 39,000 rpm. At the end of centrifugation, the total RNA content of the sample was found at the border between the cushion and the intermediate layer, the total DNA of the sample at the border between the intermediate layer and the sample, while the protein floated atop the tube. The protein is so tightly packed at the top that it is readily removed with a needle, almost like a cork from a bottle. Both the total RNA and the DNA are easily visible at the corresponding border zones.

In order to fractionate the separated macromolecules from the stepwise gradient a simple collection of fractions by piercing the centrifuge tube from the bottom is sufficient to assure quantitative and uncontaminated recovery of the corresponding fractions.

Lysis and preparation of tissue or cells prior to centrifugation. Tissue or cells were lysed in a buffer containing 4% Sarkosyl, 10% ethanol, and 2 mM DTT, facilitated with trituration. Completely lysed samples are usually viscous due to the presence of genomic DNA. The lysed material is passed through a 20 G needle four times to shear genomic DNA and thereby reduce viscosity, and followed by CsTFA salt gradient centrifugation. Fractions varying from 5 to 30 were collected after ultracentrifugation depending on need.

Agarose gel analysis of CsTFA density gradient fractions. Nucleic acids were precipitated by ethanol and re-dissolved in 100 μl of DEPC-treated H_2O. An aliquot of 10 μl from each fraction was run on 1% agarose gel.

DNAse and RNAse treatment of CsTFA density gradient fractions. A 5-μl aliquot of each re-dissolved fraction containing DNA or RNA in the volume of 100 μl was incubated with 20 U/reaction mixture of RNAse-free DNAse I (Gibco BRL) at 30 °C for 25 min and then electrophoresed on a 1% agarose gel. A 5-μl aliquot of each re-dissolved fraction containing DNA or RNA in the volume of 100 μl was incubated with 5 U/reaction mixture of ribonuclease H (Gibco BRL), at 37 °C, for 25 min. Reaction mix was run on 1% agarose gel.

Cell culture and induction. THP1 cells were maintained at 37 °C in a 5% CO_2 atmosphere in a culture medium of RPMI-1640 supplemented with 10% fetal bovine serum and 0.02 mM β-mercaptoethanol. THP1 cells proliferated in a nonadherent state. At 60% cell confluence, lipopolysaccharides (LPS) (Sigma) were added to a final concentration of 2 μg/ml. Stimulated cells were harvested at various time points by table-top centrifugation.

Cells (293) were maintained at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cell numbers were determined by hematocytometry and cells were harvested by centrifugation. The accuracy of counting cell numbers by hematocytometry is ±15%.

Real-time RT-PCR. RNA obtained by CsTFA gradient centrifugation was precipitated, washed in ethanol, and re-dissolved in 100 μl DEPC for real-time RT-PCR analysis. Real-time RT-PCR was determined with a AmpliTaq 5700 sequence detection system instrument (Applied Biosystems). In two-step RT-PCRs performed using the Taqman Reverse Transcription Reagents kit (Applied Biosystems), cDNA was synthesized from 1 μg of total RNA in each 100 μl reaction using random hexamers as primers. Real-time quantitative RT-PCR assays were conducted with 10 ng of reverse-transcribed total RNA in a final volume of 25 μl, using 300 nmol forward and reverse primers in the SYBR Green PCR master mix of reagents.

For measurements of yields of some mRNA extracted from a small number of cells the following genes were analyzed: rpL34 (ribosomal protein 34, large subunit); rpS21 (ribosomal protein 21, small subunit); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); and P53 (protein 53).

For measurements of mRNA and intron-containing RNAs, induced IL1-RN and constitutively expressed rpS21 genes were analyzed.

Primer sets were designed using the Primer Express software (Applied Biosystems). The primer sets designed for the real-time RT-PCR analysis on various genes were the following:

IL1-RA′ exon1/exon2 Forward Primer: GACCCTCTGGGAGAAAATCCA
IL1-RA exon1/exon2 Reverse Primer: TGGTTGTTCCTCAGATAGAAGGTCTT

IL1-RA exon3/exon4 Forward Primer: TCCTGTGTCAAGTCTGGTGATGA
IL1-RA exo3/exon4 Reverse Primer: CGCTTGTCCTGCTTTCTGTTCT

IL1-RA exon1/intron1 Forward Primer: CCGACCCTCTGGGAGAAAAT
IL1-RA exon1/intron1 Reverse Primer: CACCCTCACCTTC TCCTCCTT

IL1-RA exon2/intron2 Forward Primer: TTGCAAGGACCAAATGTCAATT
IL1-RA exon2/intron2 Reverse Primer: GTGACGTGATGCCCAGATACA

IL1-RA exon3/intron3 Forward Primer: TCCTGTGTCAAGTCTGGTGATGA
IL1-RA exon3/intron3 Reverse Primer: CGCTTGTCCTGCTTTCTGTTCT

IL1-RA exon4/intron3 Forward Primer: CACCTGCCCATCTTTTGATTTC
IL1-RA exon4/intron3 Reverse Primer: GCGCTTGTCCTGCTTTCTG

S21 5′ exon/exon Forward Primer: CATTTCCGCGGCACGT
S21 5′ exon/exon Reverse Primer: AGCCTCGAAATGCAGAACGA

S21 3′ exon/exon Forward Primer: GATGATTCCATTCTCCGATTGG
S21 3′ exon/exon Reverse Primer: TGTGATTCTCTCCAGTCAAAAGTTCT

S21 5′ exon/intron Forward Primer: AAGGACGGAAGAGAGGCATGT
S21 5′ exon/intron Reverse Primer: AGCCTCGAAATGCAGAACGA

S21 3′ intron/exon Forward Primer: AAATGTGAGCCCCCAACCTT
S21 3′ intron/exon Reverse Primer: CAGATGATTCCATTCTCCGATTG

L34 5′ exon/exon Forward Primer: AAGGTTGGGAAAGCACCAAA
L34 5′ exon/exon Reverse Primer: GGACGAACCCCTCGAAGTTT

P53 Forward Primer: CTGGTTAGGTAGAGGGAGTTGTCAA
P53 Reverse Primer: AGGTTCACCAAGAGGTTGTCAGA

GAPDH Forward Primer: GGATCGTGGAAGGGTTGATG
GAPDH Reverse Primer: GGCCCGTCCACTGTCTTCT

Results

Distribution of RNA, DNA, and proteins along the CsTFA stepwise gradient

A typical profile of the distribution of nucleic acids and proteins along the CsTFA gradient is shown in Fig. 1. In this case human brain tissue (60 mg) was lysed and processed by CsTFA gradient centrifugation. Eight

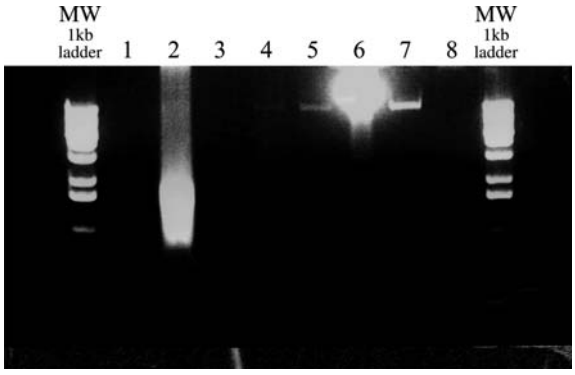

Fig. 1. Distribution of CsTFA salt gradient fractions viewed on agarose gel.

fractions were collected after centrifugation. In the figure, the contents of all fractions were analyzed by agarose gel electrophoresis. Note that in fraction #1, the cushion contained no trace of UV-absorbing material, whereas in fraction #2, the zone between the cushion and the middle layer, there is a strongly UV-absorbing material. Thereafter, there is a faint band in fraction #5, a heavy band in fraction #6, and a small band again in fraction #7. Finally, in the uppermost fraction #8, at the very entrance into the gel, there is an UV-absorbing material. Based on their relative densities these fractions were presumed to contain the total RNA in fraction #2, the majority of cellular DNA in fraction #6 (fractions #5 and #7 also contain some genomic DNA due to the overloaded gradient) and total cellular proteins in fraction #8.

Electrophoretic analysis of CsTFA gradient fractions

Electrophoretic analysis on agarose gels confirmed that fraction #2 contained the undegraded RNA (Fig. 2A). In addition to the distinct rRNA bands of defined molecular weights, diffuse UV-absorbing material corresponding to unprocessed and processed mRNA was also present.

The integrity of this diffused material was further monitored by re-isolating RNA and reanalyzing recovered RNA on gels. The integrity (high and low molecular weight RNA) was thereby reconfirmed. In Fig. 2B, the part of the gel containing RNA larger than the 28S material was cut out and divided into two portions that contained higher and lower molecular weight RNA, respectively. RNA from each portion was recovered and independently rerun on an agarose gel. Again, it was clearly shown that the high molecular weight RNA, as observed in the first lane, retained its higher molecular weight, as compared to the lower molecular weight RNA (Fig. 2C).

DNAse and RNAse treatment of CsTFA fractions

To confirm the identification of fractions #2 and #4 as RNA and DNA, respectively, samples from both were digested with either RNAse or DNAse. (Fig. 3A). Fraction #2 was thereby shown to contain completely RNAse-sensitive material (Fig. 3B) and fraction #4 to be completely DNAse sensitive (Fig. 3C).

The absorption spectra of RNA

To verify the quality and yield of isolated RNAs, the UV spectra of the isolated RNA were determined (Fig. 4). Total RNA isolated from 10, 20, and 30 mg of brain tissue was precisely proportional and each showed the same 260/280 absorbance ratio of 1.8, indicating that the total RNA was recovered consistently. The standardization of gene expression levels based on total RNA present in any given biological specimen is particularly timely and important because normalization to single housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is not recommended, since its

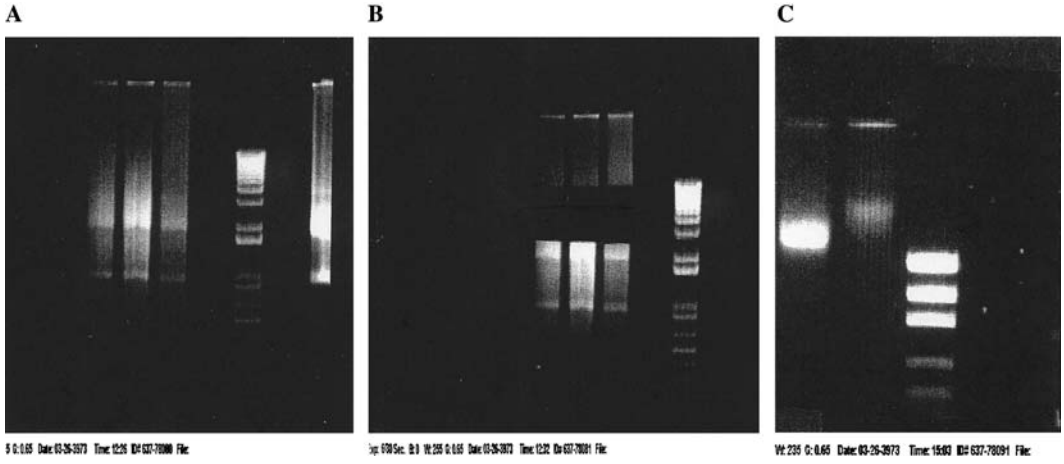

Fig. 2. Agarose gel analysis of isolated RNA fraction.

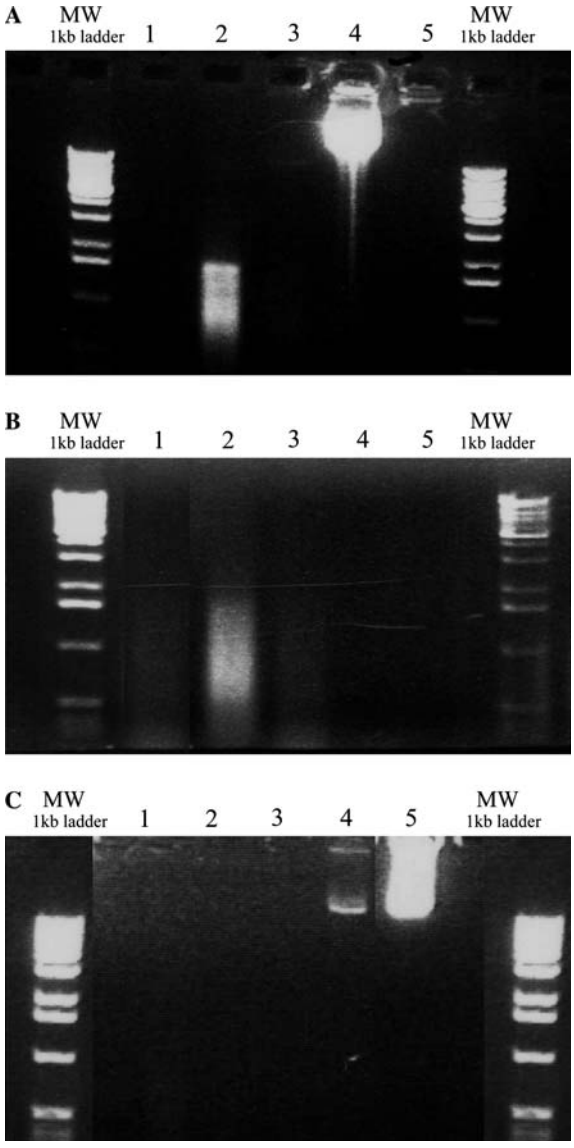

Fig. 3. DNAse and RNAse treatment of CsTFA salt gradient fractions.

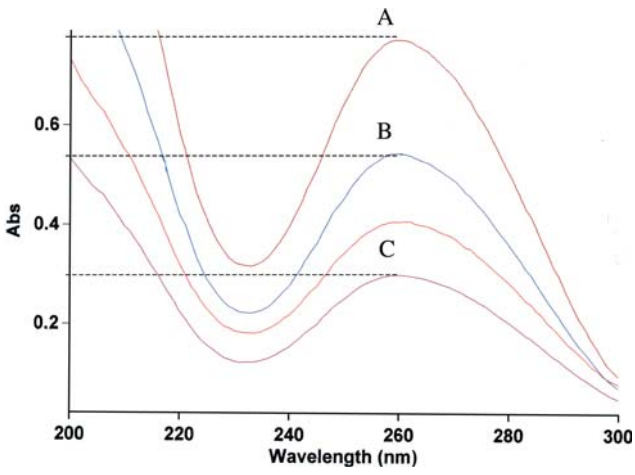

Fig. 4. UV absorbance spectra of RNA.

transcription levels can vary significantly, both in vivo and in vitro [7].

PCR and RT-PCR testing of CsTFA fractions

It was essential, for our purpose, to establish that the yield of the RNA isolated by CsTFA centrifugation is quantitative and that the RNA so isolated is not contaminated by genomic or mitochondrial DNA. Therefore, all the CsTFA fractions were further analyzed either by PCR to detect the presence of DNA or RT-PCR to detect the presence of RNA. After up to 40 PCR thermocycles, amplification of the genomic sequence of two specific genes, GAPDH, and ELF, all showed products in DNA-containing fractions, but none in RNA-containing fractions, indicating the complete absence of contaminating DNA molecules in the RNA fractions (Fig. 5A). At the same time, RT-PCR products with PCR primers that amplify exon sequences, mRNA sequences of these genes were detected in the expected size in the RNA-containing fractions, while no RT-PCR product was detected in the DNA-containing fractions, indicating also that the DNA fractions were free of RNA (Fig. 5B).

Monitoring individual mRNAs in CsTFA-isolated RNA

Assured that total RNA could be reproducibly isolated from biological specimens, we analyzed for the contents of several mRNAs in a variety of physiological settings. By calibration in a GeneAmp 5700 Sequence Detection system, one can quantify the given RNA in a sample. Although we are aware that strict quantitation for any mRNA requires calibration against its own standard, approximate quantitation can be obtained when an unrelated mRNA is used as a reference standard. Therefore, we used the message for the 1.2-kb kanamycin resistance gene prepared by in vitro transcription as a yardstick, in the range of 75–50 thousand kanamycin mRNA copies per sample (well).

Quantitative analysis showed that the lowest C^t value of 27.15 for the 50 thousand copies of mRNA per sample correlates well with the increased C^t value related to the 75 copies of mRNA per investigated sample. Our, experience and that of others are that the reproducibility and reliability of C^t measurement in the real-time RT-PCR assays varies within the range of one C^t cycle. Therefore, all our subsequent evaluations were based on this assumption (results not shown).

Quantitative extraction of total RNA from small numbers of cells

In order to see if RNA from a small number of cells could be quantitatively isolated by the CsTFA method, we extracted the RNA from human THP-1 (acute

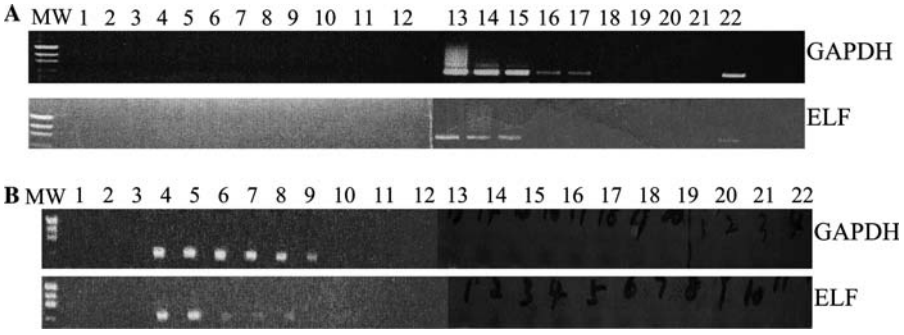

Fig. 5. PCR and RT-PCR testing of CsTFA fractions.

monocytic leukemia) and kidney-transformed 293 cells, starting with a material of 10^4, 10^5, and 10^6 cells, respectively. Since the yield of RNA from 10^4 and 10^5 cells cannot be measured accurately by photometry, we collected the fractions containing the total RNA and quantified its recovery indirectly by measuring the amount of a specific mRNA present in the total RNA by real time RT-PCR. For this purpose, the total RNA-containing CsTFA fractions were dissolved in the same volume of buffer for all three samples, and the presence of four different transcripts, ribosomal proteins S21 and L34, GAPDH, and P53, respectively, and amounts were determined by real-time RT-PCR. The results are shown in Table 1.

Table 1 compares the yield of four different mRNAs in two different human cell lines, when isolated from between ten thousand and one million cells. One order of magnitude difference in the yield of RNA corresponds to differences of 3.2 in terms of C^t value. Considering the accuracy in counting cell numbers (±15%) and of real-time RT-PCR analysis (2-fold), the recovery of RNA extracted from small amounts of cells and the content differences among the four RNAs in the cell lines would appear to be quantitative and representative. This feature of the CsTFA isolation method is particularly advantageous in cases where only localized parts of tissues (in brain, for instance) are biologically meaningful and relevant for gene expression profiling.

Monitoring the fate of transcripts upon induction

Since the CsTFA gradient centrifugation method is able to harvest the total RNA from a biological specimen, it makes it possible to monitor consistently and accurately the fate of transcripts upon induction even when the mRNA is expressed at a low level. As an example we chose to study human interleukin-1 receptor antagonist (IL1-RN) transcripts induced by LPS in THP1 cells. The induction of these transcripts was monitored at intervals between 30 min and 24 h. The mature, fully processed mRNA was monitored by analyzing the presence of the exon/exon junctions of the transcripts of IL1-RN at both 5′ and 3′ ends. However, to obtain a more general picture of the fate of transcripts upon induction, we simultaneously monitored the presence of the intron-containing RNAs by measuring the corresponding intron/exon junction sequences of the primary transcripts at both 5′ and 3′ ends as well as the intermediate intron/exon junctions between these two ends. The results of these experiments are presented in Table 2. The numbers in Table 2 represent C^t values. The threshold cycle C^t is defined as the PCR cycle number at which fluorescence is determined to be statistically significant above background. The overall accuracy of any given measurement is within ±1.0 C^t (i.e., 2-fold). The lower the C^t number, the more abundant the given RNA in the sample investigated. By analyzing the mRNA for IL1-RN at both the 5′ and 3′ exon/exon junctions, one can conclude that upon induction a dramatic increase of mature message of IL1-RN takes place. At 30 min, a 50-fold increase of mRNA over the uninduced level takes place. At 3 h the mRNA increases a 100-fold, and at 24 h, almost a 1000-fold. Here, the expression level of a constitutively expressed housekeeping gene, rpS21, which is assayed as an internal control, remains reliably and expectedly constant during

Table 1
Real time RT-PCR measurements of yields of some mRNAs extracted from a small number of cells

Number of cells	THP1 cell line			293 cell line		
	10^6	10^5	10^4	10^6	10^5	10^4
rpL34	23.5	26.2	30.1	24.5	27.1	31.1
rpS21	21.0	24.1	27.4	21.0	23.5	27.5
GAPDH	28.1	30.3	34.2	25.6	28.2	32.6
p53	29.3	32.5	36.2	23.7	26.6	31.0

The numbers represent C^t values for samples investigated.

Table 2
Real time RT-PCR analysis of mRNA and intron-containing RNAs for induced IL1-RN and constitutively expressed rpS21 genes

	Time			
	0 min	30 min	3 h	24 h
IL1-RN				
Exon1/exon2	29.3	24.7	23.8	21.0
Exon3/exon4	31.1	24.6	23.6	20.4
Exon1/intron1	30.0	28.7	28.7	27.1
Exon2/intron2	31.0	27.4	27.0	24.9
Exon3/intron3	30.7	27.0	26.7	23.7
Exon4/intron3	29.6	23.8	23.1	20.0
rpS21				
Exon1/exon2	24.2	25.3	26.0	26.0
Exon5/exon6	26.0	26.9	27.0	27.5
Exon1/intron1	30.0	31.6	30.7	31.0
Exon4/intron4	29.7	31.0	30.0	30.5

the time course of the induction, as indicated by the similar expression level of the 5′ and 3′ exon/exon junction sequences.

On the other hand, a similar analysis of the status of the premRNA for the same gene within the same time frame shows an interesting deviation from the results obtained with the mature mRNA. Although the presence of both 5′ and 3′ intron/exon junction sequences increases in the total RNA upon induction, it does so unequally. In the un-induced controls, the level of IL1-RN is very low, and both the processed and unprocessed RNA are present in the cell at an equimolar ratio. Upon induction, however, a disproportionate accumulation of unprocessed RNA takes place. By the 24th hour after induction, the 3′ intron/exon junction sequences are as abundant in the cell as the corresponding 3′ exon/exon sequences of the mature mRNA. At the same time the accumulation of the 5′ exon/intron is significantly less pronounced and differs from the corresponding 3′ exon/intron sequences by two orders of magnitude. If one compares the same expression pattern with a housekeeping gene, i.e., the ribosomal protein S21 that was assayed in parallel as an internal control, one cannot observe the discrepancies seen in the IL1-RN expression profile upon induction.

The following conclusions may be drawn from these experiments: (i) The induction of IL1-RN by LSP had, as expected, no effect on the expression levels of the investigated ribosomal structural protein (the control experiment). However, a clear and dramatic increase in the abundance of IL1-RN mRNA was observed during the time interval under investigation, reaching the 1000-fold mark by the 24th hour after induction. The reliability of this result is based also on the fact that both the 5′ and 3′ exon/exon junction sequences of the mature mRNA have increased in parallel. (ii) The picture of the presence of intron/exon junction sequences was quite different. The abundance of the 3′ exon/intron junction sequences over the corresponding 5′ junction was apparent even 30 min after induction. By the 24th hour after induction more than a 100-fold prevalence of the 3′ intron/exon sequences over the 5′ exon/intron sequences was registered. This observation then strongly indicates that the splicing of this premature RNA in the nucleus takes place from its 5′ end. Quantitation of intron/exon junctions between the 5′ and 3′ end, i.e., at exon2/intron2 and exon3/intron3, respectively, showed the tendency in the same direction. (iii) An unexpected result, which is also apparent from our experiments, is that the molar ratio of the unprocessed and processed IL1-RN RNA in the induced cell is equal to or even higher than 1. Since we know now from the human genome analysis that introns represent 24% and exons represent only 1% of the genome, our results indicate that the mass of the unprocessed RNA in the cell may reach the same prevalence as the mature, processed mRNA of any given gene, as is the case in our study. In addition to biological implications of this finding, it is important to note that this very fact may significantly influence the reliability of results obtained by gene expression profiling, regardless of what methods might be later applied in those studies.

Discussion

The CsTFA density gradient centrifugation method for quantitatively isolating an intact, DNA-free RNA is, in principle, analogous to the CsCl centrifugation method [1]. However, the trifluoroacetate anion imparts some properties that result in a higher quality nucleic acid preparation. This is because the maximum molarity of CsTFA at saturation is about 10 M compared to 7.36 M of CsCl, resulting in a maximum density of about 2.6 g/ml as compared to 1.9 g/ml for CsCl. This results in higher hydration of both the DNA and RNA in CsTFA, so that the density of these macromolecules is significantly lower than in CsCl. Thus, the density of DNA in CsTFA is about 0.1 g/ml lower than in CsCl, whereas RNA typically bands at around 1.90 g/ml. In the present work we have particularly evaluated the utility of this method for characterizing and isolating the RNA components of the cell.

In the course of developing the CsTFA method, we were able to isolate total RNA from a variety of biological samples, including cultured cell lines, human and monkey brain tissue, human tumor tissue, and sea urchin eggs, each with quantitative yield.

In summary, we would like also to emphasize that the method has broader applicability beyond RNA, since it provides from one sample, pure DNA and total protein, as well. This can find particular use when wanting to correlate gene expression profiling at the RNA level with profiling at the protein level.

Acknowledgments

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