Chapter 6

Functional analysis of

PQBP1 transcript variants

6.1 Introduction

The biological phenomenon of alternative splicing increases the repertoire of mRNA transcripts generated from a single genetic locus. This was illustrated in the previous chapter, where 15 alternative transcripts for the gene *PQBP1* were identified from various human cDNA samples. Having identified and characterised the genetic composition and expression profiles of these variants, it remains to be determined if these transcripts are capable of performing any biological function.

Some questions about the functional influence of alternative splicing can be resolved through analysis of the predicted protein sequences. Alternative splicing events frequently result in the loss or addition of domains which can, in turn, alter a protein's function. For example, alternative splicing of interleukin-4 receptor (*IL-4R*) enables the expression of either a transmembrane or extracellular isoform. The extracellular isoform is encoded by an mRNA that includes a mutually exclusive exon that contains a stop codon, which is inserted before the exons encoding the transmembrane domains (Kruse *et al.,* 1999). However, the introduction of premature termination codons by alternative splicing does not always generate functional proteins and abbreviated proteins are frequently targeted for rapid degradation through the NMD surveillance pathway.

The work described in this chapter further characterises *PQBP1* alternative transcripts in an attempt to differentiate between transcripts that are capable of producing functional proteins and those may be the result of erroneous mRNA processing. Four approaches were used to address these questions, two assessing the predicted protein products and two studying the stability of the variant transcripts. First, the predicted isoforms encoded by the *PQBP1* transcripts were analysed *in silico,* to detect premature termination codons and to predict the encoded protein domain structures. Second, the sub-cellular localisation of each unique protein was determined. Thirdly, the stability of the *PQBP1* transcripts was assessed to determine if the presence of a PTC affected the stability of the mRNA transcript. And finally, evidence was sought concerning the mechanism by which some transcripts are degraded.

Results

6.2 Identification of open reading frames in *PQBP1* alternative transcripts

Potential open reading frames within the *PQBP1* transcripts were identified using orf-finder at the NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). An alignment of the resulting peptide sequences can be found in Appendix V, while the location of predicted start and stop codons within the exons of *PQBP1* alternative transcripts are shown in Figure 6.1. Termination codons located upstream of the one that encoded the full-length protein, were classed as premature termination codons (PTCs). These were found in 56.25 percent (9/16) of the transcripts.

As discussed in section 5.3, the inclusion of the novel exon 2a in *PQBP1* transcript variants 9 - 11 introduces a premature stop codon. However, these transcripts also contain an additional open reading frame, whose start codon is located 17 bp downstream from the PTC. *In vitro* transcription/translation studies or antibody based hybridisation techniques are required to determine if these alternative proteins can be produced.

Alternative transcripts do not always encode a unique protein. Analysis of deduced amino acid sequence confirmed that the proteins putatively encoded by transcripts 7 and 8 were identical (Table 6.1). Also, the predicted proteins using the reference translation start site were identical for transcripts in 9 and 11, while the putative open reading frames using the novel translation start site where identical in transcripts 9 and 10. For this reason subsequent functional analysis was not completed for isoforms 8, 9b and 11a.

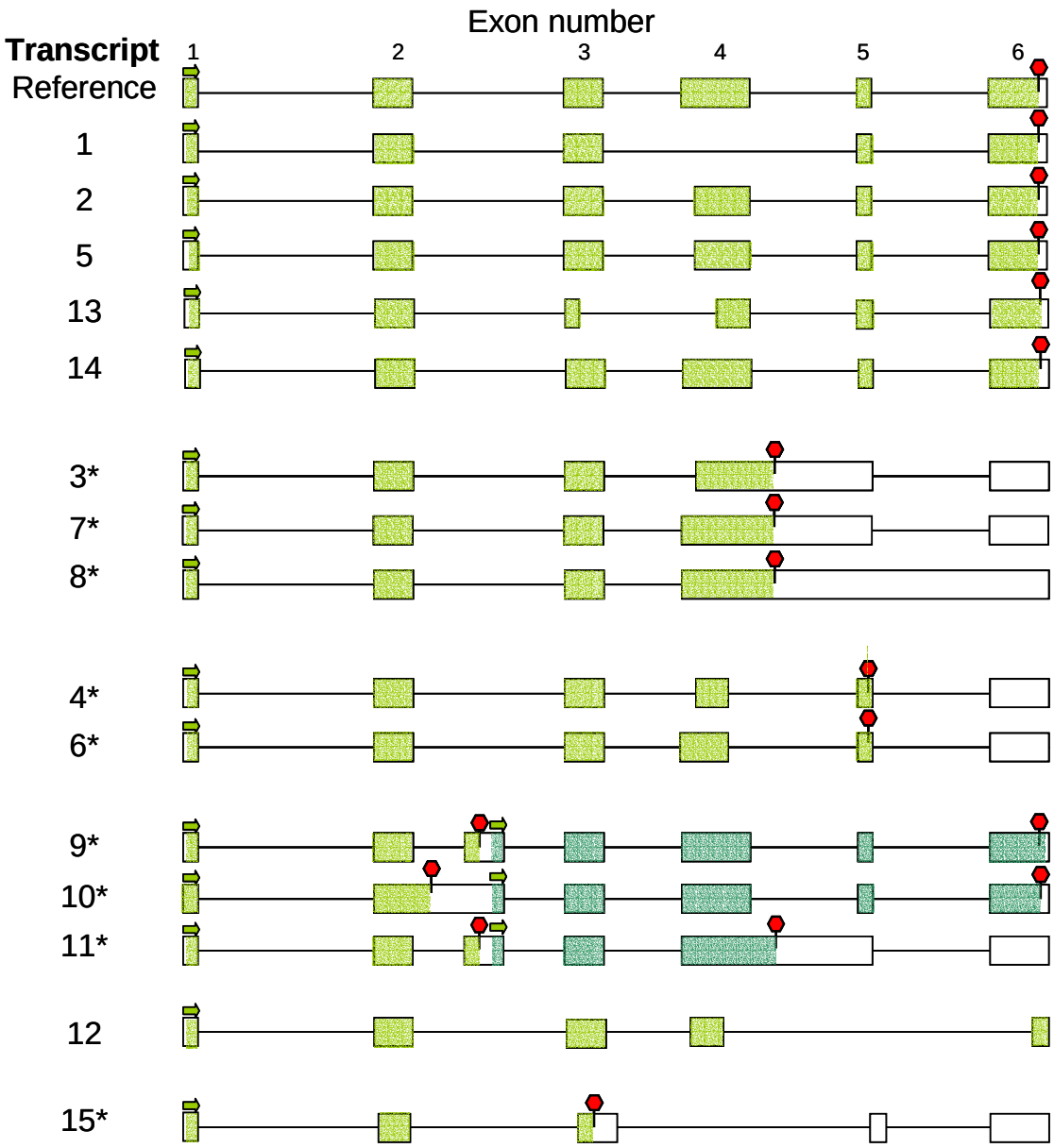

Figure 6.1 Identification of open reading frames in *PQBP1* transcripts
Open reading frames were identified using the programme orf finder. Transcript numbers refer to those outlined in section 5.2. Exons are numbered with respect to the open reading frame, (not the full length cDNA sequence), and these are displayed at the top of the figure. The location of the start codon (green arrow) and stop codon (red hexagon) in context with the exon/intron structure of the gene are shown. Transcripts with a PTC are denoted with an asterisk (*). Open reading frames that utilise the reference start codon are shaded in green. Alternative, downstream open reading frames are shaded in dark green.

6.2.1 *Predicted domains found in PQBP1 variant proteins*

Domains and motifs in all *PQBP1* isoforms were identified at Prosite (Bucher and Bairoch 1994; http://www.expasy.org/prosite/), which identifies conserved patterns (clusters of residue types) and profiles (position-specific amino acid weights) within a protein's amino acid sequence (Figure 6.2). The reference protein of *PQBP1* was predicted to contain a WW domain, an arginine rich region (ARG_RICH) and a nuclear localisation signal. WW domains are found in a number of unrelated proteins and are characterised by two highly conserved tryptophan (W) residues. The WW domain binds to proteins with particular proline-motifs, and/or phosphoserine- phosphothreonine-containing motifs (Chen and Sudol 1995; Macias *et al.,* 2002), and are frequently associated with other domains typical for proteins in signal transduction processes. The c-terminal ARG_RICH domain is rich in arginine residues and has a flexible secondary structure. It is this region of *PQBP1* that binds to homopolymeric glutamine tracts (Kumoro *et al.,* 1999b). *PQBP1* also contains a weak nuclear localisation signal which transports proteins from the cytoplasm to the nucleus.

WW domains were not identified in 25% of the *PQBP1* isoforms. Both the arginine-rich region and the nuclear localisation signal were absent from isoforms 1, 4, 6, 9a, 11a 12 and 15. Isoform 13 was predicted to contain both the WW domain, and the nuclear localisation signal but not the arginine_rich region (Figure 6.2)

Functional information could not be inferred for peptides 9a, 10a, and 15 as Prosite analysis failed to identify any domains in these sequences. Functional proteins might be produced from the alternative open reading frame found in transcripts 9, 10 and 11 (encoding proteins 9b, 10b and 11b) as they retained both the arginine rich region and the nuclear localisation signal. However, transcript 15 encodes a protein without any known domains.

Another predictive algorithm, PSORT (Nakai and Horton 1999; http://psort.nibb.ac.jp/) was employed to predict the subcellular localisation of *PQBP1* isoforms (Figure 6.3). This analysis also suggests that the isoforms encoded by transcripts 1, 4, 6, 9a, 10a 12 and 13 may not be localised to the nucleus, where the predicted likelihood of these transcripts being transported to the nucleus was

less than 50%. Interestingly, PSORT predicted that isoform 1 would be transported to the mitochondria.

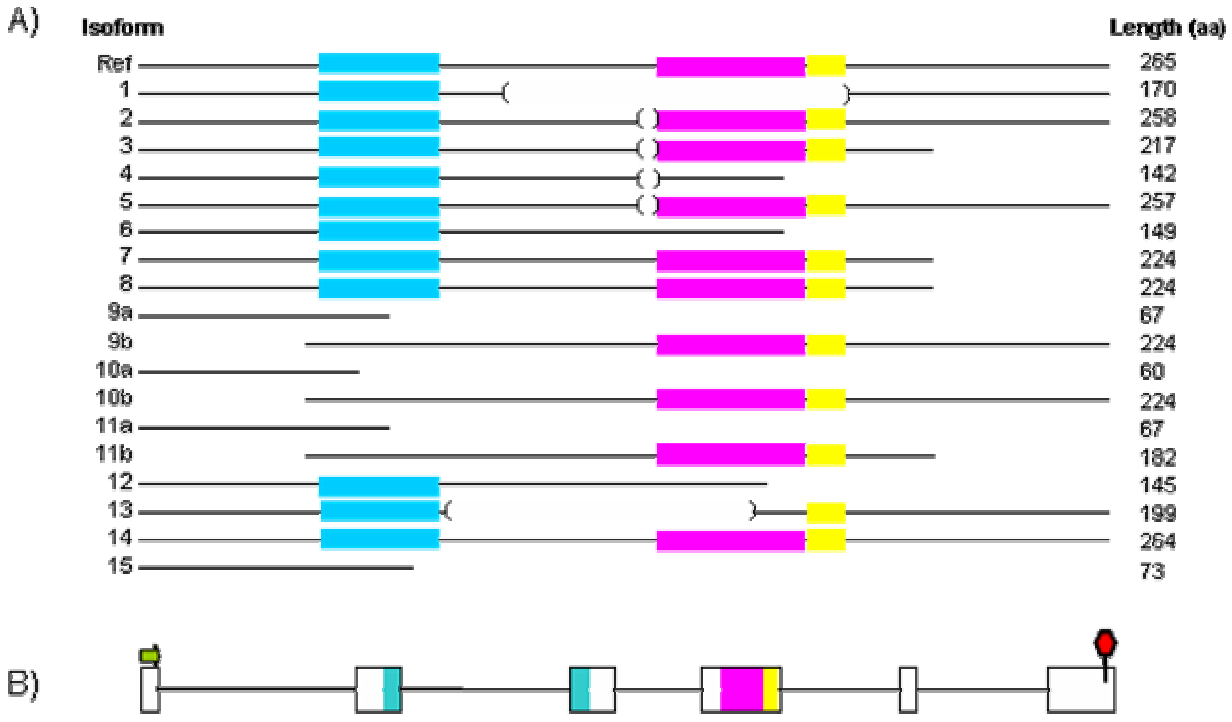

Figure 6.2 Predicted motif patterns in *PQBP1* putative proteins

A) Domain and motifs were predicted using the computational algorithim, Prosite. Deleted regions of the predicted protein sequences are flanked by brackets ( ). The domains/motifs displayed are: WW domain (blue), arginine rich region (pink), nuclear localisation signal (yellow).

B) Location of domains in exon/intron structure of *PQBP1*.

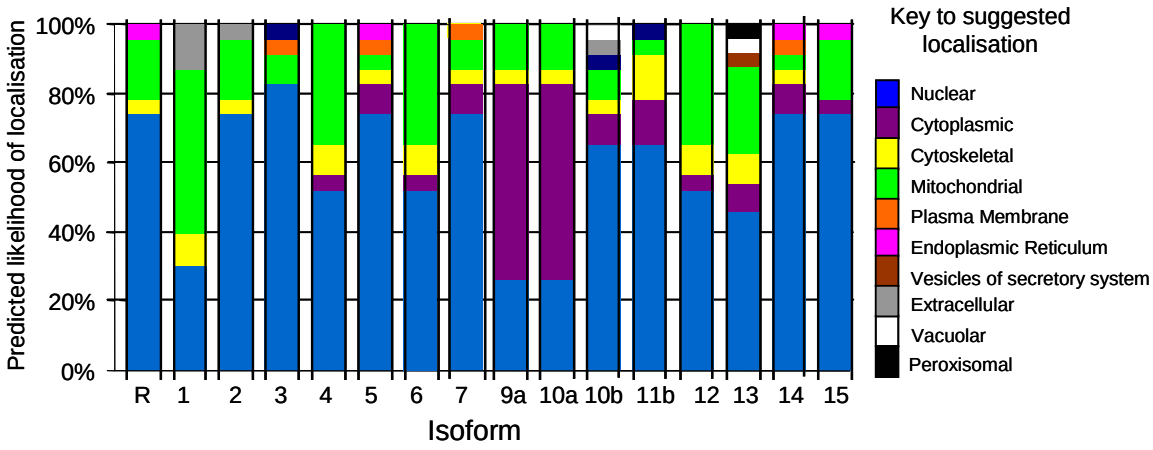

Figure 6.3 Prediction of subcellular localisation of *PQBP1* isoforms

The computational algorithim PSORT was used to predict the subcellular compartment in which the *PQBP1* proteins would be located.

Table 6.1 shows the predictions regarding nuclear localisation of the *PQBP1* isoforms by the two programmes. The majority of the isoforms (11/19) contained a nuclear localisation signal and were predicted to be localised to the nucleus by PSORT. Five isoforms did not contain a nuclear localisation signal and were therefore predicted to be found within a non-nuclear location by PSORT. However, transcripts 4, 6 and 15 did not contain a nuclear localisation signal were predicted to be localised to the nucleus by PSORT. Conversely, transcripts 13 does contain a localisation signal but was not predicted to be localised to the nucleus by PSORT.

Prosite and PSORT use sequence searches to confer similarity to identify known and previously characterised protein domains or localisation signals. Any differences observed in the comparison may be attributed to the different sensitivity and searching patterns of the computational algorithms. However, it is important to remember that these results are merely predictions and require additional experimental analysis to confirm the actual intracellular location of these proteins.

Table 6.1 Predictions regarding the nuclear localisation of *PQBP1* isoforms

Transcript	Prosite	PSORT	Transcript	Prosite	PSORT
Ref	✓	✓	9b	✓	✓
1	×	×	10a	×	×
2	✓	✓	10b	✓	✓
3	✓	✓	11a	×	×
4	×	✓	11b	✓	✓
5	✓	✓	12	×	×
6	×	✓	13	✓	×
7	✓	✓	14	✓	✓
8	✓	✓	15	×	✓
9a	×	×			

6.3 Intracellular localisation of *PQBP1* transcripts

Following *in silico* predictions of sub-cellular localisation, an experimental approach was used to study the localisation of individual isoforms in cultured cells. In order to confirm that the T7 epitope did not influence the cellular distribution of the *PQBP1* isoforms, the distribution was assessed using both amino- and carboxyl-epitope tagged proteins. Fusion proteins carrying the T7 epitope tagged to either the amino or carboxyl – terminal of each *PQBP1* isoform were expressed in both African green monkey kidney cells, cos-7, and Chinese hamster ovary, CHO-K1, cell lines. The subcellular location of the *PQBP1*-T7 isoforms was then detected using an anti-mouse T7 antibody. A schematic of the overall experimental procedure and the sequence of the T7 epitope are displayed in Figure 6.4 and the procedure is described in more detail in sections 2.23 and 2.24.

6.3.1 *Preparation of constructs*

The plasmids used in this analysis were pCDNA.3NT7 and pCDNA.3CT7 (kind gift of Dr J. Collins). These were linearised using the appropriate restriction enzymes for the location of the T7 epitope tag; pCDNA.3NT7 was digested with *Not*I and *XbaI* while pCDNA.3CT7 was digested with *Hind*III and *NheI*.

Primers were designed to amplify each of the cloned open reading frames in the holding vector pGEM®-T Easy. The primers also contained the appropriate restriction enzyme sites to permit their ligation with the pCDNA.3 vector. The primer combinations used to amplify each transcript are listed in Appendix VI. The stop codon was retained in the N-tag constructs while it was avoided in the C-tag constructs. A detailed description of the cloning procedures used in this experiment can be found in section 2.20. Sequence verification of all clones was performed by the Research and Development team at the WTSI.

PQBP1-T7 constructs were prepared for 14/15 amino-tagged isoforms and 11/15 carboxyl-tagged isoforms. Isoform 12 was not cloned as it contained a *Hind*III restriction enzyme site within the ORF which would have also been digested during the preparation for cloning. Alternative vectors of pCDNA-T7 Ntag and pCDNA-T7-Ctag without a *Hind*III site were available to carry out this experiment. These vectors used a *Xmn*I site for ligation between the insert and vector. Attempts made to clone isoform 12 using this vector were unsuccessful. In addition, isoforms 1 and

15 were not successfully cloned using the pCDNA-T7 Ctag vector, as all attempted ligations failed.

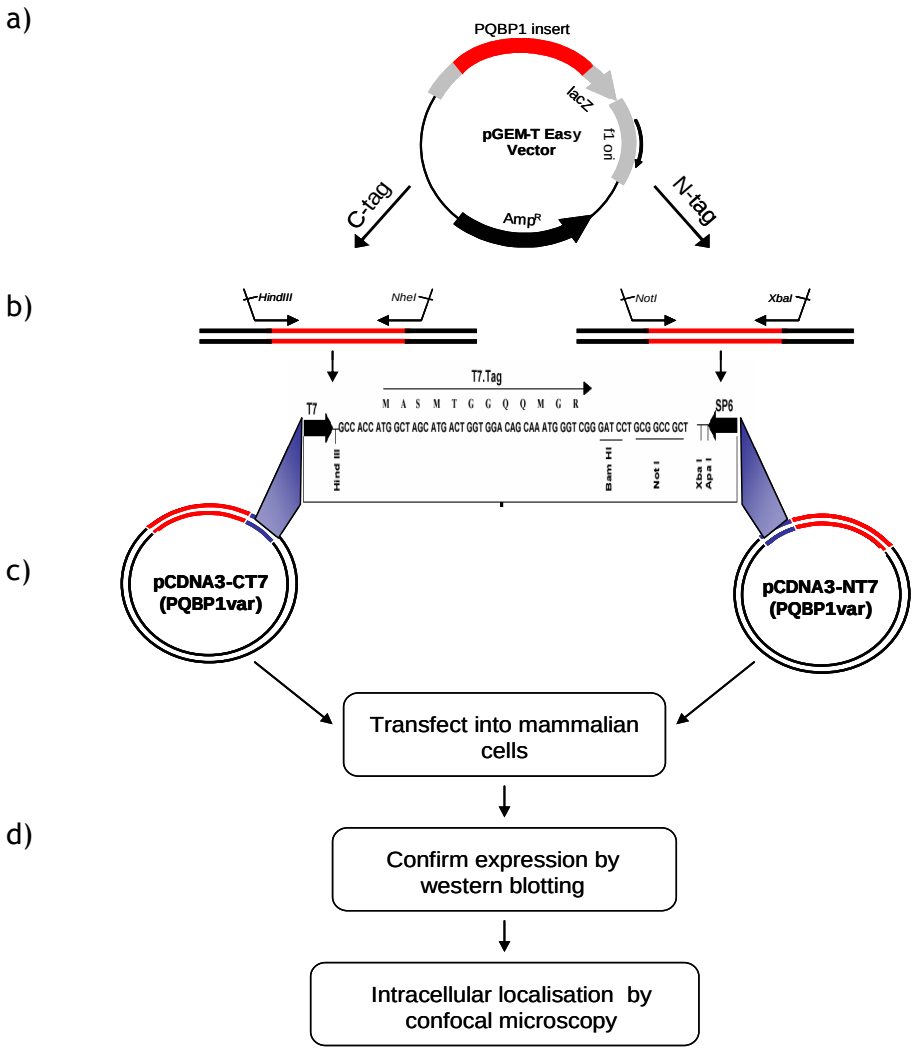

Figure 6.4 Schematic of subcellular localisation protocol
Constructs were prepared in which the T7 epitope was fused to either the N-terminal or C-terminal of *PQBP1* proteins. Following transfection into mammalian cells, the expression of the transcripts was confirmed by Western blotting. Confocal microscopy was also employed to visualise the subcellular localisation of *PQBP1* proteins.

a) Representation of the holding vector containing the cloned ORFs (section 5.2).
b) Amplification of the ORF using primers that contain a restriction enzyme linker suitable for ligation to the pCDNA3 expression vector.
c) The nucleotide and amino acid sequence of the T7 eptiope tag and its location in the pCDNA3 vectors.
d) Overview of experimental protocols carried out to determine the subcellular localisation of *PQBP1* isoforms.

6.3.2 *Western blot analysis of PQBP1-T7 fusion proteins*

Constructs containing the *PQBP1* open reading frames fused to the T7 epitope tag were transiently transfected into Cos-7 and CHO-K1 cell-lines as described in sections 2.12. Two cell lines were used in this analysis to ensure that the genetic background did not influence the cellular distribution of *PQBP1* isoforms and to ensure uniformity between this experiment and the mRNA stability experiment carried out in section 6.4.

The expression of the *PQBP1*-T7 proteins was confirmed by Western blot analysis using an anti-T7 monoclonal antibody (section 2.24). This was carried out to confirm that the predicted size of the *PQBP1* isoforms and to ensure that the isoforms were not exported from the cell. Extracellular proteins were isolated by gentle centrifugation 24 hours after transfection. After the supernatant (the fraction that included the extracellular proteins) had been removed, the cells were disrupted using chemical treatment to release the cellular proteins. Both the extracellular and cellular protein fractions were assayed.

Proteins containing the T7 epitope tag were of the imperically calculated size Table 6.2. Expression of *PQBP1* isoforms could not be confirmed for all constructs. In particular, constructs *PQBP1*-9a, -10a, -10b, 11b and 15 could not be detected. This could be attributed to inefficient transfections, or the constructs may be rapidly degraded with either the mRNA transcript or protein sequence being highly unstable.

Table 6.2 Expected sizes of T7 tagged proteins

Isoform	Predicted size (kDa)	Isoform	Predicted size (kDa)
Reference	30.5	9a	7.6
1	18.8	10a	7.0
2	29.7	10b	25.5
3	25.3	11b	21.1
4	16.4	12	16.3
5	29.6	13	18.7
6	17.2	14	30.3
7	26.2	15	8.4

* molecular weights were predicted using pepstats (emboss)

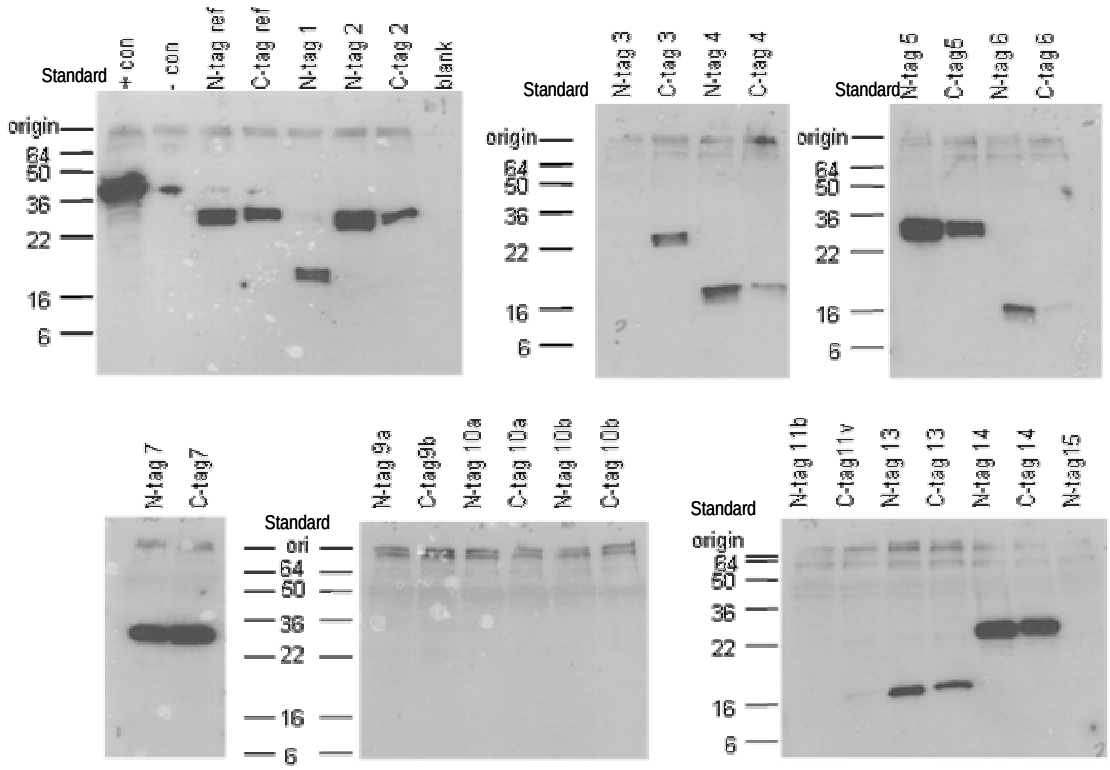

Figure 6.5 Western Blot analysis confirms *PQBP1* expression for some, but not all constructs

Cos-7 cells were transiently transfected with pCDNA3-*PQBP1*(variant)-T7 plasmids. Expression of *PQBP1*-T7 fusion proteins was confirmed using an anti-T7 antibody. Also included in the analysis are a positive control (MAPK) and a negative control (no plasmid added). Standard – protein molecular weight (kDa) standards are also displayed.

6.3.3 *Subcellular localisation of PQBP1 transcripts in cos-7 and CHO cells.*

The distribution of the *PQBP1*-T7 isoforms was assessed by confocal microscopy (Figure 6.6). This was achieved by transfecting the *PQBP1*-T7 tag into African Rhesus monkey, Cos-7, and chinese hamster ovary, CHO-K1 cells (Figure 6.6). All transfections were completed in duplicate on each cell line.

The cellular location of the *PQBP1* transcripts was ascertained for the reference *PQBP1* protein as well as 67% (10/15) of the alternative isoforms. Two isoforms were not assayed because they were not successfully cloned (due to ligation failures). Cellular expression of three other isoforms (isoforms 9, 10 and 11) was not identified by confocal microscopy. Expression of these isoforms also failed to be confirmed by Western blotting.

Visualisation by confocal microscopy localised the reference and isoforms 2, 3, 5, 7, 13 and 14 to the nucleus, while isoforms 1, 4, and 6 were not targeted to any cellular compartment (Figure 6.6). Instead, these isoforms were found to be ubiquitous throughout the cell. These observations were consistent with both the previously published localisation experiments (Okazawa *et al.,* 2001; Kalscheuer *et al.,* 2003), and the computational predictions made in section 6.2.1.

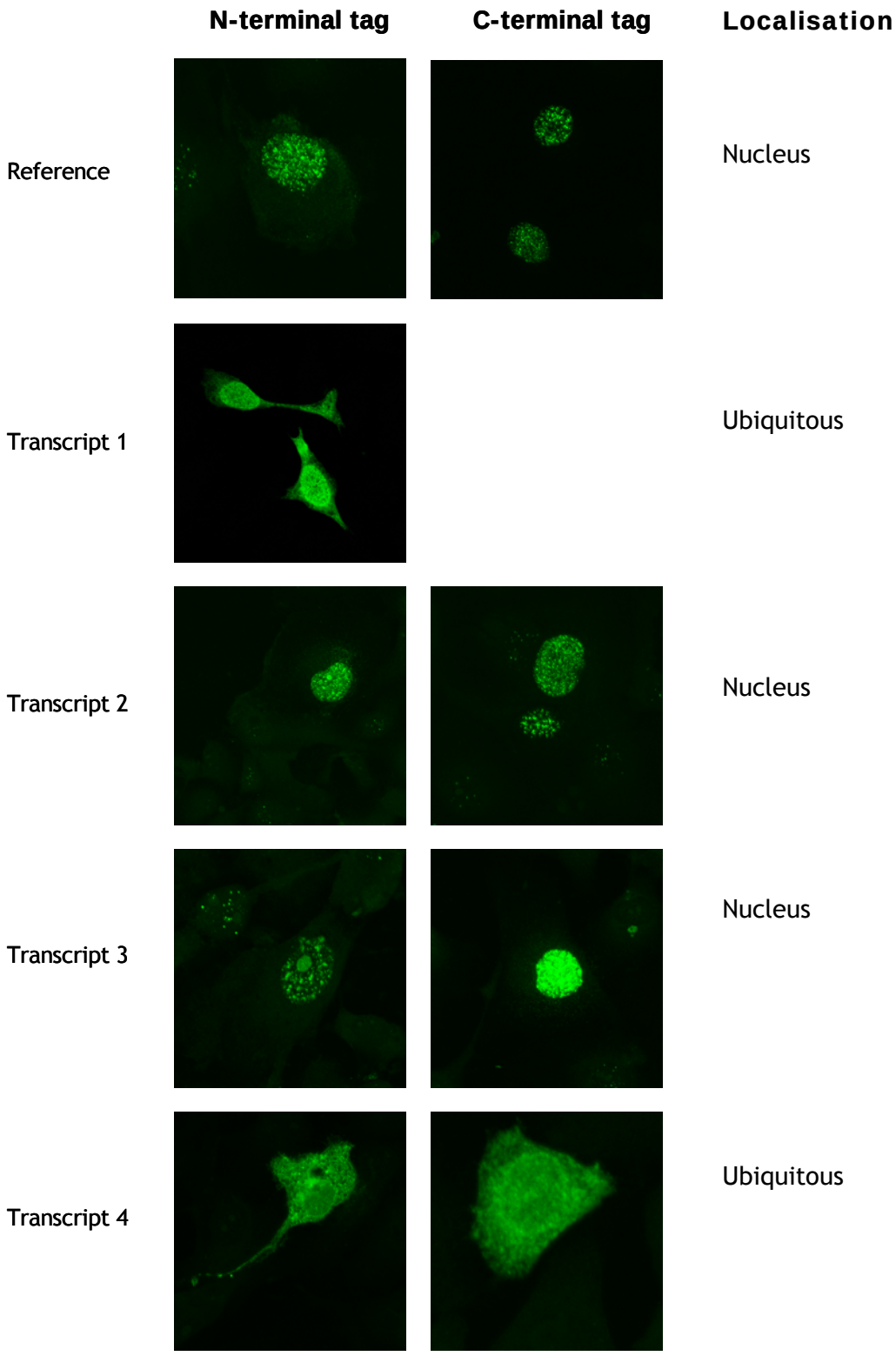

Figure 6.6 Localisation of *PQBP1* alternative isoforms in Cos-7 cells.
Isoforms encoded by *PQBP1* alternative transcripts were expressed as fusion proteins with the T7 epitope tag. The sub-cellular localisation of proteins was assessed by immunofluorescence with an anti-T7 monoclonal antibody and an FITC tagged secondary antibody. Cells were visualised by confocal microscopy. Counter-staining with DAPI confirmed the location of the nucleus (results not shown). Continued overleaf.

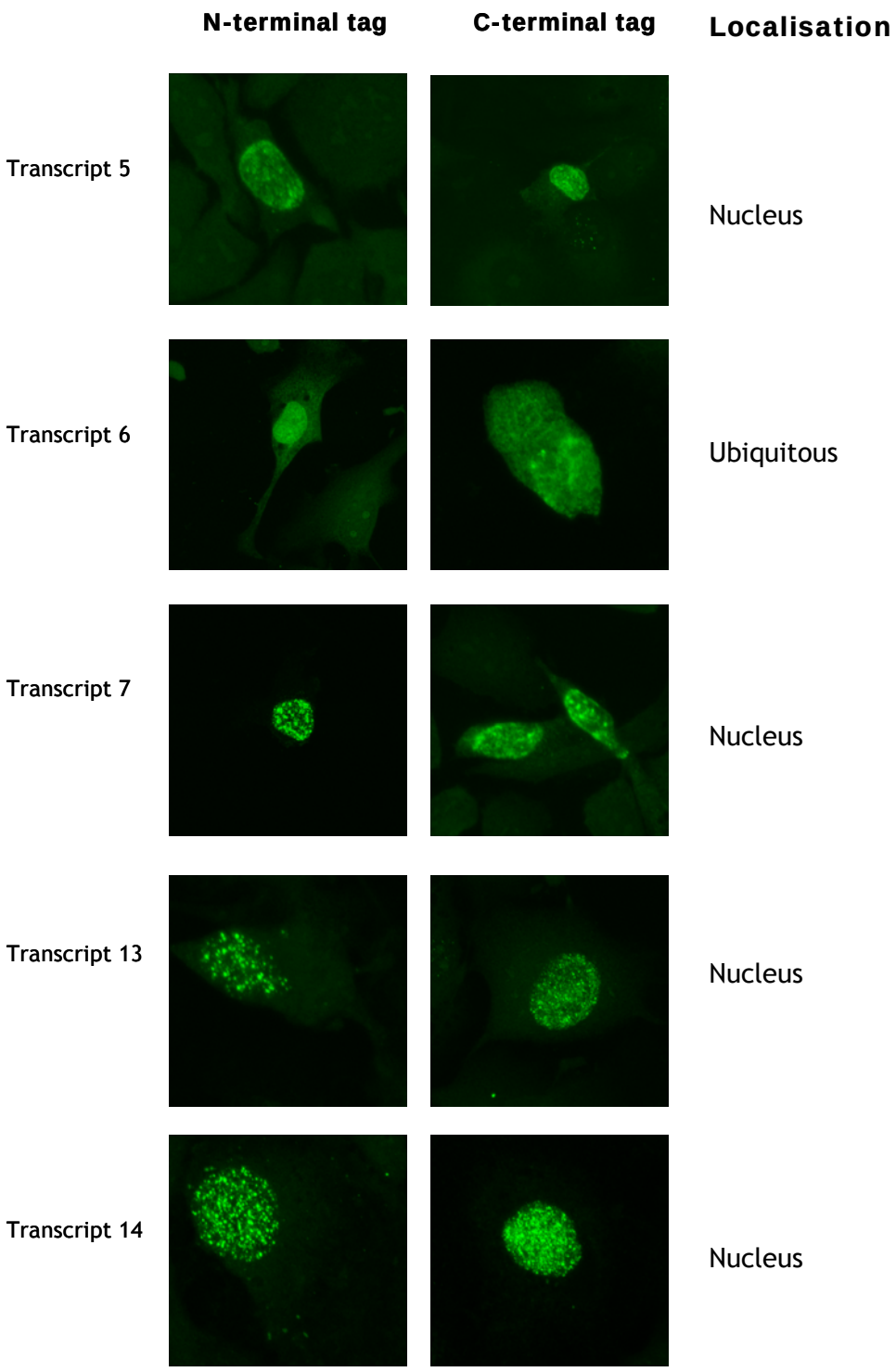

Figure 6.6 continued.

6.4 Analysis of the stability of *PQBP1 transcript variants*

The stability of an alternative transcript may also give an indirect indication of its biological importance. The NMD surveillance mechanism is thought to rid cells of potentially deleterious transcripts that are produced by mutations or errors in mRNA processing. Analysis of the open reading frames within the *PQBP1* transcripts described indicated that 9/16 variants, which represent approximately 2% of total *PQBP1* transcripts, have a PTC and therefore do not have the potential to encode a full length protein. The low expression levels of these transcripts could indicate that they are infrequently produced by the splicing machinery, or that they are removed by the NMD pathway. Furthermore, if these transcripts are rapidly removed, this may be an indication that they have no biological function. Therefore, it was decided to monitor the mRNA decay rates for the *PQBP1* transcripts. The expectation was that a difference in the stability of PQBP1 transcript would be seen in transcripts that contain a PTC and those that do not. As the expression of several *PQBP1* isoforms (isoforms 9-12) was not detected by Western blotting or immunofluorescence, it was conjectured that this may attributed to cellular stability of the relevant mRNA. Analysis of the mRNA decay patterns for these transcripts may shed further light on why expression of these transcripts could not be detected by immunofluorescence.

The method chosen to evaluate mRNA degradation kinetics was a transcriptional quantification strategy using a tetracycline (tet)-regulated promoter. Changes in mRNA levels were monitored over a defined time course, following targeted transcriptional repression with a tetracycline derivative, doxycycline. One advantage of this technique was that expression of the *PQBP1* gene could be tightly regulated without interfering with cellular physiology (Gossen & Bujard, 1992). It was hoped that this specificity would permit *in vivo* decay rates to be more closely reproduced, as pleiotropic effects that are frequently observed when using non-specific transcription/translation inhibitors would be avoided. The BD™ Tet-off system was chosen to regulate the expression of *PQBP1* alternative transcripts, and a description of this expression system follows.

The BD Tet-Off system is dependent on the integration of a tet-response element (TRE) and a regulatory element (pTet-Off) into a mammalian cell line. To control expression the pTet-off regulatory plasmid must first be stably transfected into the

cell line. This plasmid encodes the tet-response transcription activator, which is able to activate transcription by binding to tetracycline response elements (TRE). In this study, the expression of *PQBP1* transcript variants is under the control of the TRE that is found in the plasmid pTRE-TIGHT (Figure 6.6).

Following the introduction of a *PQBP1* transcript into the pTRE-TIGHT response plasmid, the construct is transfected into a CHO-AA8 Tet-Off cell line. Here, the tet-responsive transcriptional activator, expressed from the regulatory plasmid binds to the TRE, thus activating transcription. As doxycyline is added to the culture medium, transcription of the *PQBP1* insert from the TRE is turned off in a dose-dependent manner. This system has previously been used to monitor mRNA stability of the β-globin gene (Couttet and Grange 2004).

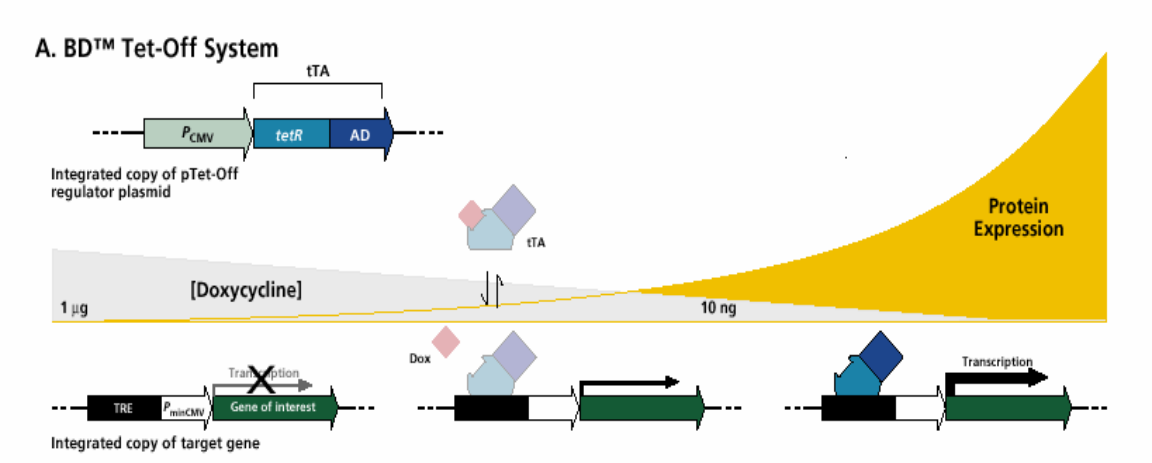

Figure 6.7 Schematic of gene regulation on the BD™ Tet-Off system
This system is dependent on both a regulatory (pTET-off) plasmid and a response plasmid (pTRE-Tight-*PQBP1*). When cells contain both of these plasmids the expression of *PQBP1* transcripts only occurs when the tet regulatory protein (tTA) is bound to the tet regulatory element (TRE). In the tet-off system tTA binds to the TRE and activates transcription in the absence of tetracycline or its analogue, doxycycline.

6.4.1 *Preparation of constructs*

PQBP1 cDNA clones (section 5.2) were subcloned into the response plasmid of the Tet-Off expression system, pTRE-TIGHT. A schematic diagram outlining the methodology employed to prepare the constructs is shown in Figure 6.8.

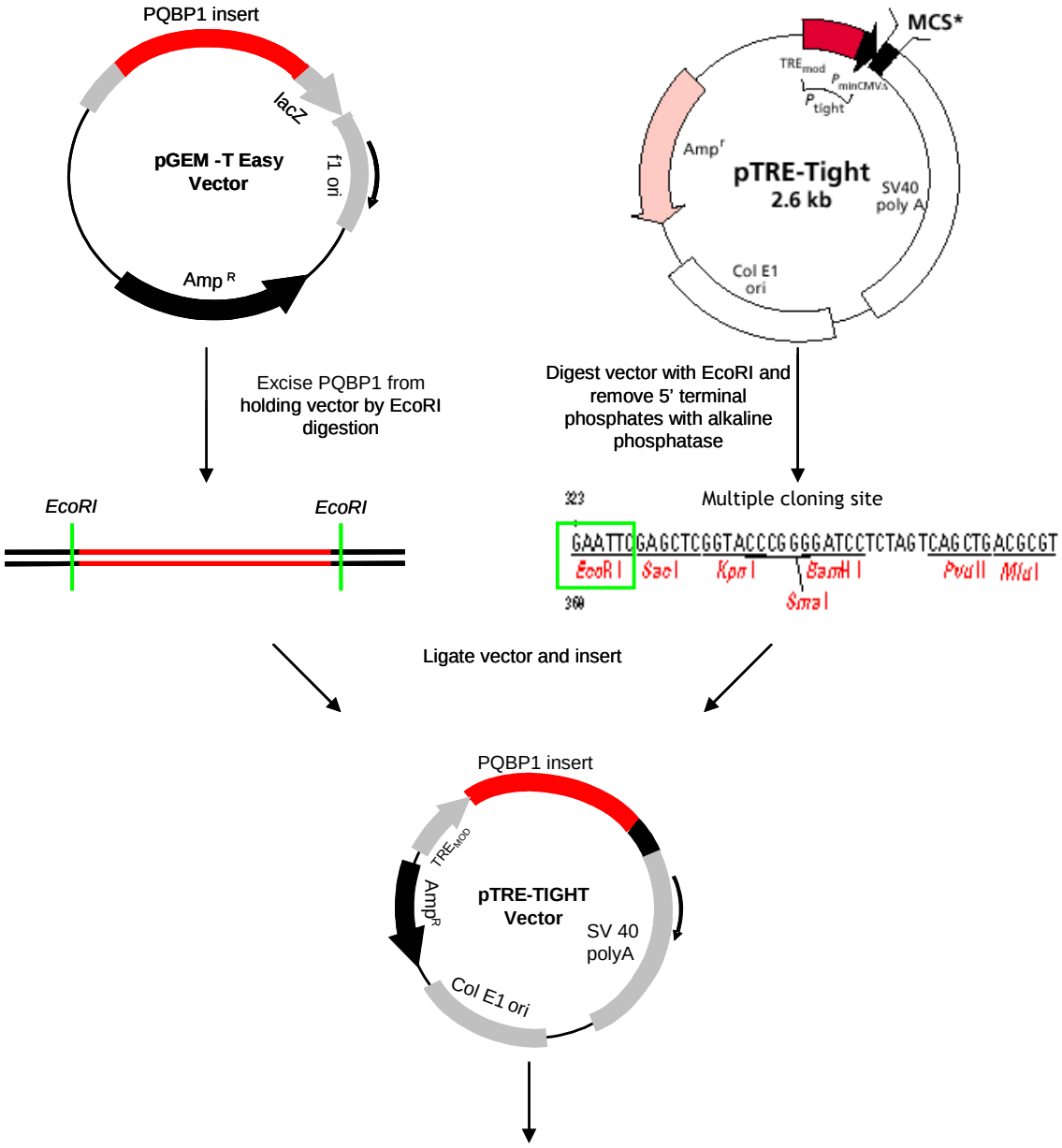

Figure 6.8 Preparation of constructs to study *PQBP1* mRNA decay rates
Cloned cDNAs were excised from the pGEM-T Easy vector by *Eco*RI digestion. The transcripts were ligated to complementary ends of the pTRE-TIGHT vector.

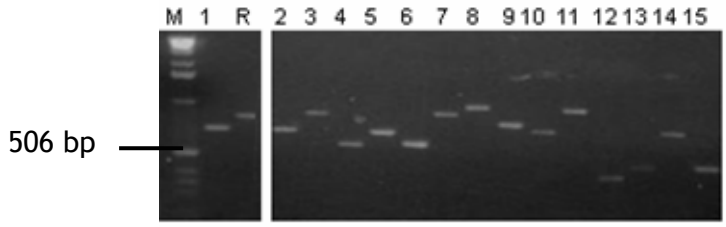

Figure 6.9 Preparation and analysis of pTRE-TIGHT-*PQBP1* plasmids
A) Transcripts (numbered accordingly) were digested from the holding vector by *EcoR*I digestion. Molecular weight marker is denoted (M). Purified plasmids are displayed.

The cloned fragments were excised from the holding vector using the restriction enzyme *EcoR*I and were purified (Figure 6.9), and treated with shrimp alkaline phosphatase prior to ligation with the plasmid pTRE-TIGHT (described in detail in section 2.21). The fidelity of the insert was confirmed by DNA sequencing, which was carried out by the Research and Development Team at the WTSI, and the pTRE-TIGHT-*PQBP1* constructs were purified using a qiagen midi-prep plasmid procedure (section 2.14.6).

6.4.2 *Optimisation of experimental protocol*

Transfection efficiencies

Transient transfection of the response plasmid in to CHO-AA8 tet-off cells was optimised using the vector pTRE-TIGHT-eGFP, where expression of the green fluorescent protein is under the control of the TRE. Transfections were completed varying the volume of transfection reagent used (GeneJuice™ (Novagen) from 1 to 8 µl), cell densities (0.3 x 10^5 to 1 x 10^5 cells per ml) and DNA concentrations (1-2 µg). The efficiency of transfection was monitored by fluorescence microscopy. The number of fluorescing cells in 3 random fields of view were counted for each transfection (results not shown). Optimal transfection results were achieved using 2 µg of plasmid DNA, 4 µl of GeneJuice and 3 x 10^5 cells in a volume of 3 ml per transfection. These conditions were used for all subsequent transfections.

Doxycycline dose response curves

The necessary concentration of antibiotic required to inhibit expression was determined by incubating CHO-AA8-luc cells (Clontech) with various concentrations of dox. The CHO-AA8-luc cells, have both the tet-off TRE and luciferase gene

stably integrated into their genome (Clontech). Expression of the luciferase gene, was under the control of the tet-off TRE and hence was repressed upon the addition of doxycycline. Doxycycline was titrated between the range of 1pg ml^{-1} and 100 ng ml^{-1} and the concentration that most effectively repressed the expression of the luciferase gene was determined using a luciferase activity assay (section 2.22.2). Results for each sample were normalised to the total protein content of each cell as determined by the Bradford assay (section 2.22.3). Approximately 500 fold repression was observed in this experiment and the optimal concentration required effectively to repress the expression of the luciferase gene was 50 ng ml^{-1} (Figure 6.10). This concentration was used in subsequent experiments.

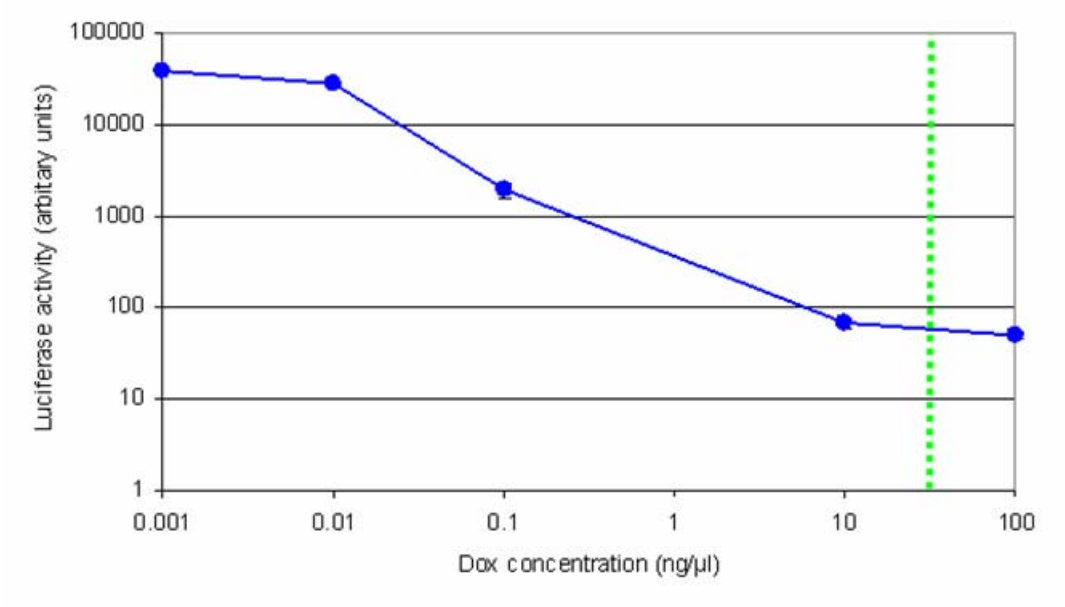

Figure 6.10 Dose response curve for the CHO-AA8-Luc control cell line
Expression of luciferase was repressed using various concentrations of doxycycline. Following an incubation of 4 hours in the presence of doxycycline luciferase activity was detected by the luciferase luminescence assay. The concentration of doxycycline used in all subsequent experiments is also indicated (green – 50 ng/µl).

6.4.3 *Quantitative analysis of mRNA stability*

Messenger RNA decay rates of the *PQBP1* alternative transcripts were determined using the tet-off expression system displayed in Figure 6.11. During transfection cells were grown in the absence of doxycycline, which was added to all cells at a final concentration of 50 ng μl^{-1} at time 0. RNAs were collected at various intervals following transcriptional arrest and cDNA was then synthesised. In total,

13 assays were performed on the following transcripts; reference 1, 2, 4, 6, 7, 8, 9, 10, 12 and 15. Five of these were carried out in duplicate from the transfection stage – they are reference *PQBP1*, 1, 6, 8 and 10. Control assays were also completed to:

- Ensure the specificity of *PQBP1* amplification. Here, CHO-AA8 cells were transfected with plasmid pCMVß to ensure that no amplification was observed using *PQBP1* primers in the CHO genetic background.
- Ensure that gene expression of the tet-regulated gene was not repressed in the absence of doxycycline.

Measures were taken to ensure that the abundance of each *PQBP1* transcript was accurately quantified throughout each experiment. Messenger RNA levels in each sample were normalised to the house keeping gene beta-actin (*Actb)*. This gene was chosen because the mRNA sequence was available in the Chinese Hamster (Embl:U20144**).**

The expression levels of the various *PQBP1* transcripts were determined using the primer pair, *PQBP1*.Q10 (section 5.4.3) which can amplify any of the *PQBP1* transcript variants. To ensure that additional splicing of the *PQBP1* transcript did not take place following its transfection, the full length *PQBP1* transcript was amplified and its size was assessed by end-point PCR using the primers that were used to amplify the entire *PQBP1* transcript (section 5.2).

The pTRE-TIGHT- transcript specific *PQBP1* construct was also co-transfected with the reporter vector, pCMVß in order to correct for varying transfection efficiencies between experiments. In this vector the expression of lacZ was under the control of the strong CMV promoter and is not regulated by doxycycline. The relative abundance of both the *PQBP1* transcript and the lacZ gene were determined by real-time PCR. The abundance of the *PQBP1* transcript was then normalised to the abundance of the LacZ gene.

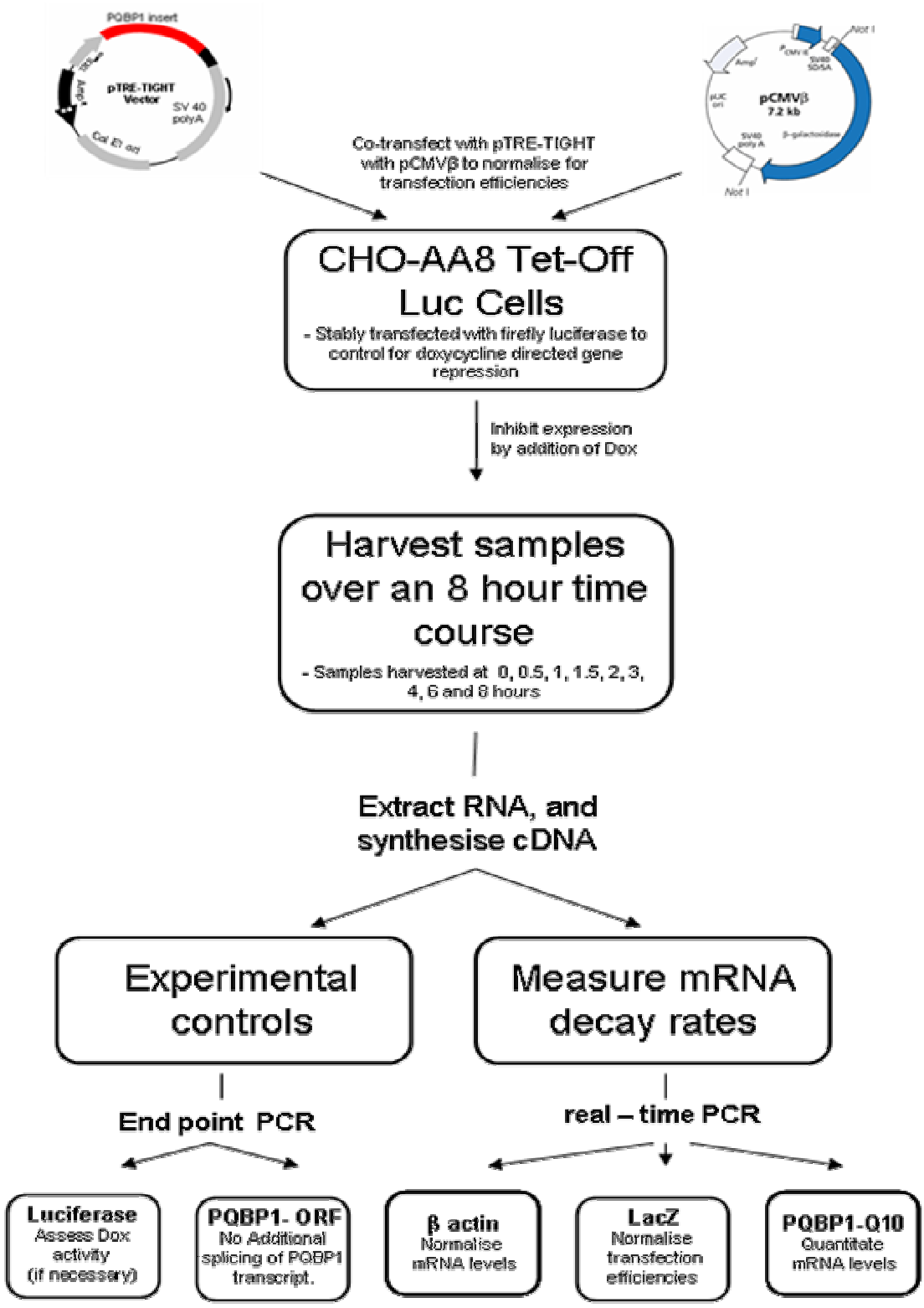

Figure 6.11 Schematic of experimental protocol used to assess mRNA decay rates

Validation of real-time PCR primer combinations

Variations in transcript abundance were determined by real-time PCR using SYBR-Green quantification. As SYBR-Green binds to double stranded DNA in an indiscriminate manner, it was necessary to ensure that all reactions amplified only the product of interest. Therefore, the samples were subjected to the heat dissociation protocol following the final cycle of the PCR. Dissociation of the PCR products consistently produced a single peak for *ActB*, lacZ and *PQBP1* demonstrating the presence of only the expected product in the reaction (data not shown). In addition, *PQBP1* expression was not observed in untransfected cells.

For the real time assay the fold change in lacZ and *PQBP1* mRNA levels were determined using the $2^{-\Delta\Delta CT}$ method (described in section 2.15.6) which assumes similar amplification efficiencies of the target gene and the internal control gene. All three primer pairs had similar amplification efficiencies (data not shown). All quantitative PCRs were performed in triplicate. Moreover, specific mRNA amplification was ensured by the inclusion of a negative control (cDNA prepared without reverse transcriptase) for each sample.

The normalised abundance of the *PQBP1* transcripts was expressed in relation to the amount present at time zero. In all cases, the level of a *PQBP1* transcript increased following the addition of doxycycline, and peaked at either 30 or 60 minutes and then declined. These decay curves are shown in Figure 6.12. The patterns of decay were similar to those described for hexosaminidase A (alpha polypeptide) (*HexA*) which also used SYBR-Green real-time PCR detection (Schmittgen *et al.,* 2000). First order mRNA decay rates were assumed to determine the mRNA half lives. These were calculated by linear regression analysis between the time points 1 and 4 hours. The correlation coefficient and standard error of the estimates were calculated from the linear regression and are shown together with the mRNA half life in Table 6.3 and Figure 6.12.

Owing to time limitations, these experiments were not completed on all *PQBP1* transcript variants. The reference and transcript variants 1, 2, 4, 6, 7, 8, 9, 10, 12, and 15 were assayed.

Table 6.3 Analysis of mRNA decay obtained from *PQBP1* alternative transcripts

Transcript	k (h^{-1})	mRNA half life (h)	r^2	Standard Error
Reference	0.6289	1.10	0.59597	0.491
1	0.4194	1.65	0.7341	0.109
2	0.523	1.27	0.9387	0.309
4	0.8784	0.79	0.9891	0.255
6	1.094	0.63	0.8696	0.156
7	0.5997	1.16	0.602	0.78
8	0.648	1.07	0.8179	0.227
9	1.122	0.62	0.9847	0.04
10	1.1437	0.61	0.8416	0.68
12	0.2088	3.31	0.9009	0.178
15	0.3902	1.78	0.6975	0.65

The amount of each *PQBP1* transcript was determined in relation to *ActB* and LacZ transcript levels for each of the transcripts listed above. Samples were collected over an 8 hour time period following transcription arrest by the addition of doxycycline, and cDNA was prepared. The first-order rate constants for *PQBP1* degradation (k), correlation coefficient (r^2) and standard error were calculated from linear regression analysis of the mRNA decay plots (Figure 6.12).

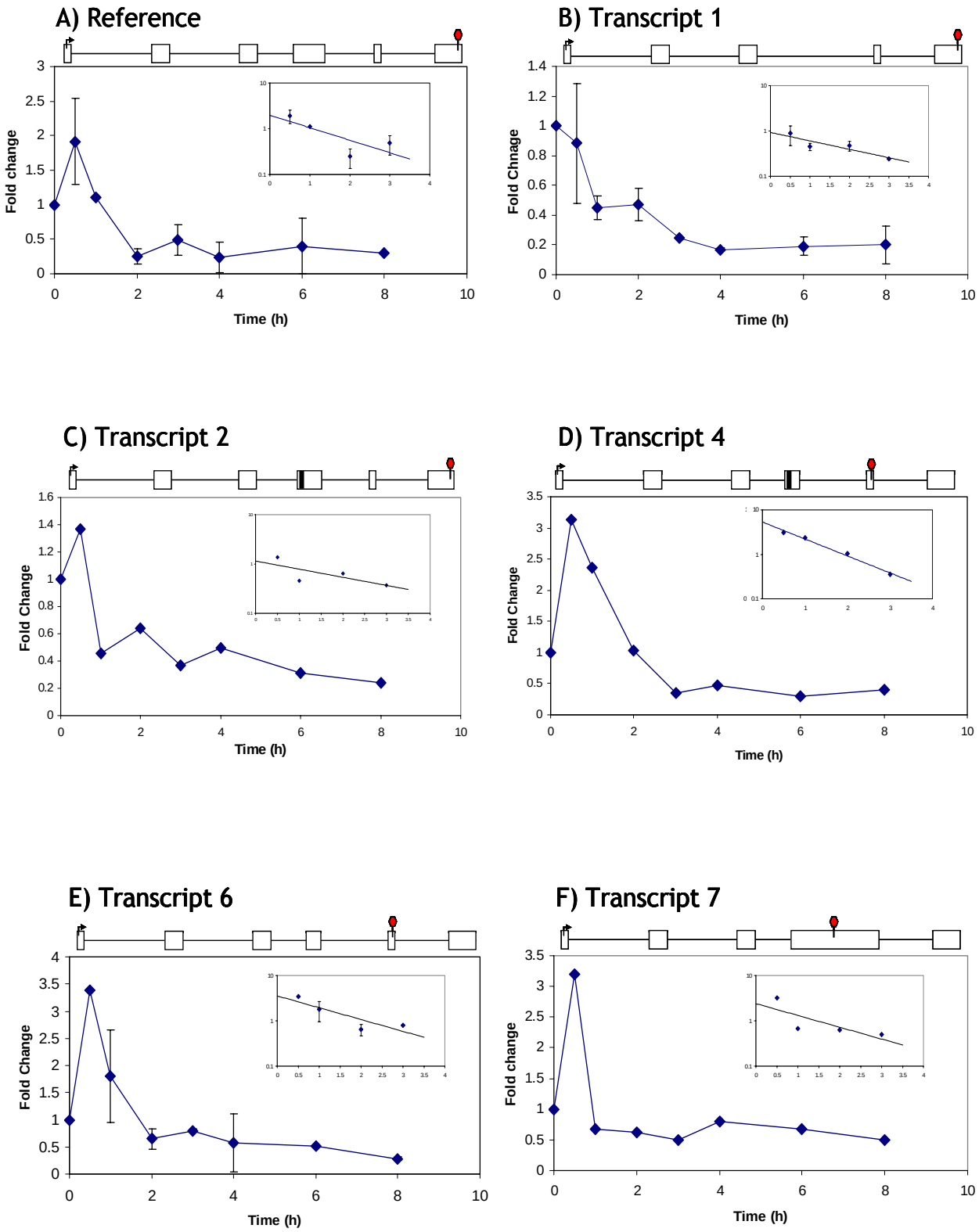

Figure 6.12 Analysis mRNA decay by real-time PCR
Plots show changes in levels of individual *PQBP1* transcripts relative to the level at time=0. mRNA expression levels were normalised to *ActB* levels as described. The exon structure of each transcript is shown, with the start and stop codons denoted by an arrow and red hexagon, respectively. First order decay plots are shown, inset. Error bars represent results from duplicate experiments.

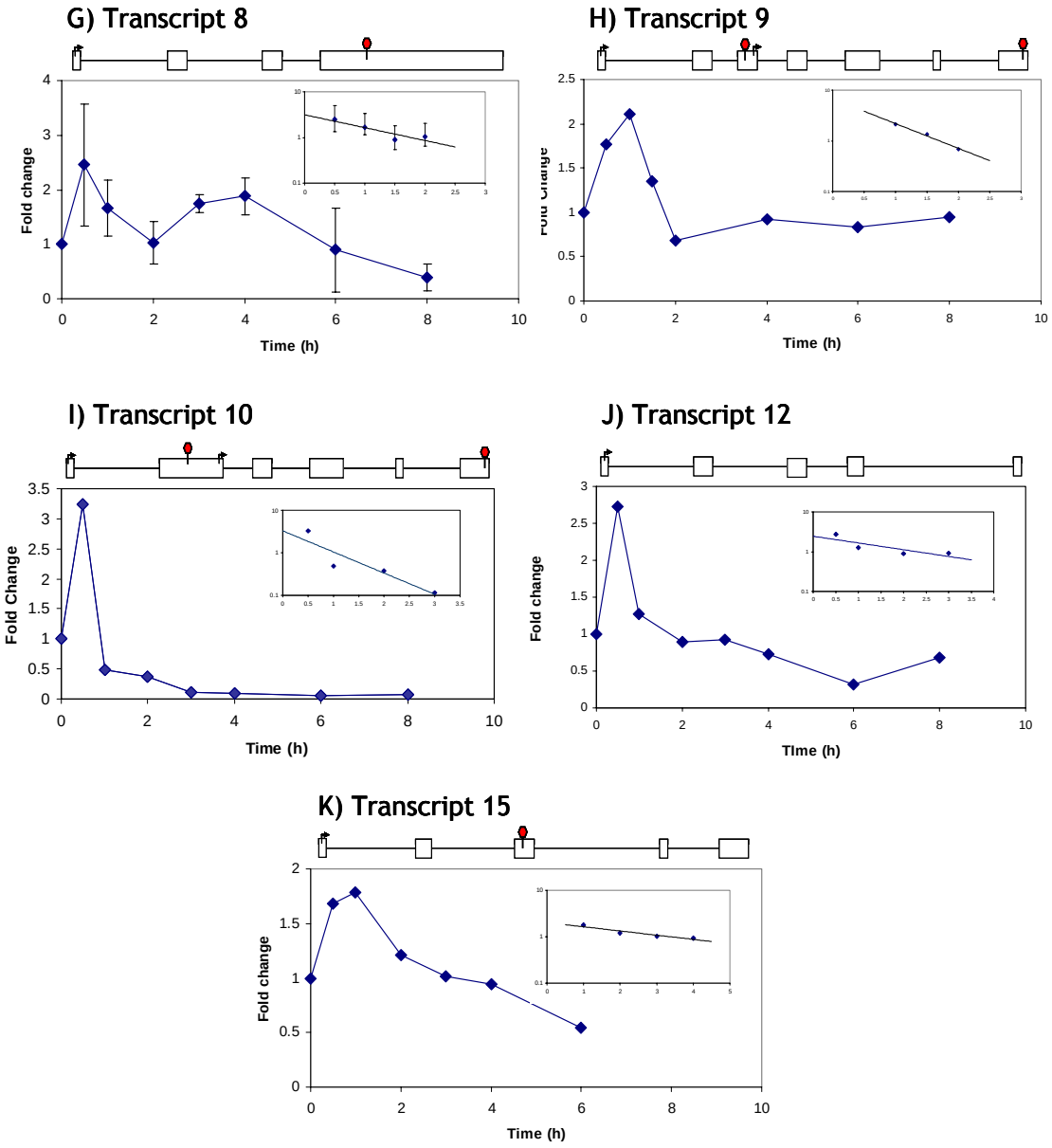

Figure 6.12 continued.

Results from Table 6.3 highlight that the 21 bp deletion identified in transcript 2, does not affect the stability of the mRNA transcripts, reference and transcript 2 being 1.10 and 1.25, hours respectively. This same deletion is also present in transcript 3, 4 and 5 and may not compromise the stability of these transcripts. The results also suggested that the intron retention (as observed in transcripts 7 and 8) did not affect the stability of the mRNA transcripts, despite the fact that this would apparently result in a PTC .

The mRNA half lives of two transcripts lacking exon 4 (transcript 1 and 15) were substantially higher than that of the reference transcript. The half life of transcript 1, where exclusion of exons is the only change, was 50% higher than that of the reference transcript (1.65 v 1.10), while transcript 15 which also has an addition to exon 2 had an mRNA half life that was 59% greater. Together these results suggest that inclusion of exon 4 in a *PQBP1* transcript may decrease the stability of the *PQBP1* transcripts.

Transcript 12 had the most significant increase in the mRNA half-life, (threefold higher than the reference transcript). The splicing pattern of this transcript results in a frame shift that removes the reference transcript's termination codon. Measures were not made to ensure that the open reading frame of the transcript was disrupted upon its introduction into the pTRE-TIGHT vector. However, an in-frame stop codon is located 12 bp downstream from the site of ligation with the pGEM-TEasy vector. This is a significant limitation that must be considered in the interpretation of these results, and it would be beneficial to introduce a stop codon into the transcript when included in the pTRE-TIGHT construct.

Finally, transcripts 4, 6 9 and 10 whose splicing patterns introduced a premature termination codon had shorter half lives than the reference transcripts. The utilisation of an alternative donor site that resulted in a 132 bp deletion in exon 4, which was found in transcripts 4 and 6, was correlated with a 40% decrease in the half life of the mRNA transcript. While the inclusion of a novel exon into the mRNA transcript for *PQBP1* also resulted in a 43% reduction in the mRNA half-lives of transcripts 9 and 10. However, the mRNA decay profile for transcript 9 requires further validation. This is because a result could not be determined for one of the time points (t=3). The inclusion of this time point in the analysis may alter its half-life.

Transcript 8 displayed an unusual, biphasic mRNA decay profile. Over the duration of the time course, two peaks of *PQBP1* transcript abundance were observed, one after 30 minutes and one 4 hours after the addition of doxycycline. While a biological explanation for this observation cannot be offered at this stage, it must be noted that the earlier quantitative analysis in human tissues failed to detect significant levels of this transcript (section 5.4). Transcript 8 was generated through the retention of two introns and may be the result of an aberrant splicing event. Additional experimental analysis is required to characterise the mRNA decay pattern of this transcript further. This could include, monitoring the stability of the mRNA using an NMD depleted system such as reducing Upf1 expression by RNAi (Upf1 is an NMD factor that is thought be to a bridge between the premature termination event and EJC.) (Mendell *et al.,* 2002).In addition, it is possible that the transcribed intron may harbour a secondary RNA structure that may somehow affect its stability. Nucleotide sequence analysis could be employed to predict this (such as mFold), but ideally the structure should be analysed by experimental analysis such as nuclear magnetic resonance.

6.5 Degradation of alternatively spliced *PQBP1* transcripts by nonsense mediated decay

The results obtained in section 6.4 indicated that some transcripts with a PTC (ie transcripts 4, 6, 9 and 10) had shorter half-lives than the reference *PQBP1* transcript and therefore may be targeted for rapid degradation. This experiment however did not identify a cellular mechanism that might be responsible for the rapid degradation of these transcripts.

In order to determine if NMD was involved in the degradation of PTC harbouring *PQBP1* transcripts, the NMD pathway was inhibited using antibiotics that block the translation which is a necessary step in the identification of transcripts harbouring a PTC. Following inhibition, the abundance of native mRNAs was determined over a six hour time course. It was hypothesised that the levels of native mRNAs without PTCs should not be targeted by the inhibited NMD pathway and are predicted to increase in abundance over the duration of the experiment.

HEK293 (human embryonic kidneys) cells were chosen for this assay. These cells were treated with antibiotics that have been shown to inhibit NMD by blocking the pioneering round of translation, a necessary step in the identification of PTCs (Figure 6.1). The antibiotics used were cycloheximde, anisomycin and puromycin and the mechanisms by which they inhibit protein translation are listed in Table 6.4. Cycloheximde and anisomycin work specifically in eukaryotic cells while puromycin blocks translation in both prokaryotes and eukaryotes. The effective doses required to induce translation inhibition without inducing apoptosis were extracted from the scientific literature.

Table 6.4 Antibiotics used to inhibit translation in HEK293 cells

Antibiotic	Concentration used in this study	Mechanism of action	Reference
Cycloheximide	100 µg/ml	Blocks translocation reaction on ribosomes	Harries *et al.*, 2004
Anisomycin	10 µg/ml	Blocks the peptidyl transferase reaction on ribosomes	Caputi *et al.*, 2002; Gatfield *et al.*, 2003; Harries *et al.*, 2004
Puromycin	20 µg/ml	Causes premature release of the nascent polypeptide by its addition to growing chain end.	Gatfield *et al.*, 2003

Unlike the mRNA stability assay carried out in section 6.4, which used cloned cDNAs, all transcripts assayed in this experiment were the native mRNAs produced from the endogenous human *PQBP1* gene. Changes in *PQBP1* expression levels were monitored over a 6 hour time course (see section 2.26). Total RNA was harvested from the cells at time points 0 (addition of antibiotic), 1, 2, 4 and 6 hours and used to synthesise cDNA. The abundance of various *PQBP1* alternative transcripts was quantified by real time PCR using SYBR green detection of double stranded DNA using the primer pairs from section 5.4 (Table 6.5).

All samples were normalised to the house keeping gene *GAPDH* and were expressed as fold-changes from the time point zero. Each real-time PCR was completed in triplicate and each experiment was repeated in duplicate. The results obtained are displayed in Figure 6.14.

Table 6.5 Primer pairs used to quantify alternative *PQBP1* transcripts

Primer Pair	Transcript	PTC	mRNA decay rates*	Predicted NMD**
Q10	All	Some	1.10	No
Q2b	1 15	1 - No 15 - Yes	1.65 1.78	No
Q3	4,6	Yes	0.79, 0.63	Yes
Q4	3,7,8,11	Yes	1.16,1.08	Yes
Q6	9,10,11	yes	0.62, 0.61	Yes

* if known
** based on location of PTC not mRNA decay rates

Throughout the time course experiment, distinct changes in the abundance of some of the *PQBP1* transcript variants were observed, and similar results were obtained with either anisomycin or cycloheximide, but different results were obtained when puromycin was used to block protein translation.

The abundance of all *PQBP1* transcripts amplified by primer pairs Q10 (all *PQBP1* variants identified in section 5.2) remained relatively constant when the cells were exposed to either anisomycin or cycloheximide. A 25% and 40% increase in the abundance of all *PQBP1* transcripts were observed over the six hour time course (anisomycin and cycloheximide respectively). As the reference transcript represents the overwhelming majority of all *PQBP1* transcripts (section 5.4), it is assumed that the expression patterns generated using Q10 primers reflect the stability of the *PQBP1* reference transcript. Slight changes in the expression profile may represent increases in the abundance of minor *PQBP1* transcripts that would normally be degraded by the NMD pathway.

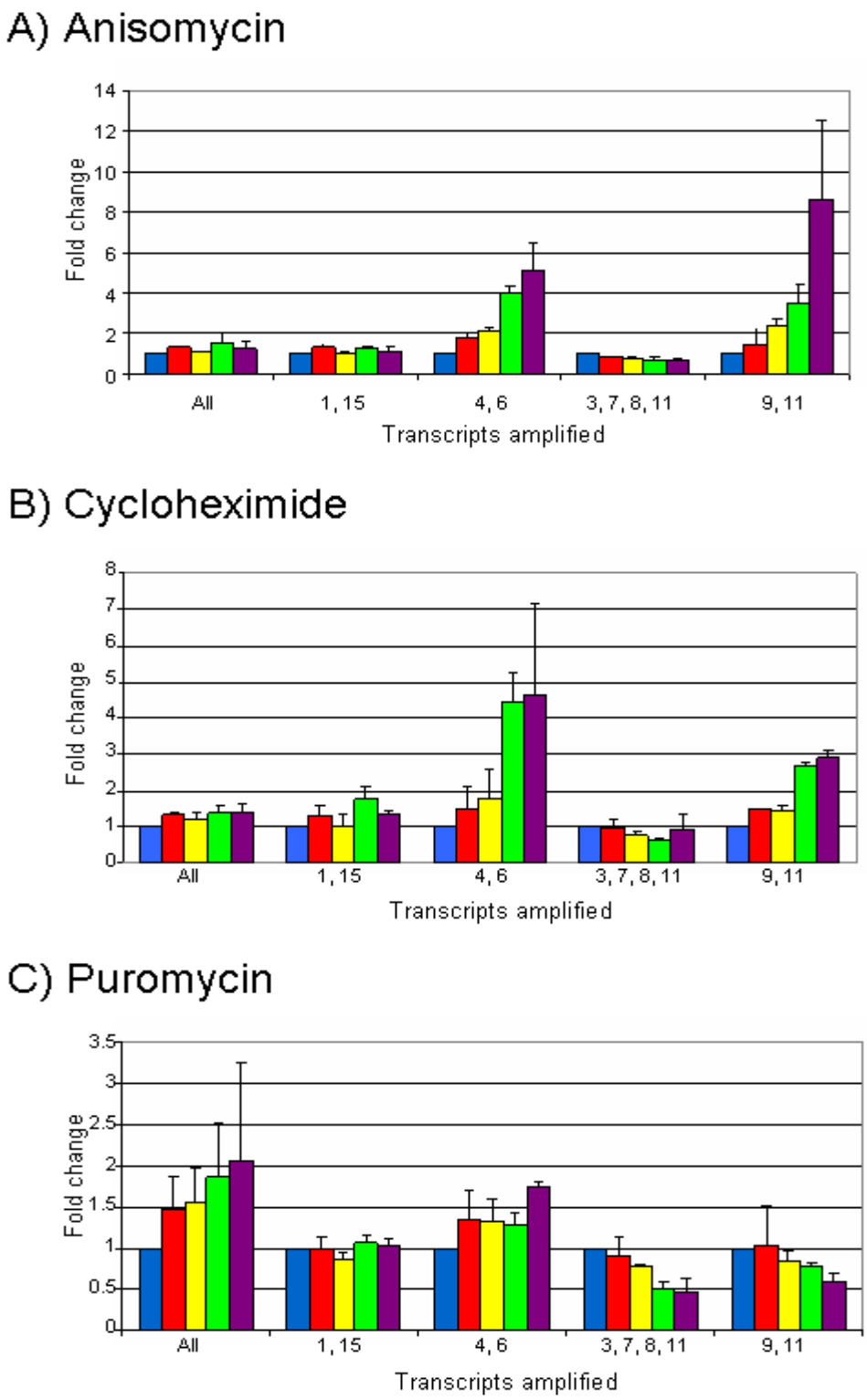

Figure 6.13 Effect on *PQBP1* transcript levels of inhibiting protein translation using anisomycin, cycloheximide and puromycin

Antibiotics were added to HEK293 cells and cell samples were taken over a 6 hour time course. At each time point 0 hr (blue), 1 hour (red), 2 hours (yellow), 4 hours (green) and 6 hours (purple). RNA was harvested and cDNA synthesised. The abundance of cDNA was measured by real-time PCR using SYBR-green fluorescence. The results were normalised to the house-keeping gene *GAPDH*.

PQBP1 transcripts amplified with primer pair Q2b (transcripts 1 and 15) also displayed very little change in their abundance throughout the six hour time course. A 35% and 13% increase in the abundance of transcripts amplified using this primer pair was observed after 6 hours incubation with either anisomycin or cycloheximide, respectively. These results suggest that the *PQBP1* transcripts amplified using primer pair Q2b are not targeted for degradation by the NMD pathway. It was anticipated that transcript 1 would be stable as it does not contain a PTC. Transcript 15 was expected to be degraded by NMD, *a priori,* as it contains a PTC. However, the data from the experiments performed in section 6.4 suggested that transcript 15 is more stable than the reference transcript and therefore may not be targeted for rapid degradation by the NMD pathway. It is possible that transcript 1 is more abundant than transcript 15, and that the changes in the abundance of transcript 15 are masked by the ability of transcript 1 to evade the NMD pathway. Further analysis is required using a primer pair that is able to differentiate between transcripts 1 and 15 in real-time PCR. Only then can the relative abundance of each transcript be described accurately.

PQBP1 transcripts amplified using primer pair Q4 (transcripts 3, 7, 8 and 11) also showed little change in transcripts abundance. The splicing variation shared by these transcripts is the inclusion intron 4. Here, a 9% (anisomycin) and 34% (cycloheximide) decrease in the transcript's abundance was observed. The slight decrease in these transcripts may be attributed to the excision of this intron from the mature mRNA transcript in the course of the experiment.

The abundance of transcripts 4 and 6 (primer pair Q3), 9 and 11 (primer pair Q6) increased in accordance with an increased time of exposure to anisomycin and cycloheximide. The increases after 6 hours were 5.2 fold (anisomycin) and 4.6 fold (cycloheximide) for primer pair Q3 and 8.7 (anisomycin) and 2.9 fold (cycloheximide) for primer pair Q6. Together, these results suggest that the abundance of transcripts 4, 6, 9 and 11 increased as a result of translation inhibition and they may be degraded by the NMD surveillance pathway. All of these transcripts contain PTCs and would be unable to encode a full length protein.

Changes in the abundance of *PQBP1* transcripts that resulted from blocking translation with puromycin had different profiles to those produced by anisomycin and cycloheximide. Here, a doubling in transcript levels after 6 hours was

observed with the Q10 primer pair, which amplifies all known *PQBP1* transcripts. The levels of transcript 1 and 15 (primer pair 2b) and transcripts 3, 7, 8 and 11 (primer pair 3) remained relatively constant over the time course of the experiment. A 3% and 75% increase was observed. Decreases in abundance were observed for transcripts 4 and 6 (primer pair Q4) and transcript 9 and 11 (primer pair Q6) 64% and 40% fold, respectively, at the 6 hour time point. None of the changes observed with puromycin are as striking as some of the changes observed with anisomycin and cycloheximide. Puromycin is known to be capable of inhibiting NMD (Gatfield *et al.,* 2003), but it may be ineffective in HEK293 cells. It is perhaps more likely that the concentration of puromycin used was not sufficient to repress translation effectively in these cells.

6.6 Discussion

This chapter describes further functional analysis of the *PQBP1* alternative transcripts that were identified in Chapter 5. Each isoform was analysed *in silico* to identify any structural alterations that would be predicted to result from alternative splicing. This was complemented by performing sub-cellular localisation of the *PQBP1* isoforms. In addition the *PQBP1* transcript variants were characterised for their ability to be produce stable mRNAs. A summary of the results obtained in this chapter is found in Table 6.6.

Overall, 56% of the *PQBP1* alternative transcripts are predicted to have an abated or altered function, while 44% of transcripts are potential targets of accelerated degradation via the NMD pathway. The functional significance of these observations is discussed in the following sections.

6.6.1 *Splicing patterns affect the sub-cellular location of PQBP1 transcript variants*

In silico analysis suggested that nine of the 15 *PQBP1* predicted isoforms had altered domain structures. One of the most striking observations was the exclusion of a putative nuclear localisation signal from eight of the 15 *PQBP1* isoforms. Sub-cellular localisation of the *PQBP1* isoforms demonstrated that this signal was required for the direction of *PQBP1* isoforms into the nucleus, which is consistent with other published investigations on four *PQBP1* transcripts (Iwamoto *et al.,* 2000).

Neither the expression nor sub-cellular localisation could be detected for isoforms 9, 10, 11 and 15. Several attempts were made to confirm the expression of these peptides and the positive results obtained with the other *PQBP1* isoforms eliminate the possibility of experimental error. The failure to confirm the expression of all *Alu* containing isoforms also suggests that this anomaly is not caused by experimental error but could be the result of the incorporation of an *Alu* fragment into the *PQBP1* transcript which destabilises the transcripts.

However, i*n silico* analysis of the isoforms encoded by transcripts 9, 10, 11 and 15 found that they all lack the WW domain (section 6.2) and it is possible that the absence of this domain may destabilise the protein. Without the WW domain it is plausible that the *PQBP1* isoforms may not fold correctly and this could lead to the

degradation of the unfolded protein rapidly. This domain is also crucial for the correct folding of other human proteins including the protein Yes-associated kinase protein, *h*Yap (Macias *et al.,* 1996). Mutational analysis of this protein has found that shorter constructs of the *h*Yap without the WW domain are either less stable than the wild-type domain or are unable to form any stable structure (Jiang *et al.,* 2001).

Additional analysis is required to determine if the variations in the domain structures of the *PQBP1* isoforms affects their biological function. This could be achieved by completing an assay specific to *PQBP1* function for example, the ability of the protein to bind polyglutamine tracts.

6.6.2 *Comparison of methods used to determine mRNA stability*

Two functionally distinct assays have been used to characterise the stability of the mRNA transcripts: the first utilised cloned cDNAs of *PQBP1* alternative transcripts to monitor mRNA decay rates, while the second interrupted cellular homeostasis by inhibiting translation, and then studied the effect on naturally occurring mRNA transcripts. The advantages of each approach are discussed below.

In the first series of experiments, general trends were observed for most of the transcripts studied, being a rapid increase in the transcript's abundance followed by gradual decay. Additional steps must to be taken to reduce the high level of inter-assay variation that was observed. This could be achieved by creating cell lines where the pTRE-TIGHT-*PQBP1* constructs are stabley integrated into the cell's genome. With this approach, exogenous effects on mRNA transcript abundance such as cell fitness or transfection efficiency would be minimised. Unfortunately, these experiments were not completed due to time constraints.

An obvious advantage of the tetracycline dependent expression system is the ability to arrest only the transcription of the exogenous *PQBP1* gene upon the addition of doxycycline, thus leaving the cell's physiology relatively undisturbed. However, the use of cloned cDNA samples in such assays has been the subject of criticism. Byers (2002) argued that recombinant DNA molecules were not biologically relevant, as they do not resemble naturally occurring spliced mRNA transcripts (Byers 2002). More specifically, cloned and transcribed cDNAs do not

bind proteins that normally bind to exon-exon junctions during splicing. The binding of spliceosomal and regulatory proteins to these junctions has been shown to have a role in identification of transcripts to be targeted by NMD (section 6.5).

Despite this argument, cDNAs containing PTCs have been shown to degrade more quickly than their wild-type counterparts. An exon junction-independent form of NMD has also been shown to degrade PTC-containing transcripts from the human genes β-globin (Couttet and Grange, 2004) Ig-u (Buhler *et al.,* 2004) and TCR-B (Wang *et al.,* 2002a). It is therefore highly probable that NMD in mammalian cells is controlled by multiple pathways that act at different levels with different efficiencies to ensure that PTC-containing mRNAs are eliminated from cells. Using intronless minigenes the transcript levels of *HEXA* harbouring PTCs were shown to be lower than the wild-type transcript (Rajavel and Neufeld 2001). However, these levels were not as low as those obtained for minigenes that contained introns and were therefore capable of binding mRNPs to spliced exon junctions. The results obtained in this chapter provide further support for exon-junction independent NMD, as in most cases transcripts harbouring a PTC degraded more quickly than full length transcripts.

In light of these observations, it was decided to seek additional support for the targeting of some *PQBP1* alternative transcripts that harbour a PTC by NMD by using naturally occurring mRNA species that are bound by mRNPs at exon junctions. Translation inhibitors were used to identify those transcripts that are stabilised when the ribosomal-associated PTC identification mechanism was suppressed. The problem associated with this experimental approach is the potential for pleiotropic effects of general translation inhibition. For example, it is crucial that the appropriate antibiotic concentrations are used to inhibit translation without inducing apoptosis. These figures were obtained from previous experiments on mammalian cells, but further optimisation of the puromycin concentration used in this assay is required to inhibit translation and therefore NMD. NMD was not inhibited for any of the transcripts using a puromycin concentration of 20 ng/µl.

Table 6.6 Summary of results obtained in this chapter

Transcript	Translation confirmed by Western Blotting	Subcellular localisation	mRNA half life (h)	Stabilisation with translation inhibitors	Altered /Abated Function?	NMD candidate?
Reference	Yes	Nucleus	1.10	No	No	No
1	Yes	Ubiquitous	1.65	No	Yes	No
2	Yes	Nucleus	1.27	No	No	No
3	Yes	Nucleus	N.C	No	No	No
4	Yes	Ubiquitous	0.79	Yes	Yes	Yes
5	Yes	Nucleus	N.C	N.C	No	No
6	Yes	Ubiquitous	0.63	Yes	Yes	Yes
7	Yes	Nucleus	1.16	No	No	No
8	N.C.	N.C	1.07	No	No*	No
9	No	Not detected	0.62	Yes	Yes	Yes
10	No	Not detected	0.61	Yes	Yes	Yes
11	No	Not detected	N.C.	Yes	Yes	Yes
12	Yes	Not detected	3.31	N.C	Yes	Yes
13	Yes	Nucleus	N.C	N.C	Yes*	No*
14	Yes	Nucleus	N.C	N.C	No	No
15	No	Not detected	1.78	No (partial)	Yes	Yes*

*Prediction based on *in silico* analysis.
NC - not completed
'Altered means relative to the function of the reference transcript.

The results obtained using cycloheximide and anisomycin supported the results obtained using the tet-off expression system, in that most transcripts containing a PTC were targeted for NMD. A discussion about the stability of the alternative *PQBP1* transcripts follows.

6.6.3 *Not all PQBP1 transcripts containing a PTC are targeted for rapid degradation.*

It has been proposed that susceptibility of transcripts containing PTCs to NMD can be predicted from sequence data alone (Hillman *et al.*, 2004). That is, transcripts containing a PTC 50-55 bp upstream from the ultimate exon junction would be identified by components of the NMD pathway and rapidly degraded. Results obtained in this chapter have demonstrated that a single set of rules cannot be applied to predict the stability of all mRNA transcripts, and that multiple mRNA

surveillance mechanisms probably serve to remove aberrantly spliced transcripts from eukaryotic cells.

Sequence analysis suggested that transcript 7 would be subjected to NMD, as it contains a PTC more than 50 bp upstream from the ultimate exon-junction. However, experiments described here did not provide evidence for decreased mRNA stability; the mRNA half life of the transcript is similar to that of the reference transcript (1.16 v 1.10 hours respectively) and also the transcript was not stabilised by translational inhibitors. *In silico* analysis of the protein encoded by this transcript predicted that it should contain the same structural motifs as the reference protein.

Experimental evidence did, however, demonstrate that on certain occasions the mRNA degradation profile for some of the *PQBP1* alternative transcripts could be predicted from sequence alone. A 132 bp deletion from exon 4 that was identified in transcripts 4 and 6 introduced a frameshift and a PTC. The mRNA half lives of these transcripts were reduced and the transcripts were also stabilised by the addition of anisomycin and cycloheximide to the cell media. Functional changes induced by alternative splicing of these transcripts were also observed experimentally, when the proteins produced from them failed to localise exclusively to the nucleus. Taken together the data suggests that these transcripts are unlikely to have a function and are removed by NMD.

In contrast, transcript 1, which has a deletion of exon 4 was not subjected to NMD. This was predicted as the deletion event did not introduce a PTC into the *PQBP1* variant. However, the functional impact of this alternative splicing event requires further investigation. This functional importance of this observation remains to be solved as the known functional roles of *PQBP1* are performed in the nucleus. Work carried out in chapter 5 suggested that exon 4 may have been acquired after divergence of lineages leading to mammals and fish. If exon 4 was acquired by an already established gene, it is possible that this acquisition introduced an additional nuclear function to the *PQBP1* gene and that non-nuclear *PQBP1* isoforms may also be capable of performing a biological function.

An unexpected result was the increased stability of transcript 15 whose half life was longer than that observed for the reference transcript (1.78 v 1.10 hours), but

whose cognate protein was not detected by either Western blotting or confocal microscopy. The predicted open reading frame encoded by this transcript was significantly shorter than the reference protein, being 76 amino acids in length, and computational analysis failed to identify any structural domains. This suggested that the encoded protein may not be capable of forming structural domains. Together, these results indicate that this transcript appears to be stable and perhaps the problem is more likely to lie with the stability of the encoded protein.

Additional experimental evidence is required to support enhanced stability of transcript 15. This could be obtained on two fronts; firstly the stability of transcript must be confirmed by additional experimental analysis, for example, using transcript specific primers to determine if the transcript is indeed subject to NMD. The transcript stability assay should be repeated and the transcript's stability should be determined using a functionally distinct assay to monitor the mRNA decay, for example by monitoring the rate of mRNA decapping, another indicator of mRNA stability (Couttet and Grange, 2004).

From the results obtained, it appears that the incorporation of an exon located within an *Alu* repeat (transcripts 9-11) has no functional relevance. Computational analysis identified two potential open reading frames for each transcript, but the potential proteins were not detected by Western blotting or sub-cellular localisation. Moreover, both transcript stability assays suggested that these transcripts were less stable than the reference transcript.

It has been hypothesized that the incorporation of *Alu* repeat fragments into mature mRNA species is facilitated by internal sequence motifs that resemble splice sites. Coupled with experimental validation, bioinformatic analysis of exons located within *Alu* repeats have determined that in many cases a point mutation can create the necessary sequence requirements to ensure efficient incorporation the *Alu* fragment into an mRNA transcript (Sorek et al., 2004a). Functional implications of the inclusion of *Alu* in transcripts suggest that apart from contributing to additional primate specific transcript diversity these transcripts may have the potential to cause genetic disorders including Dent's disease (Claverie-Martin *et al.,* 2003), Alzheimer's disease (Clarimon *et al.,* 2003) and Hunter disease (Ricci *et al.,* 2003).

6.6.4 *Conclusions*

This work extends upon expression analysis carried out in chapters 4 and 5, which illustrated that detailed cDNA screening is able to identify transcripts that are found at very low levels in the cell- levels that are much lower than the reference *PQBP1* transcript.

In this chapter it has been shown that some of the *PQBP1* transcript variants are less stable than the reference transcript. At least five of the *PQBP1* transcript variants were degraded rapidly, probably by the nonsense mediated decay pathway. *In silico* analysis of the *PQBP1* isoforms demonstrated that at least 8 isoforms lack domains that appear to the one known function of *PQBP1* (section 6.2). Isoforms without the WW domain failed to produce a protein in a transfection assay (section 6.3), while isoforms without a nuclear localisation signal were not directed to the nucleus following translation. Based on these diverse observations, the picture that seems to be emerging, for *PQBP1* is that much of the transcript diversity that can be detected does not create functional diversity and is more likely to be a result of aberrant splicing.