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# Expression Patterns of Splicing Factors in Four Cell Lines

## Abstract

Heterogeneous ribonucleoprotein particles (hnRNPs) are mainly nuclear complexes of RNA and protein that are responsible for the proper splicing of immature RNA molecules. The protein components of these complexes, termed heterogeneous nuclear ribonucleoproteins (hnrp), also exhibit additional functions that may influence their expression patterns in various cancers. In particular, hnrpC has been found to associate with telomeres. In addition, hnrpM comprises the N-acetylglucosamine-specific receptor and is involved in the recycling of thyroglobulin, a glycoprotein which has been found to be elevated in the blood of patients with thyroid cancer. HnrpR exhibits two known isoforms which are expressed differently and believed to play a role in brain development. Finally, hnrpU has been observed to be cleaved as part of the pro-apoptotic caspase pathway. In all, the relative importance of these proteins as splicing factors and as members of other metabolic pathways has led to the question of whether these proteins are expressed at different levels in cancer cells. Thus, the expression patterns of such splicing factors were assessed in four distinct cell lines.

## Introduction

In eukaryotes, the presence of introns within the transcribed region of a gene adds a level of complexity to the expression patterning of genes. Proper splicing of introns out of an immature RNA is required to ensure that a proper message will exit the nucleus and be translated into a functional protein. Heterogeneous nuclear ribonucleoproteins (hnrp)

are the protein molecules that are involved in this function. Complexed with RNA, these proteins have also been implicated in the shuttling of mature messenger RNAs to the cytoplasm and may also play roles in their subsequent translation (Caporali *et al.* 2005).

Hnrp molecules are the most abundant proteins found in the nucleus; interestingly, most members of this family possess additional functions besides the ability to splice pre-mRNA. The hnrpC gene encodes two known isoforms, C1 and C2, which form a tetramer containing three units of the C1 isoform and one unit of the C2 isoform (Nakielny and Dreyfuss 1996). HnrpC is required for the assembly of the 40S hnRNP particle (Huang *et al.* 1994). A second member of this family, hnrpM comprises the N-acetylglucosamine-specific receptor that has been implicated in the recycling of the glycoprotein thyroid hormone precursor thyroglobulin. Importantly, elevated blood levels of thyroglobulin have been associated with thyroid cancer (Bajenova *et al.* 2003). With regards to a third member of this family, hnrpR, a previous study conducted by J Huang *et al.* (2005) established that expression of two isoforms of hnrpR vary widely and may be involved in neural development. Specifically, whereas the R1 isoform is expressed robustly in nearly all tissues, R2 is much more lowly expressed and is found only in neural tissues (Hassfield *et al.* 1998). Finally, hnrpU also possesses a scaffold-associated region (SAR)-specific DNA-binding domain in addition to the RNA binding domain characteristic of all hnrp proteins (Gupta *et al.* 1998). HnrpU plays a significant role in the apoptotic pathway as it is cleaved in the caspase pathway and subsequently can no longer bind DNA (Kipp *et al.* 2000). Thus, these proteins exhibit a wide variety of functions that make each a valuable marker in the assessment of the health of a cell.

HEK293 is a cell line derived from embryonic kidney cells that were transformed using adenovirus DNA. Similarly, CF2789 cells are derived from lymphoblast cells transformed via infection with the Epstein Barr virus. Using these cell lines as well as the nonmalignant LAI-5S neuroblastoma cell line and the malignant LAI-55N neuroblastoma cell line, the aim of the study presented here was to assess the expression patterns of these four splicing factors to more fully grasp the gene expression pattern of these cells.

**Materials and Methods**

Materials. RNA extracts from four cancerous cell lines (HEK293, LAI-55N, 5S, and CF2789) as well as primers for GAPDH, hnrpC, hnrpM, hnrpR and hnrpU were kindly provided by Dr. Sylvia Anderson.

Primers. Five pairs of primers were designed to amplify a segment of human hnrpC mRNA, hnrpM mRNA, hnrpR mRNA, hnrpU mRNA, and GAPDH mRNA (table 1). HnrpC primers were predicted to amplify a 130 bp product from hnrpC RNA and a 373 bp product from genomic DNA; hnrpM primers were expected to generate a 142 bp product from hnrpM RNA and a 3822 bp product from genomic DNA. HnrpR primers were predicted to amplify a product of 170 bp from hnrpR RNA and a product of 4950 bp from genomic DNA; hnrpU primers were expected to generate a product of 217 bp from hnrpU RNA and a product of 891 bp from genomic DNA. Finally, GAPDH primers were predicted to amplify a 244 bp product from GAPDH RNA and a 2095 bp product from genomic DNA.

Table 1. Primers Used in RT-PCR.

| Primer | | Sequence (5'⟶3') | Position in mRNA* | Size of Intron(s) Located between Primers |
|---|---|---|---|---|
| hnrpC | Forward | GTTGAAAGGAGATGACCTTC | 752-771 | 243bp |
| | Reverse | CATTCTTCATCTCTACTGC | 864-882 | |
| hnrpM | Forward | CAATGCACGTCAAGATGGATG | 1048-1068 | 2 Introns: 1189bp, 2491bp |
| | Reverse | CAGGTGATTGGCATCAATGG | 1171-1190 | |
| hnrpR | Forward | AGGATCACAAGTCAGCAGCA | 980-999 | 4780bp |
| | Reverse | CTGTCACCGTAGTAGCCAAG | 1131-1150 | |
| hnrpU | Forward | GGAAATTTCAGAGGAGGAGC | 2367-2386 | 2 Introns: 295bp, 379bp |
| | Reverse | CGTCCTCTGAAGTTCTGG | 2567-2584 | |
| GAPDH | Forward | AGGTGAAGGTCGGAGTCAACG | 110-130 | 3 Introns: 1632bp, 90bp, 129bp |
| | Reverse | TTTGGAGGGATCTCGCTC | 337-354 | |

*hnrpU mRNA, GenBank Accession No. NM_031314; hnrpM mRNA, GenBank Accession No. NM_005968; hnrpR mRNA, GenBank Accession No. NM_005826; hnrpU mRNA, GenBank Accession No. NM_031844; GAPDH mRNA, GenBank Accession No. NM_002046.

RT-PCR. Ten nanograms of each RNA sample were amplified in 25µl reactions (5µl 5x RT buffer, 1µl 10mM dNTPs, 1µl RT enzyme, 1.25µl 10pmol/µl forward primer, 1.25µl 10pmol/µl reverse primer, 14.5µl $dH_2O$) using the One Step RT-PCR kit (Qiagen). Additionally, a GAPDH RT-PCR reaction was run using 1ng of RNA in 25µl reactions (5µl 5x RT buffer, 1µl 10mM dNTPs, 1µl RT enzyme, 1.25µl 10pmol/µl forward GAPDH primer, 1.25µl 10pmol/µl reverse GAPDH primer, 14.5µl $dH_2O$). Finally, negative controls were run with each primer pair to test for an absence of contamination (5µl 5x RT buffer, 1µl 10mM dNTPs, 1µl RT enzyme, 1.25µl 10pmol/µl forward primer, 1.25µl 10pmol/µl reverse primer, 16.5µl $dH_2O$). One step RT-PCR was performed as follows: one cycle of 50ºC for 30 min and 95ºC for 15 min, followed by 40 cycles of

94ºC for 15 sec, 58ºC for 30 sec, and 72ºC for 30 sec, and then a final extension at 72ºC for 2 min. The amplified products were fractionated on a 1.5% agarose gel.

RT-PCR Product Purification. RT-PCR products were purified using the Marligen Biosciences Inc. Rapid PCR Purification System protocol.

DNA Sequencing. The concentrations of the purified RT-PCR products were determined using a UV spectrophotometer. Approximately 80ng of each sample were added to tubes containing either the respective forward or reverse primer used in the RT-PCR reaction and sent to Genewiz® for sequencing. The homologies between the sequences determined and those expected were assessed using BLAST.

**Results**

**RT-PCR.** Reverse transcriptase PCR was performed under the conditions described (see Materials and Methods). The products of the reaction were run on an agarose gel and are shown in Figure 1. HnrpC appears to be expressed at a lower level in the LAI-55N neuroblastoma cell line than in the other three cell lines (top row). Additionally, hnrpU is weakly expressed in the LAI-5S neuroblastoma cell line, and is only slightly higher in the malignant LAI-55N cell lines, with the highest levels of expression exhibited by the HEK293 and CF2789 cell lines (fourth row). HnrpM and hnrpR appear to be expressed at comparable levels in all four cell lines (second and third rows, respectively). Finally, GAPDH was included as a control (bottom row).

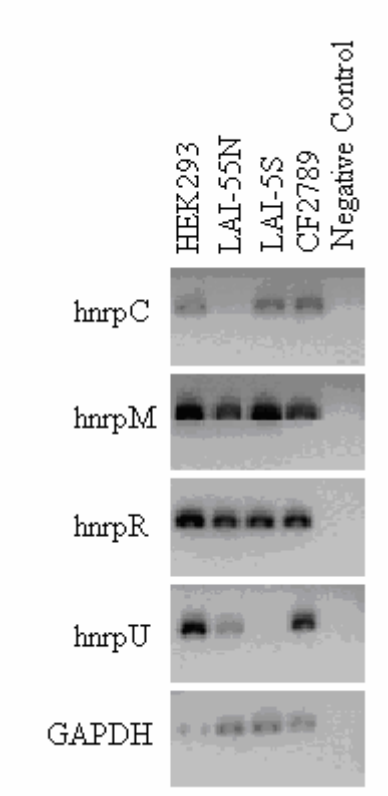


**Figure 1.** Analysis of RT-PCR products. RT-PCR was performed as described, and the resultant products were run on a 1.5% agarose gel. The labels on the left indicate the mRNA species that was reverse transcribed and amplified. The labels on the top indicate the cell line from which the RNA was extracted and subjected to RT-PCR. Negative control indicates the absence of added RNA in the RT-PCR reaction.

**Sequencing.** RT-PCR products were purified and sequenced to confirm the identity of the RT-PCR products. As shown in figure 2, the amplified sequences shared a great deal of similarity with the published sequences, thus establishing that the sequences amplified were in fact the desired products.

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RT-PCR (hnrpC R)   1    TTTTTTCAGGTTTTCCAGGAGAGAATCCACATTTTTGTTTTATCTGGGTCAGCTCCTTCT  60
                        ||||| ||||||||||||||||||||||||| |||||||||||||||||||||||||||||
hnrpC               797  TTTTTCCAGGTTTTCCAGGAGAGAATCCAC-TTTTTGTTTTATCTGGGTCAGCTCCTTCT  739

RT-PCR (hnrpC R)   61   TAATGGCCTGAAGGTCATCTCCTTTCA  87
                        ||||||||||||||||||||||||||
hnrpC               738  TAATGGCCTGAAGGTCATCTCCTTTCA  712
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**Figure 2.** Alignment of sequenced RT-PCR products with published sequences. RT-PCR products were purified and prepared for sequencing as described (see Materials and Methods). The resulting sequences were then compared to established sequences using BLAST. The RT-PCR product obtained using the hnrpC primer set (red) matched the established hnrpC mRNA sequence (accession number NM_031314). The hnrpM product (blue) matched the published hnrpM mRNA sequence (NM_005968); the hnrpR product (purple) matched the published hnrpR mRNA sequence (NM_005826), and the hnrpU product (green) matched the established hnrpU sequence (NM_031844). The GAPDH product (black) completely matched known GAPDH mRNA sequence (NM_002046.3). Parenthetical terms shown in the alignments indicate the primers used to obtain the respective RT-PCR product sequence (i.e. F indicates the forward primer, and R indicates the reverse primer). Vertical dashes indicate identical nucleotides; horizontal dashes in a sequence indicate a gap in that sequence; mismatched nucleotides are indicated by a space.

**Discussion**

The expression patterns of four splicing factors were assessed in four different cell lines. Further studies should focus on the expression pattern of other splicing factors in these as well as in other cell lines. In an era when the ability to use gene expression patterns to determine the type and stage of a given cancer has become a reality, determination of the expression patterns of various genes in a normal versus a diseased state is critical.

Various experiments involving these hnrp proteins may shed light on the results obtained in this study. Interestingly, a study conducted by Waterhouse *et al.* revealed that whereas apoptotic cells exhibit a pathway which involves the proteolytic cleavage of hnrpC by enzymatic relatives of interleukin 1β-converting enzyme, such a process does not occur in cells which are resistant to apoptosis (1996). As shown in figure 1, hnrpC is lowly expressed in only the malignant LAI-55N cell line. Taking into consideration that cancerous cells generally do not adhere to the same rules as normal cells in regards to the occurrence of apoptosis, it is perhaps possible that cleavage of hnrpC does not occur in cancerous cells because the protein is simply absent. Despite this intriguing possibility, it is necessary to conduct more extensive studies concerning the half-life of the hnrpC mRNA and the resulting protein in order to more fully assess the roles that this molecule may play in normal and cancer physiology.

A study conducted by Schepens *et al.* detailed the role that hnrpC plays in the increased expression of the upstream of Ras protein (Unr), particularly during the G2 to M transition of the cell cycle. Unr is a transcription factor that activates the transcription of cyclin-dependent kinase 11p110 (2007). Thus, it would be interesting to study the

expression patterns of Unr and of this kinase in cells such as those from the LAI-55N cell line, that have a relatively low level of hnrpC.

Furthermore, Ford *et al.* (2000) observed the ability of hnrpC to colocalize with telomerase as well as with telomeres. While the researchers did not find that hnrpC was required for the telomerase to function, they report data that suggest that association of hnrpC with telomerase may somehow allow the enzyme to access the telomere (Ford *et al.* 2002). Such findings should prompt a future study that assesses the stability of telomeres in cancers that have relatively low expression of hnrpC.

The intricate relationship between cancer and splicing is not limited to hnrpC. HnrpM has been implicated as a specific receptor of the carcinoembryonic antigen diagnostic of colorectal cancers (Bajenova *et al.* 2003, Laguinge *et al.* 2005). The current study outlined here demonstrated a lack of differential expression of the mRNA amongst the four cell lines studied. In light of the study conducted by Bajenova *et al.,* these results may not be surprising. Based on this study, perhaps a differential expression study involving a colorectal cancer cell line may reveal increased expression of this gene in such a cell line.

Huang *et al.* (2005) proposed that that expression of the two isoforms of hnrpR may play a role in neural development. The current study revealed similar expression patterns of this gene amongst all four cell lines.

Interestingly, hnrpU has been found to reduce activation of Bax transcription. Bax is a protein that complexes with Bcl2 and subsequently serves as an apoptotic activator (Howell *et al.* 2004). The mRNA encoding HnrpU appears to be present at a low level in the benign LAI-5S cancer cell line as opposed to the more malignant LAI-

55N neuroblastoma line. Further studies are necessary in order to elucidate the impact such expression patterns of hnrpU have on the occurrence of apoptosis in these two neuroblastoma cell lines.

The relationship between the aberrant expression of various genes and the onset of cancer has been well established (Swendeman and La Quaglia 1996, Amatschek *et al.* 2004). However, much remains to be elucidated regarding the precise pathways that lead to the onset or the progression of various cancers. The data presented in this study attempts to illuminate aspects of gene expression in various cell lines.

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